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Research Article

Phenotypic and molecular diagnosis of *Trichoderma* spp. isolated from the local soil of Nineveh Governorate, Iraq

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Abstract
Trichoderma spp. is a common soil fungus that penetrates plant roots and engages with various microorganisms and plants. The present study aimed to determine the phylogenetic relationships of rhizosphere isolates of *Trichoderma* spp. obtained from the local soil in Mosul in the north of Iraq utilizing the sequence of the internal transcribed spacer-1 (ITS-1) region of the ribosomal DNA of eight *Trichoderma* spp.. Approximately 192 samples were collected between March and October 2023 from the rhizospheric soil of Potato)*Solanum tuberosum*), Tomato (*Solanum lycopersicum*), Wheat (*Triticum aestivum*), Barley (*Hordeum vulgare*), Eucalyptus (*Eucalyptus globulus*) and Citrus (*Citrus limon* (.Only 5.8% of the rhizosphere samples belonged to *Trichoderma* spp. The samples were identified based on morphological characteristics and molecular techniques. All the selected isolates amplified a similar band (660 bp). The nucleotide sequences were then analyzed, and a phylogenetic tree was constructed to compare the present isolates with the closest isolates submitted to the National Center for Biotechndogy Information (NCBI). Isolates were submitted to NCBI as new strains under the accession numbers: *Trichoderma harzianum* (PP968131), *T. longibrachiatum* (PP977534), *T. harzianum* (PP977576), *T. longibrachiatum* (PP989798), *Trichoderma longibrachiatum* (PP989800), *T. longibrachiatum* (PP989803), *T. longibrachiatum* (PP989804), and *T. harzianum* (PP989805). This study expands the NCBI database with novel *Trichoderma* strains, enhancing fungal genetic resources globally. These new entries provide unique genomic data, potentially revealing valuable traits, resolving taxonomic ambiguities within *Trichoderma*, and improving the reliability of the NCBI database for future studies.

Keywords: Transcribed spacer-1, *Trichoderma longibrachiatum, Trichoderma harzianum*, ITS1 and ITS4

INTRODUCTION

Trichoderma spp. are widely distributed soil fungi, recognized as key components of many microbial communities due to their ability to colonize plant roots and interact with other microorganisms and host plants (Alfiky and Weisskopf, 2021; Kubiak *et al*., 2023). These fungi are ubiquitous across diverse soil types and are frequently regarded as plant symbionts. Studies by Tang *et al*. (2022) and Tyśkiewicz *et al*. (2022) have documented the presence of *Trichoderma* spp. in various plant parts, including leaves, decaying wood, bark, endophytic tissues, and soil. *Trichoderma* spp. contribute to plant disease suppression by producing secondary metabolites and competing for space and nutrients, showcasing their remarkable symbiotic and competitive capabilities (Allaga *et al*., 2021; Kredics *et al*., 2021). These traits enable them to thrive in diverse soil environments and support host plants. Given their role as effective biocontrol agents, extensive research has focused on the biology of *Trichoderma* spp., emphasizing the importance of accurate species identification for their application in managing plant diseases (Kubicek *et al*., 2019; TariqJaveed *et al*., 2021).

The history of *Trichoderma* taxonomy demonstrates that defining species at the genus and species levels is extremely challenging, relying solely on morphological definitions. *Trichoderma* spp. can be identified by traditional methods that use the fungus's large and small features. Examining the fungus under a light microscope reveals how the colonies develop in the growth medium, including their colours, the number of spores they produce, the conidiophores and phialides, and the shape and size of the conidia (Susila *et al*., 2023). To

overcome this difficulty, scientists have developed molecular identification techniques. Previously, classification was based solely on morphological traits; however, progress in science over the last 100 years, including the development of accurate tools such as the polymerase chain reaction (PCR), the scanning electron microscope (SEM), and the transmission electron microscope (TEM), has significantly enhanced classification accuracy. As taxonomy evolved, it ceased relying on simple morphological traits and began using more accurate and multiple diagnostic factors, such as molecular diagnosis (Stace, 1989). Molecular information also changed many previous classification concepts, especially morphological ones (Turner, 1998). Kindermann *et al*. (1998) conducted the first evolutionary analysis of the entire genus *Trichoderma* using the ITS-1 region of rDNA. For the accurate identification of Trichoderma spp., Druzhinina et al. (2006) recommended using molecular techniques such as DNA sequencing, genealogical concordance, and phylogenetic species recognition with multiple genes that are not linked.

