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Genetic Diversity of *Trichoderma* spp. Isolated from Soils in the Iraqi Environment

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ABSTRACT

Background: *Trichoderma* species are biocontrol fungi of great significance in sustainable agriculture soils worldwide. Despite their global importance, little is known about the genetic diversity of local strains, particularly those from extreme habitat Iraqi desert soils. The study utilizes precise molecular techniques (PCR/ ITS analysis), avoiding classical identification constraints, to explore the genetic diversity of Najaf and Karbala *Trichoderma* isolates. Through comparisons with worldwide records (National center for biotechnology information, NCBI), the research aims at understanding local biodiversity and determining their prospects for sustainable agricultural applications.

Methods: Fungal isolates from desert soils were molecular identified using PCR, amplifying the ITS genetic region with the ITS1-ITS4 primers. Resulting DNA sequences were compared to the NCBI database through BLAST to identify –species. Phylogenetic analysis was carried out using MEGA-X. Distinct isolates were identified and accession numbers were assigned. This molecular approach successfully enabled assessment of genetic diversity within *Trichoderma* spp. populations.

Result: Genetic analysis revealed two novel, previously unregistered isolates: *T. reesei* (4) and *Trichoderma harzianum* (5), which were then deposited in NCBI under the accession numbers PV112458 and PV114821, respectively. The other identified species, *Trichoderma viride* (3), *Trichoderma longibrachiatum* (1) and *Trichoderma harzianum* (2), were also recorded in NCBI, with the accession numbers PV113441, PV114839 and PV114819, respectively.

Key words: Fungal isolates, Genetic diversity, PCR sequencing, *Trichoderma* species.

INTRODUCTION

Fungi comprise a large diversity of eukaryotic life forms that have major environmental functions as decomposers and living symbiotically with plants (Hawksworth and Lücking, 2017). They are found in almost all environments, including soil, water and air, as well as on and within living and non-living surfaces. According to estimates, there are millions of fungal species on Earth but a small fraction of them have been formally named until now (Blackwell, 2011; Al-Abedy *et al.,* 2020).

Fungi are important to plant health, but can also be beneficial or harmful. Pathogenic fungi are among the main causes of significant of severe economic losses in agriculture, with widely reported damage to agricultural crops in general and food production and food safety in particular (Strange and Scott, 2005). In order to reduce the effect of fungal diseases, the cause requires accurate and rapid identification of the pathogen, which enables the selection of appropriate and effective control strategies, whether chemical, biological, or cultural (Agrios, 2005). Rapid diagnosis also facilitates the implementation of preventive measures to curb disease spreading in other areas or crops. In addition, it helps to maintain crop health and reduce the damage caused by fungal infections, increasing the dividend and improves the quality of the product (Kamaluddin *et al.,* 2021).

The morphological characteristics of fungi such as appearance of fungal colonies, the size and shape of spores and reproductive structures are used for traditional fungal identification (Leslie and Summerell, 2006; Al-Salami *et al.,* 2019; Odeh *et al.,* 2021; Mnati *et al.,* 2021). Morphological identification is a simple and low-cost method, requiring no advanced equipment or specialized materials but it comes with some restrictions. Most fungal species share morphological characteristics, making accurate morphological diagnosis highly dependent on extensive expertise and detailed knowledge of fungal taxonomy. Fungi features are also affected by environmental conditions like temperature, humidity and the growth medium, so identification can be difficult and less accurate. Additionally, morphological identification involves intensive laborious procedures, including the cultivation of fungi in the laboratory, continuous monitoring of growth and development and macroscopic and microscopic examination (Bhunjun *et al.,* 2021).

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In recent years, molecular diagnostic methods have quickly developed into fast and reliable alternatives to the morphological identification of fungi. DNA analysis forms the basis of these techniques, allowing for discriminatory and speedy identification at the fungal species level (Bahar *et al.,* 2020; Al-Abedy *et al.,* 2020). One of these techniques, polymerase chain reaction (PCR), is especially noteworthy because of its accuracy in differentiating between fungal species and strains. This type of analysis is based on genetic material, a unique genetic fingerprint for each species and strain (Bridge and Spooner, 2001).

