TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2023) 47: 595-605
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doi:10.55730/1300-008X.2787

Trichoderma indica: a new species in the *Longibrachiatum* clade from Western Ghats, India

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Received: 01.03.2023 • **Accepted/Published Online:** 27.09.2023 • **Final Version:** 22.11.2023

Abstract: *Trichoderma* is a hyperdiverse genus that comprises species showing a wide range of applications. In this study, we have found an isolate of the genus *Trichoderma* from a soil sample collected from Vetal Hill, Pune, Maharashtra State, India. The isolate was found to be morphologically distinct compared to other isolates of the *Longibrachiatum* clade showing longer phialides and shorter conidia. Moreover, a phylogenetic analysis based on combined sequence data of the second-largest nuclear RNA polymerase subunit (*rpb2*), and the translation elongation factor 1-α (*tef1-α*) confirmed the uniqueness of the present isolate, showing an independent branch supported by strong bootstrap values (SH-like approximate likelihood ratio and ultrafast). The comprehensive morphology and multigene phylogeny confirmed the identity of the novel isolate as *Trichoderma indica* belonging to the clade *Longibrachiatum*.

Key words: Soil, hypocreaceae, *tef1-α*, *rpb2*, taxonomy

1. Introduction

Persoon established the genus *Trichoderma* belonging to the order Hypocreales in 1794. The members of the genus are found to be saprophytes, parasites, and endophytes. The genus comprises species that are commonly isolated from soil, but they have also been reported from decaying wood, leaves, and plant endophytes. The genus can survive in various environments with effective reproductive ability and diverse metabolic patterns (Lopes et al., 2012). Many *Trichoderma* species have been reported for their use as biocontrol agents, biofertilizers, plant growth promoters, food processors, and producers of antibiotics and enzymes. Some of the species have been reported to be pathogenic.

The genus *Trichoderma* has been characterized by branched treelike conidiophores with cylindrical to subglobose phialides and globose to ellipsoidal conidia. However, variations in phialides and chlamydospores are not enough to distinguish the species of *Trichoderma* (Chaverri et al., 2015; Qin and Zhuang, 2017; Qiao et al., 2018). Conventional taxonomic studies of the genus *Trichoderma* were merely based on morphology. Moreover, the morphology of most of the species was found to be highly similar. Therefore, species distinction based on morphology alone was insufficient to resolve the chaos in the taxonomy of the *Trichoderma* genus. Thus, multi-gene phylogeny has been used in addition to morphological features for the correct identification of *Trichoderma* species. Different molecular markers like RNA polymerase II subunit (*rpb2*), the translation elongation factor 1-α gene (*tef1-α*), ATP citrate lyase (*acl1*), actin (*act*), and the internal transcribed spacer (ITS) region have been recently used for accurate identification of the *Trichoderma* species. Phylogenetic analysis based on combined sequence data of these molecular markers allows the accurate identification of the species in the genus *Trichoderma* (Jaklitsch and Voglmayr, 2015; Bustamante et al., 2021).

As per the current classification, the species of *Trichoderma* are grouped into the 10 phylogenetic lineages of *Longibrachiatum*, *Harzianum*, *Viride*, *Polysporum*, *Hypocreanum*, *Brevicompactum*, *Deliquescens*, *Psychrophilum*, *Stromaticum*, and *Semiorbis* based on combined sequence data of tef*1*, *rpb2*, and *acl1* (Chaverri et al., 2015; Jaklitsch and Voglmayr, 2015). Among them, *Longibrachiatum* is one of the lineages comprised of many species with promising bioactive potential use in agriculture (Bustamante et al., 2021). Colonies of members of the *Longibrachiatum* clade are sporulating and showing diffusing yellow pigment on potato dextrose agar (PDA) media. The conidiophores arising from aerial mycelium and phialides are majorly solitary and arise singly, while in some species, they are found in whorls. The phialides are lageniform to

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cylindrical, hooked, or sinuous. The conidia are smooth, ellipsoidal to oblong, and rarely subglobose to tuberculate to roughen (Samuels et al., 2012).

Among the 12 megadiverse countries in the world, India is one of the biodiversity-richest. The Western Ghats region of India is one of the global hotspot regions among the four hotspot regions in India. It is known for its species richness and diversity of flora, fauna, and microbes. During our exploration of the biodiversity of the Western Ghats, we isolated a new species of *Trichoderma* in soil collected from Vetal Hill, Bhamburda, Pune. We have confirmed the present taxon as a novel species based on morphology and multilocus phylogenetic analysis.

2. Materials and methods

2.1. Collection and isolation

The soil sample was collected in a sterile ziplock bag from Vetal Hill, Bhamburda, Pune, Maharashtra, India (18.5255°N, 73.8154°E) and stored at 4 °C. Fungi of different taxonomic groups were isolated from the collected soil sample using the serial dilution method (Waksman, 1916). For this, higher dilutions (10^{-3}, 10^{-5}, 10^{-7}) of the sample were spread plated on PDA plates and incubated at 25 °C for the growth of fungal colonies. Fungal colonies showing different morphological features were selected and transferred onto fresh PDA slants.