These days, most people agree that molecular methods, such as the ITS rDNA region, can be used to create fingerprints and examine the sequences of multiple genes. The use of ITS regions (ITS1 or ITS2) revealed that closely related *Trichoderma* spp. have the same ITS phylotypes (Hillis *et al*., 1991; White *et al*., 1991; Yurkov *et al*., 2020). The spacer regions and genes, either alone or together, act as molecular markers to determine phylogenetic relationships (Wheeler *et al*., 1993). Determining phylogenetic relationships among higher taxonomic groups typically involves using 28S rDNA and 18S rDNA, which are characterized by their length-conservative regions. Throughout evolution, closely related nucleotide sequences indicate phylogenetic links. Technological advancements have rendered nucleotide sequencing comparatively straightforward. When examining variation at the species level, researchers often utilise areas with different lengths (Prahl et al., 2021; Harnelly et al., 2022), such as internal transcribed spacers (ITS1 and ITS2). Researchers such as Marcilla *et al*. (2001), Damgaard et al. (2005), and Calleros et al. (2010) frequently use these regions. The ITS1 and ITS2 regions are widely used in the molecular diagnosis of fungi due to their high variability, which enables accurate species identification and phylogenetic analysis. ITS1 sequences were analyzed in this study to determine the phylogenetic relationships of *Trichoderma* spp. Isolates, consistent with numerous studies employing ITS1 and ITS2 for fungal taxonomy and diversity assessments (Cai and Druzhinina, 2021). These regions facilitate the identification of closely related species, such as *T. harzianum* and *T. longibrachiatum*, enhancing the reliability of National Center for Biotechnology Information (NCBI) deposits for future research.

This study aimed to expand *Trichoderma* spp. knowledge by registering novel, unrecorded local strains (e.g., *T. harzianum* PP968131, *T. longibrachiatum* PP977534) isolated from the rhizospheric soil of Mosul, Iraq, in the NCBI database, using ITS-1 sequences for phylogenetic analysis. *Trichoderma's* utility as an effective biocontrol agent against phytopathogenic fungi, as well as an enzyme producer such as cellulases, and as a biofertilizer, supports sustainable agriculture and bioremediation. The novelty lies in contributing unique ITS-1 sequences to NCBI, while accurate molecular characterization addresses taxonomic challenges and enhances the database's reliability for future phylogenetic research

MATERIALS AND METHODS

Collection of samples

Samples were collected from various areas of Mosul city and its suburbs; isolates were isolated from the soil rhizosphere of Potato)*Solanum tuberosum*), Tomato (*Solanum lycopersicum*), Wheat (*Triticum aestivum*), Barley (*Hordeum vulgare*), Eucalyptus (*Eucalyptus globulus*) and Citrus (*Citrus limon* ()Fig. 1). Soil was obtained from a depth of 15 cm (Hastuti *et al*., 2016; Rashmi *et al*., 2020), with each sample representing 500 g (Kale *et al*., 2018). The samples were organized, prepared, and stored in labelled plastic bags indicating the plant name, collection location, and date. Then, they were transported to the College of Science laboratory at the University of Mosul, where they were dried and sifted to remove impurities.

Isolation and identification

Soil samples underwent serial dilutions to isolate and purify *Trichoderma* spp. using the serial dilution technique (Bensons, 2005; Almashhadani and Qassim, 2025). Diluted samples were then spread on potato dextrose agar (PDA) and incubated at 28°C for 7 days. The fungal samples were preserved by culturing on slants at 4°C for future use (Xue *et al*., 2021; Alwadai *et al*., 2024; Qassim *et al*., 2024).

Colonies were primarily identified using a combination of morphological and microscopic characteristics. These characteristics included colony shape, diameter, color, background, and the presence and arrangement of fungal phialides. The identification process was carried out in accordance with the *Trichoderma* spp. Identification key (Ranga *et al*., 2017; Prameeladevi *et al*., 2018 ; Haque *et al*., 2020).

Determination of percentage frequency of occurrence

To calculate the frequency of occurrence for various fungal isolates, the formula used elsewhere was followed (Al-Ameri, 2022; Al-healy and Al-Taee, 2023).