Molecular diagnosis can be completed within a few hours, allowing for rapid action to control fungal diseases (Schena *et al.,* 2004; Renuka *et al.,* 2025). Additionally, molecular diagnostic techniques are not influenced by surrounding environmental conditions, making them more reliable than morphological diagnosis (Al-Sharmani *et al.,* 2019; Aljuaifari *et al.,* 2019; Dewan *et al.,* 2020).A recent study demonstrated that polymerase chain reaction (PCR) technology is highly effective in diagnosing various fungal pathogens, including *Fusarium oxysporum*, the causative agent of Fusarium wilt in tomatoes. This approach enabled precise and rapid identification of the pathogen, facilitating the implementation of effective measures to control disease spread (Kumar *et al.,* 2024). Another study illustrated how sequencing technology can be used to detect rare and/or unfamiliar fungal species that are challenging to identify by morphology methods (Irinyi *et al.,* 2023). Molecular diagnostic methods are being applied for the early detection of plant-pathogenic fungi and to implement preventive actions before disease symptoms appear (Mahmoudi *et al.,* 2025).

Given the importance of accurately identifying both pathogenic and non-pathogenic fungi, this study aims to characterize fungal isolates belonging to the genus *Trichoderma* collected from different soil types. Polymerase chain reaction (PCR) technology and nucleotide sequence analysis of the amplified DNA products will be utilized to determine the genetic similarities and differences between the isolates in this study and globally documented *Trichoderma* isolates.

MATERIALS AND METHODS

Molecular identification of fungal isolates

The genomic DNA of the fungal isolates was extracted using the Favorgen DNA extraction kit (Cat. No: FAPGK100), according to the manufacturer's procedures (Favorgen, Taiwan-China). The extracted DNA concentration and purity were evaluated using a spectrophotometer at 260 and 280 nm wavelengths (Williams *et al.,* 1997). DNA was extracted and stored at -20°C until PCR analysis.

Polymerase chain reaction (PCR)

The partial ITS region of each DNA extracted from each *Trichoderma* isolate was PCR-amplified using the universal primer pair: ITS1 (TCCGTTGGTGAACCAGCGG) and ITS4 (TCCTCCGC TTATGATATGC) (White *et al.,* 1990). PCR amplification was prepared using *Taq* DNA polymerase (Roche, Cat. No. 11 146 173 001) in a final volume of 20 µl PCR reaction mixture containing 2 µ1 10X PCR buffer, 1 µl each primer (10 pmol), 3 µl template DNA (30 ng/µ1), 2 µl dNTPs (2 mM) and 1 unit *Taq* polymerase. Each sample volume was then completed to 20 µl by adding nuclease-free water.

The amplification of DNA from fungal isolates was carried out using the following polymerase chain reaction (PCR) conditions: An initial denaturation step at 98°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 45 seconds, primer annealing at 55°C for 45 seconds and an initial elongation step at 72°C for 1 minute. The PCR process was concluded with a final elongation step at 72°C for 5 minutes.

Agarose gel electrophoresis

Agarose gel preparation: 1 g of agarose powder was dissolved in 100 mL of 1 × TBE buffer (Tris-Boric acid-EDTA buffer) until the solution became clear. When the solution had cooled to about 45°C, 5 microliters of ethidium bromide dye were added. A gel casting tray with a comb on one side for well formation was prepared. Agarose containing ethidium bromide was poured into the tray and left to solidifyat room temperature. Following polymerization, the comb was gently pulled and the tray returned to the electrophoresis tank. The tank was filled with 1 × TBE buffer, covering the agarose gel with about 70 mm height.

Ten microliters of the PCR-amplified DNA product were added to each well of the previously prepared agarose gel. Additionally, five microliters of a molecular-weight size marker were added to the well on the left side of the sample wells to determine the sizes of the amplified DNA fragments. The electrodes of the power supply were connected to the electrophoresis tank and the system was run at 150 milliamps for one hour. After the electrophoresis process was completed, the agarose gel containing the PCR products was examined under ultraviolet (UV) transillumination and images were captured.

Analysis of PCR-amplified DNA sequences

To identify the fungal isolates, the PCR-amplified DNA products from the fungal isolates, obtained using the ITS1 and ITS4 primers, were sent to Macrogen (South Korea) for bidirectional sequencing of the nucleotide sequences.