2.2. Morphology of culture

For the morphological studies, agar blocks (0.5 mm diameter) of pure culture of *Trichoderma* were inoculated from five-day-old PDA culture plates on 3 different media (in triplicate): PDA, cornmeal dextrose agar (CMD), and synthetic low-nutrient agar (SNA) (Chaverri et al., 2015). Plates were incubated at 25 °C with 12 h in natural light and 12 h in darkness. The colony radius was measured daily until the plates were covered entirely with mycelium. After 7 days of incubation, the cultural characteristics were studied. The asexual stage was described based on its morphotaxonomic features like macromorphology (growth pattern, colony diameter, front, and reverse colour) and micromorphology (formation of conidiophores, branching pattern, development of phialides and conidia, shape, size, colour, ornamentation, etc.) and followed key to the sections of *Trichoderma* (Bisset, 1991; Samuels et al., 1998). The *Methuen Handbook of Colour* (Kornerup and Wanscher, 1978) was used to record the colour codes. Slides were prepared in lactophenol cotton blue mount, and microphotographs of the different morphological structures were taken using a Carl Zeiss AXIO-10 microscope. For the average dimensions of conidia and phialides, 30 measurements were taken, and the mean value of the length and width of the conidia and phialides were calculated. A pure culture was deposited in the NFCCI (National Fungal Culture Collection of India), Pune (NFCCI 5214).

2.3. DNA extraction, PCR, and sequencing

The fungal genomic DNA was extracted from the mycelia growing on a five-day-old PDA culture plate as the protocol reported by Aamir et al. (2015). Amplification of the translation elongation factor 1 alpha (*tef1-α*) and RNA polymerase II second largest subunit (*rpb2*) gene region was done by polymerase chain reaction (PCR) using a ProFlex PCR machine (Applied Biosystems, USA). The primer pairs used for amplification of the *tef1-α* gene region are EF1-728F (Carbone and Kohn, 1999) and TEF1LLErev (Jaklitsch et al., 2005), while fRPB2-5f and fRPB2-7cr (Liu et al., 1999) are used for the *rpb2* gene region. For *rpb2*, the PCR conditions were an initial step of 5 min at 94 °C, 40 cycles of 1 min at 94 °C, 30 s at 55 °C, and 2 min at 72 °C, followed by 7 min at 72 °C. For *tef1-α,* the PCR conditions were an initial step of 3 min at 94 °C, 40 cycles of 30 s at 94 °C, 50 s at 55 °C, 1 min at 72 °C, followed by 10 min at 72 °C. The amplified PCR products were purified using a FavorPrep™ PCR purification kit (Favorgen Biotech Corporation, Taiwan). The purified PCR products were subjected to sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and an ABI Avant 3100 automated DNA sequencer machine. The obtained sequences were edited manually and deposited in the NCBI nucleotide sequence database [GenBank Acc. No. *tef1-α*– ON951920, *rpb2*– ON952501 (NFCCI 5214) and *tef1-α*– OR192144, *rpb2*– OR192143 (NFCCI 5549)].

2.4. Sequence alignment and phylogenetic analysis

The *tef1-α* and *rpb2* sequences of the present taxon were subjected to MegaBLAST searches, and a combined dataset of *tef1-α*, and *rpb2* sequences comprising genetically related species of the genus *Trichoderma* belonging to the *Longibrachiatum* clade was prepared. Multiple sequence alignment was performed using MAFFT version 7.520 (Katoh and Standley, 2013), and the ends of the aligned sequences were manually edited in MEGA 7 (Kumar et al., 2016). A phylogenetic tree was generated using the maximum likelihood method based on combined *tef1-α* and *rpb2* sequence data of 51 closely related isolates, including the present taxon (Table 1). *Trichoderma longisporum* HMAS 248843 belonging to the *Spirale* clade (Chen and Zhuang, 2017) was used as an outgroup. A best-fit evolutionary model was selected for the aligned sequence dataset using the ModelFinder algorithm (Kalyaanamoorthy et al., 2017) implemented in IQ-TREE multicore v. 1.6.11 (Nguyen et al., 2015). An IQ-TREE-supported edge-proportional partition model with proportional branch lengths was used for the multigene alignments so that each partition had its own substitution models and evolutionary rates (Chernomor et al., 2016). A phylogram was constructed in IQ-TREE using a combined *tef1-α* and *rpb2* aligned sequence dataset based on the maximum likelihood method employing an edge-

Table 1. Taxa used for phylogenetic analysis and their corresponding GenBank accession numbers.