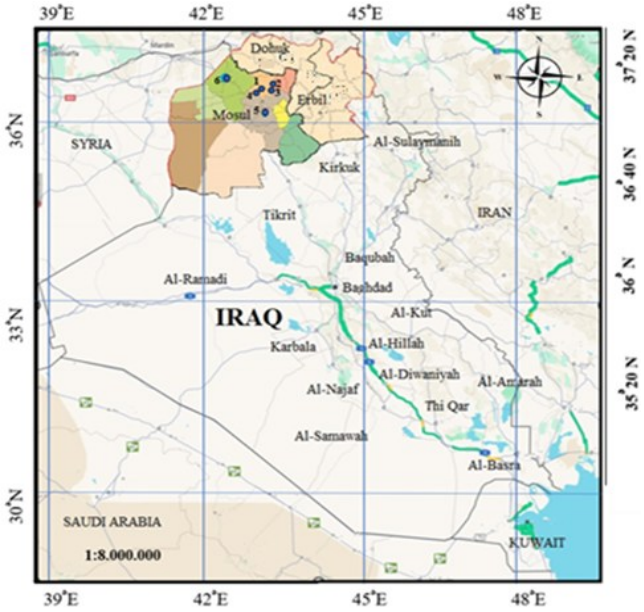


Fig. 1. *Map showing sample collection areas*

The frequency of fungal colonies in the samples, as a percentage, is

% Frequency =The number of fungus colonies in the sample s/ Total number of colonies .....Eq. 1

Molecular identification using Polymerase Chain Reactions (PCR)

DNA extraction from the mold isolates

Eight isolates of fungi previously identified morphologically were selected. DNA was meticulously extracted from eight mycelia, utilizing the Geneaid Genomic DNA Mini Kit (Taiwan) and following the detailed instructions provided by the Geneaid company (Taiwan).

PCR amplification

Universal primers were purchased from Macrogen, Korea, and used to amplify the ITS region. The primers used were ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). The polymerase chain reaction (PCR) was conducted in 20 µL reaction volume. The reaction contained 10 µL of 1X GoTaq G2 Green Master Mix (Promega), 2 µL of each primer (1 µL), 1 µL of DNA template, and 5 µL of nuclease-free water. The PCR protocol consisted of one cycle at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, an additional cycle at 72°C. The PCR products were analyzed on a 2% agarose gel stained with Redsafe dye, and the amplicon sizes were determined using a Biolaps 100 bp DNA ladder.

DNA sequencing of amplified fragments

PCR bands were excised from the gel and purified using a Geneaid kit. The concentration of the extracted DNA was measured precisely using a Nanodrop device, a sensitive instrument for quantifying nucleic acids, at the Laboratory of the College of Science, University of Mosul. Nucleotide sequencing of the amplified DNA fragments was performed using Sanger sequencing at Psomagene company (Maryland, USA).

Phylogenetic tree

The Nucleotide Basic Local Alignment Search Tool (BLASTn) program (Altschul *et al.,* 1990) was used to search for homology between the input sequences and the entire sequences available in the NCBI GenBank database. The MEGA-11 software (Tamura *et al*., 2021) conducted a bootstrap (100X) analysis to construct the phylogenetic tree.

RESULTS AND DISCUSSION

The distribution of fungal genera in this study is significantly influenced by environmental factors, with a strong correlation to variations in temperature, relative humidity, and soil pH (Aghajani *et al*., 2018; Mohammed and Al-Taie, 2024; Qassim *et al*., 2024). Phenotypic characterization was initially conducted on fungal isolates collected from diverse regions of Mosul to identify the genera present. The isolates were systematically classified following preliminary identification, and their relative abundance across sampled sites was quantified. Subsequently, isolates of *Trichoderma* spp. were selectively isolated and subjected to detailed molecular analysis to identify the specific species, which constituted the primary focus of this investigation. The results revealed that *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. were the predominant genera, representing 46.8%, 21.3%, and 14% of the isolates, respectively (Allawi and Hmoshi, 2022). Notably, *Trichoderma* spp. was detected at a frequency of 5.8%, and unlike other fungal genera, it was consistently present across all samples from the diverse crops examined, highlighting its widespread distribution and strong association with agricultural ecosystems. This ecological prominence, likely attributable to its adaptability to prevailing environmental conditions, underscores the need for further research into its role in enhancing crop health and development.