Sr. No.	Fungal strain	Strain	GenBank accession no.		Reference
			tef1-α	*rpb2*	
1	*Trichoderma aethiopicum*	PPRC H5	EU401616	-	Druzhinina et al., 2008
2	*Trichoderma andinense*	COAD 2431	MK044155	MK044248	Rodríguez et al., 2021
3	*Trichoderma andinense*	GJS 09-62	JN133570	JN133559	Chaverri and Samuels, 2013
4	*Trichoderma andinense*	DAOM 220821	KJ713205	KJ842208	Druzhinina et al., 2012
5	*Trichoderma aquatica*	YMF 1.04624	MK775506	MK775511	Zheng et al., 2021
6	*Trichoderma aquatica*	YMF 1.04625	MK775507	MK775512	Zheng et al., 2021
7	*Trichoderma awajun*	CP 24-7	MW480138	MW480147	Bustamante et al., 2021
8	*Trichoderma awajun*	CP 24-8	MW480139	MW480148	Bustamante et al., 2021
9	*Trichoderma bissettii*	SZMC 25718	MN641029	MN650681	Hatvani et al., 2019
10	*Trichoderma capillare*	GJS 06-66	JN175585	JN175530	Druzhinina et al., 2012
11	*Trichoderma centrosinicum*	HMAS 252910	KX066257	KX066267	Qin and Zhuang, 2016
12	*Trichoderma citrinoviride*	S27	KJ665450	KJ665251	Jaklitsch and Voglmayr, 2015
13	*Trichoderma citrinoviride*	S20	KJ665449	KJ665250	Jaklitsch and Voglmayr, 2015
14	*Trichoderma effusum*	C.P.K. 254	KJ665473	KJ665260	Jaklitsch and Voglmayr, 2015
15	*Trichoderma ghanense*	GJS 95-137	AY937423	JN175559	Samuels, 2006
16	*Trichoderma ghanense*	DAOM 165776	JN175610	JN175560	Druzhinina et al., 2012
17	*Trichoderma gillesii*	GJS 00-72	JN175583	JN175527	Druzhinina et al., 2012
18	*Trichoderma gracile*	GJS 10-263	JN175598	JN175547	Druzhinina et al., 2012
19	***Trichoderma indica***	**NFCCI 5214**	**ON951920**	**ON952501**	**Present study**
20	***Trichoderma indica***	**NFCCI 5549**	**OR192144**	**OR192143**	**Present study**
21	*Trichoderma konilangbra*	C.P.K. 132	JN258681	JQ513367	Druzhinina et al., 2012
22	*Trichoderma kunigamense*	TAMA 0193	AB807645	AB807657	Yabuki et al., 2014
23	*Trichoderma longibrachiatum*	CBS 816.68	AY865640	DQ087242	Druzhinina et al., 2005
24	*Trichoderma longibrachiatum*	C.P.K. 1707	-	JN182315	Druzhinina et al., 2012
25	*Trichoderma longibrachiatum*	C.P.K. 744	JN182276	JN182308	Druzhinina et al., 2012
26	*Trichoderma longibrachiatum*	S328	JQ685867	KJ665291	Jaklitsch and Voglmayr, 2012
27	*Trichoderma longisporum*	HMAS 248843	KY688043	KY687982	Chen and Zhuang, 2017
28	*Trichoderma novae-zelandiae*	GJS 81-265	AY937448	JN133563	Samuels, 2006
29	*Trichoderma novae-zelandiae*	GJS 99-113	JN175582	JN175526	Druzhinina et al., 2012
30	*Trichoderma orientale*	G.J.S. 88-81	EU401581	-	Druzhinina et al., 2008
31	*Trichoderma orientale*	CP15-1	MW541138	MW541111	Bustamante et al., 2021
32	*Trichoderma parareesei*	COAD 2482	MK044153	MK044246	Rodríguez et al., 2021
33	*Trichoderma parareesei*	COAD 2483	MK044154	MK044247	Rodríguez et al., 2021
34	*Trichoderma parareesei*	COAD 2485	MK044082	MK044265	Rodríguez et al., 2021
35	*Trichoderma pinnatum*	GJS 04-100	JN175571	JN175515	Druzhinina et al., 2012
36	*Trichoderma pinnatum*	GJS 02-120	JN175572	JN175516	Druzhinina et al., 2012
37	*Trichoderma pluripenicillatum*	YMF 1.6174	MT070159	MT070160	Zheng et al., 2021
38	*Trichoderma pseudokoningii*	GJS 81-300	AY937429	HM182985	Atanasova et al., 2010
39	*Trichoderma reesei*	GJS 00-89	JN175599	JN175548	Druzhinina et al., 2012
40	*Trichoderma reesei*	GJS 00-09	JN175600	JN175549	Druzhinina et al., 2012
41	*Trichoderma reesei*	GJS 06-138	GQ354370	HM182972	Atanasova et al., 2010

Table 1. (Continued).

42	*Trichoderma rugosum*	HMAS 254548	MH612379	MH612373	Zhang and Zhuang, 2018
43	*Trichoderma rugosum*	HMAS 254536	MH612378	MH612372	Zhang and Zhuang, 2018
44	*Trichoderma saturnisporopsis*	S19	JQ685869	JQ685885	Jaklitsch and Voglmayr, 2012
45	*Trichoderma saturnisporum*	ATCC 28023	JN388897	JN175524	Druzhinina et al., 2012
46	*Trichoderma sinense*	C.P.K. 530	JN182273	JN182310	Druzhinina et al., 2012
47	*Trichoderma sinense*	C.P.K. 531	JN182274	JN182311	Druzhinina et al., 2012
48	*Trichoderma solani*	GJS 08-81	JN175597	JN175546	Druzhinina et al., 2012
49	*Trichoderma thermophilum*	HMAS 252912	KX066249	KX066261	Qin and Zhuang, 2016
50	*Trichoderma tsugarense*	TAMA 0203	AB807647	AB807659	Yabuki et al., 2014
51	*Trichoderma xanthum*	HMAS 247202	MF371226	MF371211	Kai and Wen-Ying, 2017

proportional partition model with proportional branch lengths using the respective best-fit models obtained for the partitions (K2P+I+G4 as the best suitable model for *tef1-α*; TIM3e+I+G4 for *rpb2*). A total of 1000 bootstrap replicates of the trees were analysed to get both SH-like approximate likelihood (SH) and ultrafast bootstrap (UF) values, and values above 50% were represented on nodes in the tree.

3. Results

3.1. Taxonomy

Trichoderma indica A.C. Lagashetti, S.K. Singh, & P.N. Singh sp. nov. (Figures 1 and 2)

Etymology: The specific epithet refers to the place where it was isolated.

MycoBank: MB 847645

Material examined: INDIA. MAHARASHTRA: Pune, Bhamburda, Vetal Hill, saprophyte from the soil, 1 Oct 2020, A.C. Lagashetti and S.K. Singh (AMH 10506, holotype; NFCCI 5214, ex-type living culture).

Description: On PDA after 72 h, 60 mm, the mycelium covers the plate after 4 days at 25 °C. Colony circular, slightly raised, cottony, abundant aerial hyphae. Colour from front white (1A1) to light yellow (1A4) and reverse light yellow (1A4) at the centre and white (1A1) at the periphery. **Conidiophores** trichoderma- to verticillium-like, formed on aerial hyphae. **Phialides** commonly solitary or rarely in whorls of 2, lageniform, subulate to spatulate, straight to curved, 4–18.8 × 1.3–2.9 µm ($\overline{x}$ = 8.6 × 1.9 µm, n = 30), l/w 1.8–11.7 µm ($\overline{x}$ = 4.7 µm, n = 30), 1.0–1.9 µm ($\overline{x}$ = 1.4 µm, n = 30) wide at the base. **Conidia** solitary or in groups, hyaline to green, globose to subglobose, oval to ellipsoidal, smooth, 2.6–5.0 × 1.9–3.1 µm ($\overline{x}$ = 3.5 × 2.6 µm, n = 30), l/w 1.0–1.7 ($\overline{x}$ = 1.3 µm, n = 30). **Chlamydospores** abundant, terminal, or intercalary, globose to subglobose, oval to ellipsoidal, 5.8–10.8 × 4.8–9.0 µm ($\overline{x}$ = 8.0 × 6.5 µm, n = 30), l/w 1.0–1.9 ($\overline{x}$ = 1.2 µm, n = 30). No distinct odour; slightly yellow diffusing pigment observed.

On CMD after 72 h, 70 mm, the mycelium covers the plate after 4 days at 25 °C. Colony slightly raised, not zonate, numerous aerial hyphae, colour from front white (1A1) to light yellow (2A4) and reverse light yellow (1A4). **Conidiophores** trichoderma- to verticillium-like, formed on aerial hyphae. **Phialides** solitary or commonly divergent in whorls of 2–3, lageniform, curved, 4.5–20 × 1.4–3 µm ($\overline{x}$ = 8.9 × 2.2 µm, n = 30), l/w 2.2–10.9 µm ($\overline{x}$ = 4.3 µm, n = 30), 1.0–2.2 µm ($\overline{x}$ = 1.6 µm, n = 30) wide at the base. **Conidia** hyaline to green, globose to subglobose, oval, ellipsoidal, smooth, 1.5–4.2 × 1.3–3.3 µm ($\overline{x}$ = 3.4 × 2.6 µm, n = 30), l/w 1.0–1.6 ($\overline{x}$ = 1.3 µm, n = 30). **Chlamydospores** terminal or intercalary, globose to ellipsoidal, 3.8–11.6 × 3.3–9.3 µm ($\overline{x}$ = 7.5 × 6.3 µm, n = 30), l/w 0.8–1.6 ($\overline{x}$ = 1.2 µm, n = 30). No distinct odour; no diffusing pigment was observed.

On SNA after 72 h, 35–39 mm, the mycelium covers the plate after 4 days at 25 °C. Colony nearly transparent, flat, aerial hyphae indistinct or not common, margin slightly wavy and filamentous. **Conidiophores** trichoderma- to verticillium-like, formed on aerial hyphae. **Phialides** solitary or commonly divergent in whorls of 2–3, lageniform, subulate to spatulate, straight to curved, 4.4–18.6 × 1.7–3.3 µm ($\overline{x}$ = 9.9 × 2.4 µm, n = 30), l/w 1.5–10.9 µm ($\overline{x}$ = 4.3 µm, n = 30), 0.75–2.1 µm ($\overline{x}$ = 1.3 µm, n = 30) wide at the base. **Conidia** solitary or in groups, hyaline, globose to subglobose, oval to ellipsoidal, smooth, 2.6–4.6 × 2.1–3.3 µm ($\overline{x}$ = 3.7 × 2.8 µm, n = 30), l/w 1.0–1.7 ($\overline{x}$ = 1.3 µm, n = 30). **Chlamydospores** not observed. No distinct odour; no diffusing pigment was observed.

A comparison of the present taxon with allied taxa showed that *Trichoderma indica* NFCCI 5214 is morphologically distinct compared to other species of *Trichoderma* (Table 2). Phialides of *T. indica* NFCCI 5214 [4.4–18.6