Analysis of Table 1 reveals that while certain fungal genera exhibited regional variability, appearing in some areas and absent in others, *Trichoderma* spp. were consistently detected across all sampled locations, affirming their extensive environmental distribution. Belonging to the Hypocreaceae family, *Trichoderma* spp. demonstrates remarkable diversity and adaptability, thriving in various niches such as soils, plant roots, stems, and leaves (Pokhrel *et al*., 2022; Li *et al*., 2025). The highest prevalence of *Trichoderma* spp. was observed in the rhizosphere of tomato crops, with a detection rate of 10%, followed by citrus crops at 8%, barley at 6.45%, wheat at 6%, potatoes at 3.84%, and

eucalyptus at 2.5%. These variations in prevalence across crop rhizospheres suggest that host-specific factors, such as root exudates, pH, and soil microenvironmental conditions, play a pivotal role in shaping the distribution of *Trichoderma* spp.

The elevated occurrence of *Trichoderma* spp. in the rhizospheres of tomato and citrus plants is primarily driven by the abundance of organic matter and root exudates, which provide an optimal nutritional environment for fungal proliferation (Matei and Matei, 2024). Additionally, the favourable soil conditions in these regions, including moderate moisture levels, a slightly acidic to neutral pH (4.5–7), and temperatures ranging from 20 °C to 30°C, significantly enhance the growth and persistence of *Trichoderma* spp. (Shao *et al*., 2025). The consistent detection of *Trichoderma* spp. across all sampled areas further emphasizes its ecological resilience and adaptability to fluctuating environmental conditions, reinforcing its significance in agricultural systems (Li *et al*., 2025). These findings underscore the importance of ongoing research into the ecological and functional roles of Trichoderma spp., particularly its potential to facilitate sustainable crop production through its interactions with the soil microbiome and plant hosts (Eman *et al*., 2023).

Molecular identification of *Trichoderma* spp. Isolates.

Molecular characterization of *Trichoderma* spp. isolates obtained from the rhizospheric soil of various crops, as shown in Table 1, was conducted to ensure precise identification. Amplification of the internal transcribed spacer (ITS) region using universal primers generated a 660 bp PCR product, aligning with the expected size for *Trichoderma* spp. (Fig. 2). These findings corroborate prior research by Shahid *et al*. (2014) and Al-Rubaiey and Al-Juboory (2020), confirming the reliability and efficacy of the ITS region for the accurate molecular identification of *Trichoderma* species.

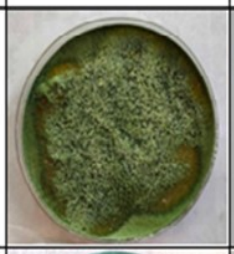
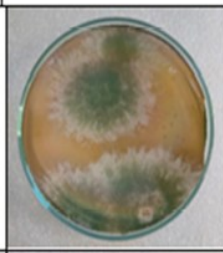
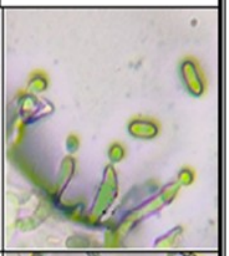
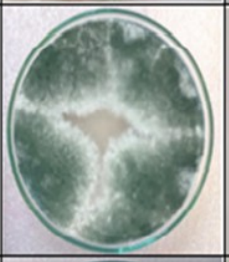
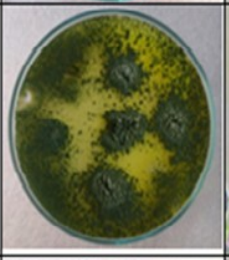
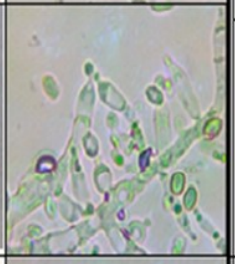
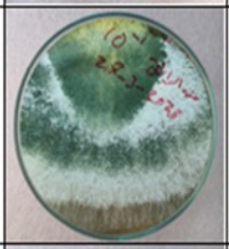
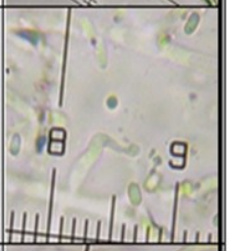
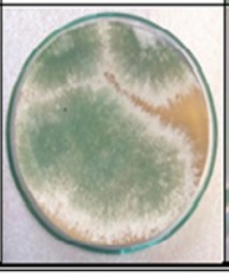
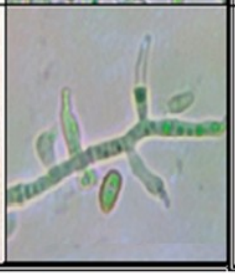
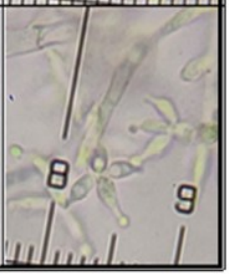
DNA sequencing

The findings demonstrated that the fungi exhibited the highest identity, aligning with the isolates identified in the Basic Local Alignment Search Tool (BLAST) database. The sequences were submitted, accompanied by

Table 1. Frequencies of fungal isolation from different areas of Mosul city

Soil rhizosphere of isolation		Source of isolation	Fungal species	No.	Frequency%
1	Mosul forest	Eucalyptus	*Aspergillus niger*	14	35%
			A. flavus	12	30%
			penicillium chrysogenum	9	22.5%
			Geotrichum candidum	4	10%
			Trichoderma sp.	1	2.5%
Sum of isolates				40	100%
2	House garden	Citrus	*A. niger*	12	48%
			P. chrysogenum.	8	32 %
			Alternaria sp.	3	12%
			Trichoderma sp..	2	8%
Sum of isolates				25	100%
3	Hamam Al-allel	Wheat	*A. niger*	16	32%
			Fusarium oxysporium	15	30%
			P. chrysogenum.	10	20%
			Mucor sp.	6	12%
			Trichoderma sp.	3	6%
		Sum of isolates		50	100%
4	Al-Gubba	Tomato	*A. niger*	9	45%
			F. solani.	6	30%
			Mucor sp.	3	15%
			Trichoderma sp.	2	10%
Sum of isolates				20	100%
5	Al-Rashidiya	Potato	*A. niger*	12	46.16%
			F. solani	9	34.62%
			Rhizopus sp.	4	15.38%
			Trichoderma sp.	1	3.84%
Sum of isolates				26	100%
6	Rabeea	Barley	*A. flavus*.	15	48.38%
			F. oxysporum	11	35.48%
			Mucor sp.	3	9.64%
			Trichoderma sp..	2	6.45%
Sum of isolates				31	100%

Table 2. Local isolates of *Trichoderma* spp. strains under study

Sample s name	Colonies	Phialides	Samples name	Colonies	Phialides
TMW1			TMW5		
TMW2			TMW6		
TMW3			TMW7		
TMW4			TMW8		

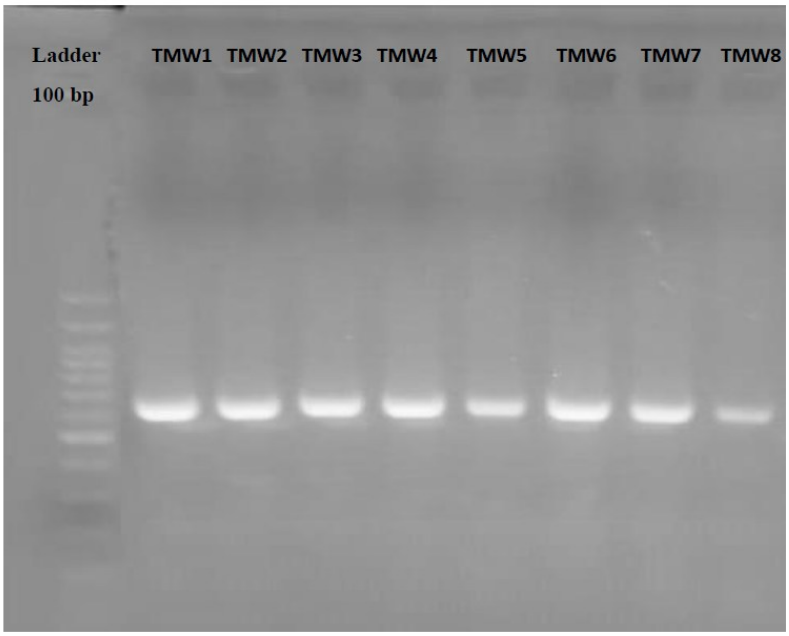


Fig. 2. *PCR amplicons for the ITS region of Trichoderma spp. studied for the ITS region and a reaction product of 660 bp, carried over by a 2% agarose gel*

Table 3. Local Iraqi strains of *Trichoderma* spp.