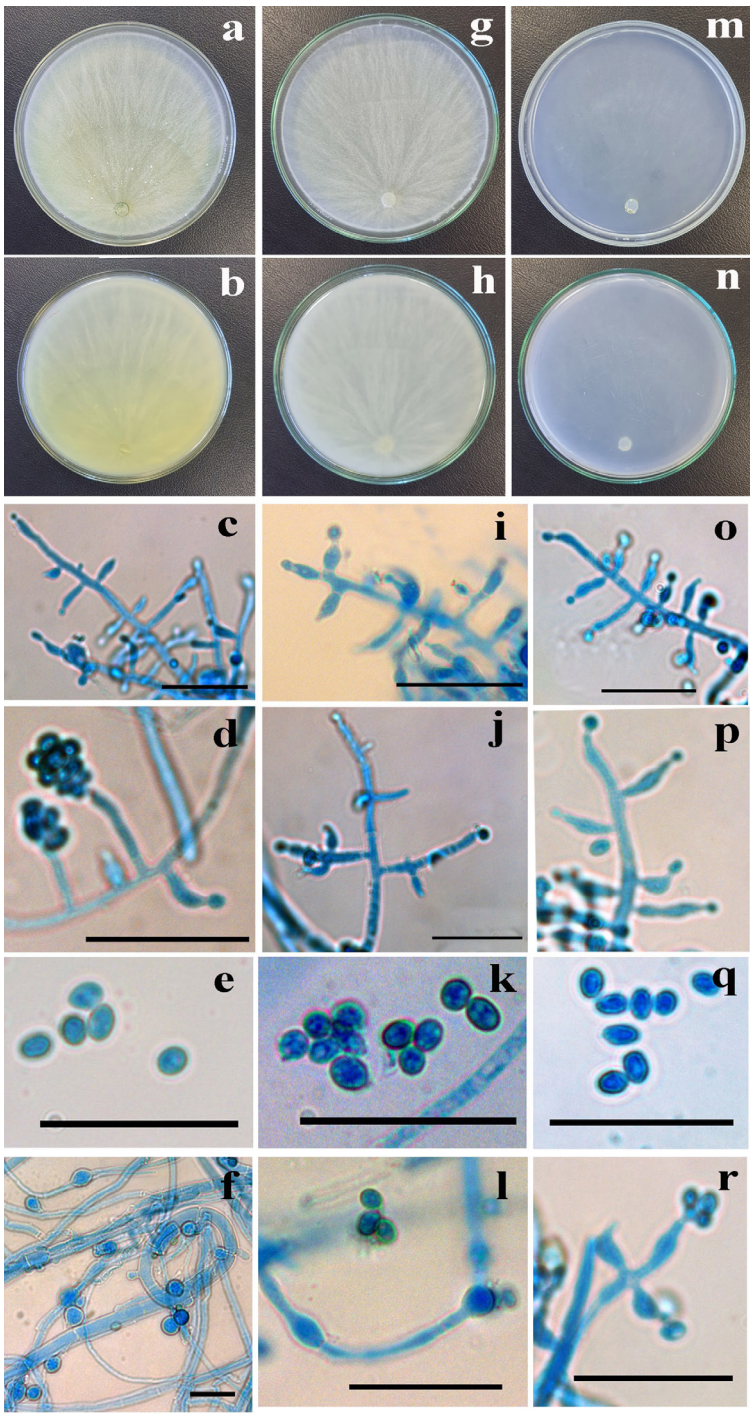

Figure 1. *Trichoderma indica* NFCCI 5214 on different media: (a,b) culture on PDA after 7 days at 25 °C (front and reverse), (c) conidiophores on PDA, (d) phialide-bearing group of conidia on PDA, (e) conidia on PDA, (f) chlamydospores on PDA, (g,h) culture on CMD after 7 days at 25 °C (front and reverse), (i,j) conidiophores with phialides on CMD, (k) conidia on CMD, (l) chlamydospores on CMD, (m,n) culture on SNA after 7 days at 25 °C (front and reverse), (o,p) conidiophores with phialide-bearing conidia on SNA, and (q) conidia on SNA, (r) phialide-bearing group of conidia on SNA. Scale bar: (c–f) = 20 µm, (i–l) = 20 µm, and (o–r) = 20 µm.

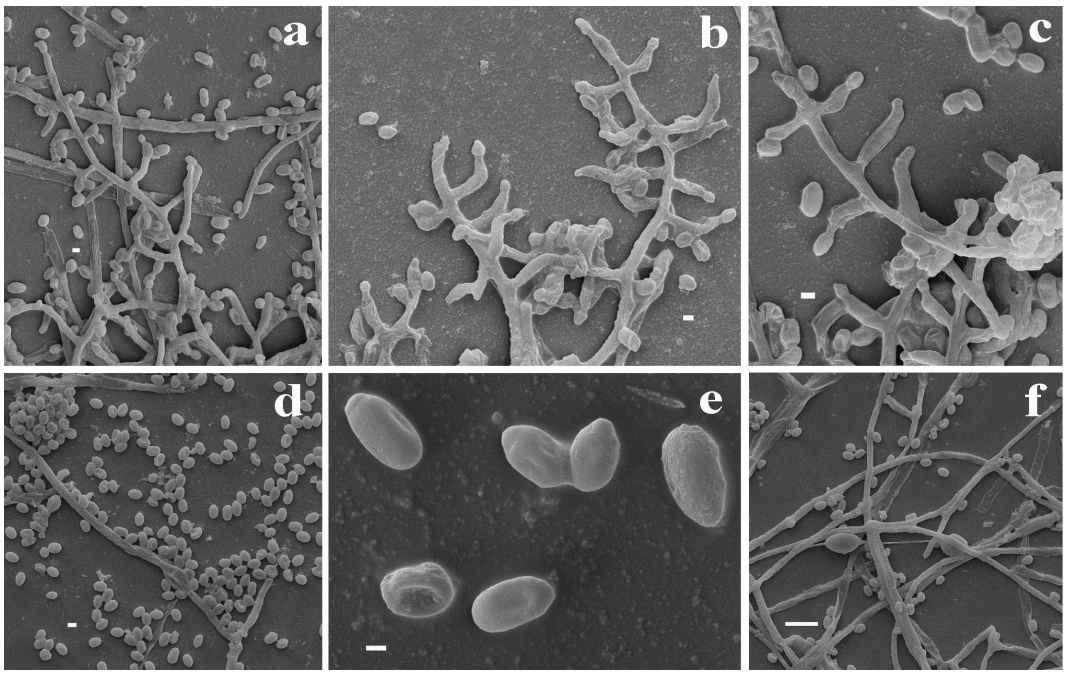

Figure 2. SEM images of *Trichoderma indica* NFCCI 5214: (a–c) conidiophores with phialide-bearing conidia, (d,e) conidia on PDA, and (f) chlamydospores on PDA. Scale bar: (a–d) = 2 µm, (e) = 1 µm, and (f) = 10 µm.