Name	Scientific name	Accession No.
TMW1	*Trichoderma harzianum* SF_847	PP968131.1
TMW2	*Trichoderma longibrachiatum* Voucher PDA N189-2	PP977534.1
TMW3	*Trichoderma harzianum* SF_847	PP977576.1
TMW4	*Trichoderma longibrachiatum* ACP-T116	PP989798.1
TMW5	*Trichoderma longibrachiatum* 18APLE013	PP989800.1
TMW6	*Trichoderma longibrachiatum* TF7	PP989803.1
TMW7	*Trichoderma longibrachiatum* LMBC166	PP989804.1
TMW8	*Trichoderma harzianum* SF_847	PP989805.1

Table 4. Most closely related strains of local *Trichoderma* spp. with *Trichoderma* spp. recorded in the National Center for Biotechnology Information (NCBI)

strains	Most Closely Strain	Accession No.	Similarity (%)
TMW1	*Trichoderma harzianum* strain ARCF479	(MZ452060.1)	98.4%
TMW2	*Trichoderma longibrachiatum* strain PDA N189-2	(MF102210.1)	96.8
TMW3	*Trichoderma harzianum* strain T7	(MG707199.1)	95.2%
TMW4	*Trichoderma longibrachiatum* strain ACP-TI16	(MH208263.1)	94.4%
TMW5	*Trichoderma longibrachiatum* strain TRS708	(KP009307.1)	99.3%
TMW6	*Trichoderma longibrachiatum* strain XZ N140-1	(MF102195.1)	98%
TMW7	*Trichoderma longibrachiatum* strain LMBC 166	(MN249170.1)	99.6%
TMW8	*Trichoderma harzianum* strain SF 847	(MT530123.1)	97%

the strain name and accession number listed in Table 3.

Phylogenetic tree analysis

Table 4 presents a detailed summary of the closest related strains, including their accession numbers and similarity percentages. This study examined Iraqi *Trichoderma* strains, specifically *T. harzianum* (TMW1, TMW3, TMW8) and *T. longibrachiatum* (TMW2, TMW4, TMW5, TMW6, TMW7), comparing these locally isolated strains with sequences in the NCBI GenBank database. A comprehensive analysis of the data elucidated the genetic similarities between the local isolates and their GenBank counterparts, underscoring the significance of these isolates in advancing *Trichoderma* research and establishing a foundation for future investigations (Carro-Huerga *et al*., 2023).

A neighbor-joining phylogenetic tree is shown in Fig. 3. It links the strains of *T. longibrachiatum* TMW2, TMW4, TMW5, TMW6, and TMW7 (shown in black circles) to *T. harzianum* TMW1, TMW3, and TMW8 (shown in blue circles) with the closely related strains based on ITS rRNA sequences using MEGA-11 software with a scale length of 0.01. The percentage of replicate trees where the associated strains clustered together in the bootstrap test (100 replicates) is shown next to the branches.

Conclusion

Morphological and molecular characterization of locally isolated *T. harzianum* and *T. longibrachiatum* strains, isolated from the rhizospheric soil of diverse crops in Mosul, Iraq, using PCR revealed a 660-bp band, confirming their affiliation with the genus *Trichoderma*. Comparing the sequences in the NCBI records revealed a genetic similarity of 94.4 to 99.6% with the isolates known worldwide. There are now eight new strains of *Trichoderma* with scientific names and accession numbers in the NCBI. They are named *Trichoderma harzianum* PP968131, *T. longibrachiatum* PP977534, *Trichoderma harzianum* PP977576, *T. longibrachiatum* PP989798, *Trichoderma longibrachiatum* PP989800, *T. longibrachiatum* PP989803, *T. longibrachiatum* PP989804, and *Trichoderma harzianum* PP989805. The novelty lies in contributing unique ITS-1 sequences to NCBI, while accurate molecular characterization addresses taxonomic challenges and enhances the database's reliability for future phylogenetic research.