× 1.7–3.3 µm ($\overline{x}$ = 9.9 × 2.4 µm)] are longer compared to *T. longibrachiatum* [2.8–3.2 × 2.5–2.8 ($\overline{x}$ = 2.97 × 2.69) µm], *T. bissettii* [(6.0)7.0–10.4(13.0) × (2.0)2.1–2.9(3.5) um], *T. awajun* [3.1–10.0 × 6.4–9.6 µm], *T. xanthum* [5.0–11.1(15.3) × 2.8–3.9 µm], *T. pinnatum* [(4.2)5.5–9.0 (12.0) × (2.0)2.5–3.5(4.2) µm], and *T. aethiopicum* [(3.0)5.7–9.5(12.7) × (1.7)2.2–2.7(3.2) µm]. However, the width of the phialides is similar to that of the other closely related taxa except *T. awajun*. The width of the phialide in the present taxon is observed to be shorter compared to those of *T. awajun* (3.1–10.0 × 6.4–9.6 µm). Conidia of *T. indica* show variations in their shape from globose to subglobose and oval to ellipsoidal. In addition, the phialospore dimensions of *T. indica* NFCCI 5214 [2.6–4.6 × 2.1–3.3 µm ($\overline{x}$ = 3.7 × 2.8 µm)] are noticeably shorter compared to *T. bissettii* [(3.5)3.9–5.1(6.0) × (2.0)2.3–2.9(4.0) um], *T. longibrachiatum* (3.6–6.5 × 2.2–3.0 um), *T. xanthum* [(3.6)4.4–5.6 × 2.5–3.6 µm], and *T. pinnatum* [(2.2)2.5–3.5(5.0) × (1.7)2.5–3.0(3.5) µm]. Similarly, the conidia of *T. indica* (length/width ratio, $\overline{x}$ = 1.3) are found to be less slender than the conidia of other closely related species. These distinct morphological variations from other closely related species confirm the novelty of the present taxon.

Additional specimen examined: INDIA. MAHARASHTRA: Pune, Bhamburda, Vetal Hill, saprophyte from the soil, 1 Oct 2020, A.C. Lagashetti and S.K. Singh (NFCCI 5549, living culture).

3.2. Phylogenetic analysis

The MegaBLAST search showed that the *tef1-α* sequence of the present taxon shows 98% identity with *Trichoderma longibrachiatum* Tloum3 (1211/1237), *T. longibrachiatum* S328 (1170/1191), *T. awajun* CP 24-7 (1175/1200), and *T. awajun* CP 24-8 (1174/1199). Similarly, the *rpb2* sequence of the present taxon showed 98% identity with *T. longibrachiatum* S328 (1065/1084), *T. longibrachiatum* HL167 (1141/1165), and *T. longibrachiatum* ATCC 18648 (1004/1023), 99% identity (1003/1017) with *T. orientale* GJS 09-784, and 98% identity (996/1017) with *T. orientale* GJS 10-230.

The length of the final combined *tef1-α* and *rpb2* aligned sequence dataset of 51 taxa is found to be 996 base pairs, which include 557 conserved sites, 419 variable sites, 343 parsimony informative sites, and 69 singleton sites. The estimated base frequencies (as percentages) for the concatenated data set are as follows: A = 23.2, C = 27.8, G = 26.3, and T = 22.8.

The resulting phylogram clearly shows that the *T. indica* NFCCI 5214 & *T. indica* NFCCI 5549 form a separate clade (99.6% SH/100% UF) and a distinct monophyletic branch under the *Longibrachiatum* clade with species of *T. longibrachiatum*, *T. awajun*, *T. bissettii*, *T. pinnatum*, *T. xanthum*, and *T. aethiopicum* (92.4% SH/98% UF) (Figure 3). This confirms the genetic resemblance or closeness of *T. indica* NFCCI 5214 & *T. indica* NFCCI 5549 with the species of *T. longibrachiatum*, *T. awajun*, *T. bissettii*, *T. pinnatum*, *T. xanthum*, and *T. aethiopicum* in the *Longibrachiatum* clade.

Table 2. Morphological comparison of *T. indica* NFCCI 5214 with closely related species.

Sr. No.	Species	Media	Phialides				Conidia			Chlamydospores		
			Shape	Length & width	l/w	base	Shape	Length & width	l/w	Shape	Length & width	l/w
1	***T. indica* NFCCI 5214**	SNA	Solitary or commonly divergent in whorls of 2–3, lageniform, subulate to spatulate, straight to curved.	4.4–18.6 × 1.7–3.3 µm ($\overline{x}$ = 9.9 × 2.4 µm).	1.5–10.9 µm ($\overline{x}$ = 4.3 µm).	0.75–2.1 µm ($\overline{x}$ = 1.3 µm).	Solitary or in groups, hyaline, globose to subglobose, oval to ellipsoidal, smooth.	2.6–4.6 × 2.1–3.3 µm. ($\overline{x}$ = 3.7 × 2.8 µm).	1.0–1.7 µm. ($\overline{x}$ = 1.3 µm).	Not observed.		
		PDA	Solitary or rarely in whorls of 2, lageniform, subulate to spatulate, straight to. curved.	4.0–18.8 × 1.3–2.9 µm. ($\overline{x}$ = 8.6 × 1.9 µm).	1.8–11.7 µm. ($\overline{x}$ = 4.7 µm).	1.0–1.9 µm. ($\overline{x}$ = 1.4 µm).	Solitary or in groups, hyaline to green, globose to subglobose, oval to ellipsoidal, smooth.	2.6–5.0 × 1.9–3.1 µm. ($\overline{x}$ = 3.5 × 2.6 µm).	1.0–1.7 µm. s($\overline{x}$ = 1.3 µm).	Terminal or intercalary, globose to subglobose, oval to ellipsoidal.	5.8–10.8 × 4.8–9.0 µm ($\overline{x}$ = 8.0 × 6.5 µm).	1.0–1.9 µm. ($\overline{x}$ = 1.2 µm).
		CMD	Solitary or commonly divergent in whorls of 2–3, lageniform, curved.	4.5–20 × 1.4–3 µm. ($\overline{x}$ = 8.9 × 2.2 µm.	2.2–10.9 µm. ($\overline{x}$ = 4.3 µm).	1.0–2.2 µm. ($\overline{x}$ = 1.6 µm).	Hyaline to green, globose to subglobose, oval, ellipsoidal, smooth.	1.5–4.2 × 1.3–3.3 µm. ($\overline{x}$ = 3.4 × 2.6 µm).	1.0–1.6 µm. ($\overline{x}$ = 1.3 µm).	Terminal or intercalary, globose to ellipsoidal.	3.8–11.6 × 3.3–9.3 µm ($\overline{x}$ = 7.5 × 6.3 µm).	0.8–1.6 µm ($\overline{x}$ = 1.2 µm).
2	*T. bissettii*	–	Singly and laterally on the main axis or from side branches, or divergent in small whorls of 2–3, cylindrical to lageniform, often curved or flexuous, hyaline, smooth-walled.	(6.0)7.0–10.4(13.0) × (2.0)2.1–2.9(3.5) µm.	(2.0)2.6–4.6(6.5) µm.	(1.4)1.5–1.8(2.0) µm.	Broadly ellipsoidal to nearly oblong, smooth and thin-walled.	(3.5)3.9–5.1(6.0) × (2.0)2.3–2.9(4.0) µm.	(1.3)1.5–2.1(3.0) µm.	Terminal and intercalary, subglobose or ellipsoidal, smooth and thick-walled.	(6.5)6.8–8.4(9.5) × (5.5)6.6–8.2(8.5) µm.	–

Table 2. (Continued).

3	*T. longibrachiatum*	MEA	Subglobose or short obovoid, often with a broad truncate base, perfectly smooth-walled.	2.8–3.2 × 2.5–2.8 ($\overline{x}$ = 2.97 × 2.69) µm.	–	–	Elliptical to obovoid to ellipsoidal.	3.6–6.5× 2.2–3 um.	–	Terminal and intercalary, globose.	10 µm diameter.	–
4	*T. awajun*	PDA	Commonly in whorls of 2–5, inequilateral, often slightly curved, distinctly enlarged, plump with a mostly short neck and base.	3.1–10.0 × 6.4–9.6 µm.	–	–	Subglobose to globose, rarely oblong, smooth to faintly verruculose.	1.5–3.0 × 1.3–2.6 µm.	–	Terminal and intercalary, globose to subglobose.	6.2–10.4 × 7.3–11.2 µm.	–
5	*T. xanthum*	SNA	Straight or curved, ampulliform to lageniform	5.0–11.1(15.3) × 2.8–3.9 µm.	1.5–4.6 µm.	1.4–2.8 µm.	Ellipsoidal, smooth.	(3.6)4.4–5.6 × 2.5–3.6 µm.	1.4–1.8 µm.	Terminal or intercalary, globose, subglobose or ellipsoidal.	6.2–10.3(14.5) × 4.8–7.2 µm.	1.0–1.5(2.1) µm.
6	*T. pinnatum*	SNA	Solitary and intercalary, lageniform, straight, sinuous, or hooked.	(4.2)5.5–9.0 (12.0) × (2.0)2.5–3.5(4.2) µm.	(1.3)1.5–3.5(5.0) µm.	(1.2–)1.5–2.2(–2.7) µm.	Ellipsoidal.	(2.2)2.5–3.5(5.0) × (1.7)2.5–3.0(3.5) µm.	(1.2)1.3–1.7(1.0) µm.	Not observed.		
7	*T. aethiopicum*	SNA	Cylindrical to lageniform.	(3.0)5.7–9.5(12.7) × (1.7)2.2–2.7(3.2) µm.	(1.2)2.2–4.2(6.2) µm.	(1.0–)1.5–2.0(–2.5) µm.	Ellipsoidal to nearly oblong.	(2.5)3.0–4.0(4.5) × (1.7)2.0–2.5(3.0) µm.	(1.0–)1.2–1.7 (–2.3) µm.	Subglobose, terminal and intercalary.	5–10 µm diameter.	–