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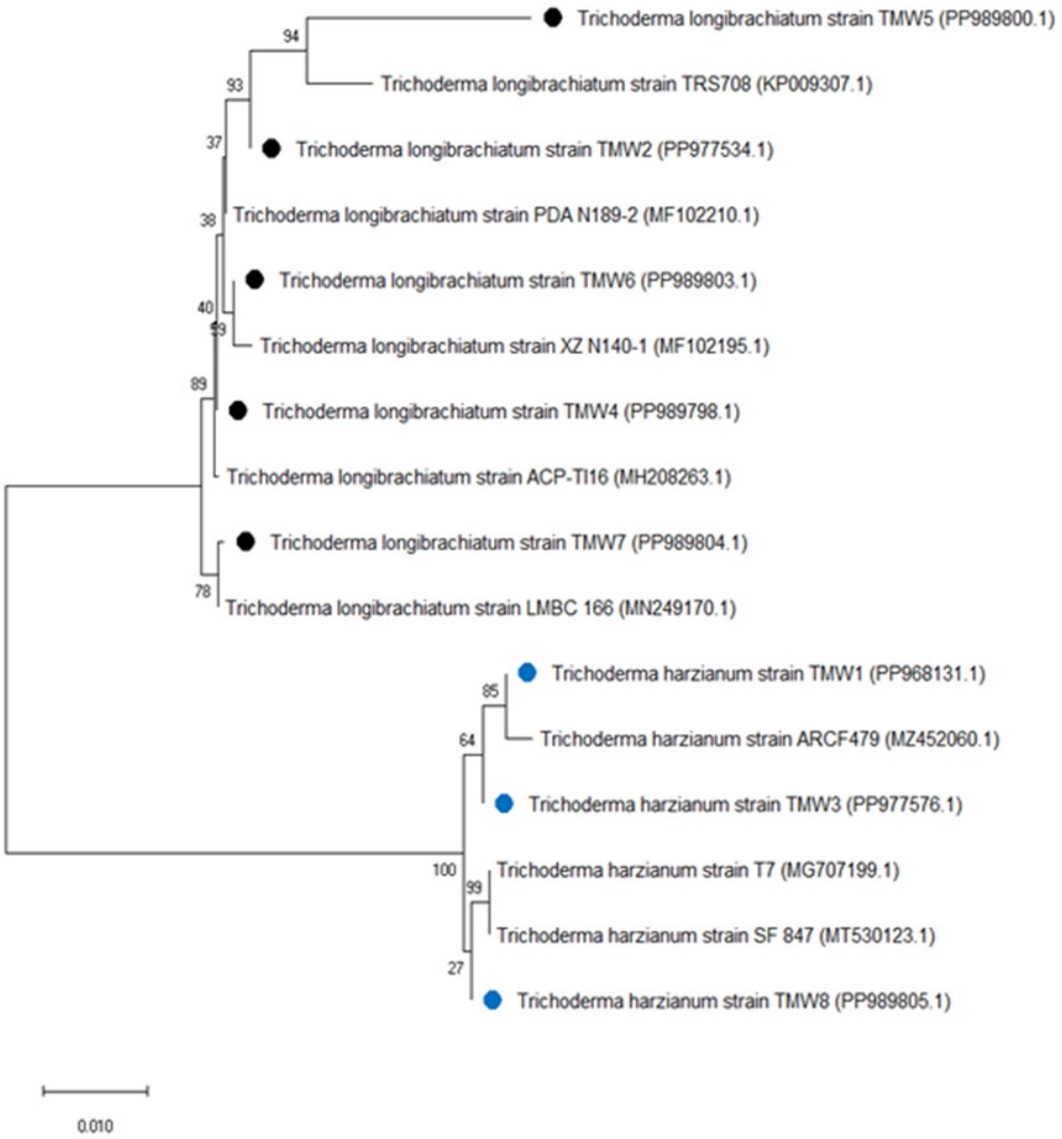

Fig. 3. *Phylogenetic relationship of Trichoderma spp.*

technical support.

Conflict of interest

The authors declare that they have no conflict of interest.

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