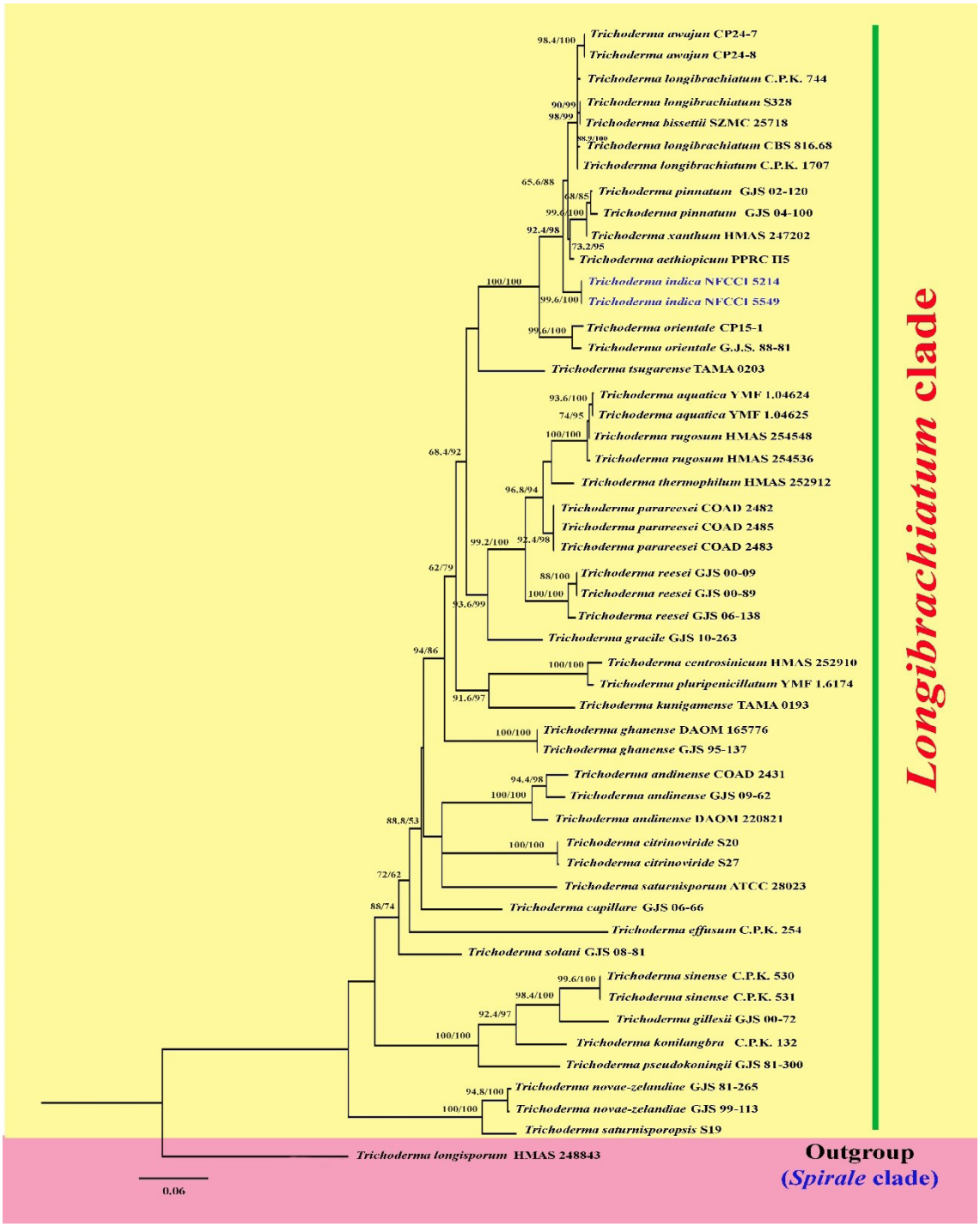

Figure 3. A phylogram of *Trichoderma indica* NFCCI 5214 using the maximum likelihood method based on the combined sequences data of *tef-1α* and *rpb2*. Support values SH-aLRT (≥ 50%), UFBoot (≥ 50%) are indicated at the nodes. The newly proposed species are indicated in blue.

4. Discussion

Differentiation of species of the genus *Trichoderma* based on morphology alone is insufficient due to its structural simplicity and the absence of morphological variations among the species of *Trichoderma*. Uncertainty in the taxonomy of *Trichoderma* can be resolved by molecular analysis in addition to morphological characteristics. Sequences of *rpb2* and *tef1-α* are currently used to resolve the taxonomy of the genus *Trichoderma* due to their interspecific variations (Gu et al., 2020).

The present study proposes the novel species *T. indica* based on both morphological variations and phylogenetic analysis. The detailed morphotaxonomic study revealed that *T. indica* is morphologically distinct as compared to *T. longibrachiatum*, *T. awajun*, *T. bissettii*, *T. pinnatum*, *T. xanthum*, and *T. aethiopicum* (Table 2). Distinctive morphological variations were observed in the dimensions of the phialides and conidia of the *T. indica* compared to other related taxa. Especially, the length of the phialides of *T. indica* is observed to be longer compared to *T. longibrachiatum*, *T. bissettii*, *T. pinnatum*, *T. xanthum*, and *T. aethiopicum*. However, the width of phialides is quite similar to that of the other related taxa except for *T. awajun*. Similarly, the conidia of *T.* indica are observed to be shorter compared to other closely related species of *Trichoderma*. All these morphological variations demonstrate the uniqueness and novelty of the present taxon.

The novelty of the present taxon was also confirmed by molecular analysis and multigene phylogeny. The MegaBLAST analysis of the *tef-1α* and *rpb2* sequences reveals the similarity of the present taxon with *T. longibrachiatum*, *T. awajun*, and *T. orientale*, representing the genetic relatedness of the present taxon with the species of the *Longibrachiatum* clade. A multilocus phylogenetic tree generated from the combined sequence data of *tef-1α* and *rpb2* shows that *T. indica* forms a sister branch to *T. longibrachiatum*, *T. awajun*, *T. bissettii*, *T. pinnatum*, *T. xanthum*, and *T. aethiopicum*. This confirms the position of the present taxon in the *Longibrachiatum* clade and its phylogenetic relationship with the other species of the *Longibrachiatum* clade. In addition, having a distinct separate branch from the other closely related species supported by strong SH (99.6) and UF (100) values reveals that *T. indica* is genetically different from the other species and confirms the identity of the present taxon as a novel species of the genus *Trichoderma* in the *Longibrachiatum* clade (Figure 3).

Therefore, based on comprehensive morphology and multigene phylogeny, the present taxon is confirmed as novel as *T. indica.*

Acknowledgment

A.C. Lagashetti and colleagues thank the director of the MACS-Agharkar Research Institute, Pune, for providing the necessary facilities. A.C. Lagashetti thanks CSIR, New Delhi, for a Senior Research Fellowship and S. P. Pune University for granting permission to register for a Ph.D. degree.

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