All nucleotide sequences were analyzed using the BLAST (basic local alignment search tool) program to compare them with available sequences in the National center for biotechnology information (NCBI) database, specifically those of globally identified fungal species. Based on the nucleotide sequences of *Trichoderma* spp. obtained through PCR, a phylogenetic tree was constructed using the MEGA-X software (Kumar *et al.,* 2018).

Analysis of DNA sequences with PCR amplification

For identification of the fungal isolates, the PCR amplified DNA products of the fungal isolates using the ITS1 and

ITS4 primers were sent to Macrogen (South Korea) for bidirectional sequencing of the nucleotide sequences. Nucleotide sequences were compared to globally identified fungal species by BLAST (basic local alignment search tool) program with reference sequences available from the National Center for Biotechnology Information (NCBI) database. According to the nucleotide sequences of *Trichoderma spp*. PCR and a phylogenetic tree was constructed with the MEGA-X software (Kumar *et al.,* 2018).

RESULTS AND DISCUSSION

Molecular identification of *Trichoderma* spp. isolates

The results of the polymerase chain reaction (PCR) demonstrated the successful amplification of DNA products ranging in size from 600 to 650 base pairs (bp) after extracting the DNA from the fungal isolates and amplifying it using PCR with the ITS1 and ITS4 primer pair (Fig 1).

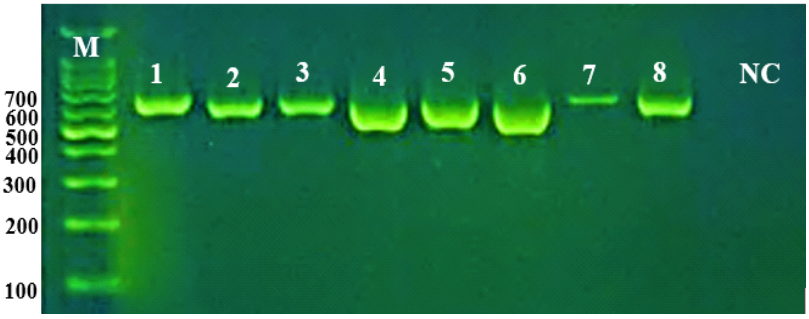
Sequence analysis of PCR products, conducted using BLAST, indicated that all isolates fell within the *Trichoderma* genus, though representing distinct species. Isolates 2 and 5 of *Trichoderma* were identified as *T. harzianum*, yet showed genetic dissimilarity despite a 93% sequence similarity. These two isolates shared 91-100% similarity with other *T. harzianum* isolates already documented in the NCBI database (Fig 2).

Comparison of the ITS1 and ITS4 nucleotide sequences, amplified from *T. harzianum* isolate 1, revealed a maximum similarity of 100% with a *T. harzianum* isolate (EOD AEe2 s, OR435192) collected and identified in Russia. The lowest similarity (90%) was observed with isolates from Singapore (OQ789693 and OQ789699), India (PQ277020) and the United States (PP336463). When compared to other isolates registered at the NCBI, isolate 1 showed similarity ranging from 92-99% (Fig 3).

Regarding *T. harzianum* isolate 2, identified in this study, its closest genetic similarity (98%) was with *T. harzianum* isolates EOD AEe2 s (OR435192), from Russia and those documented in Saudi Arabia (OK636078) and India (EF552703). Conversely, it showed greater genetic distance from isolates such as those from the United States (PP336463) and Iran (KT351798). Other isolates registered at the NCBI exhibited similarity ranging from 92-97% with isolate 2. Both *T. harzianum* isolates 2 and 5 were deposited in the NCBI under accession numbers PV113441 and PV112458, respectively (Fig 4).

The results also indicated that fungal isolates 1, 6 and 8 all belonged to *T. longibrachaitum*, exhibiting 100% similarity (Fig 5 and 6). When the nucleotide sequences of the amplified products from these isolates were compared with sequences of the same gene region from isolates of the same fungus previously registered at the NCBI, 100% similarity was observed with a *T. longibrachaitum* isolate (KY764813) isolated and identified in China. Other isolates registered at the NCBI, including those identified in China (MF116273, KY750397, MH284177 and KY76481) and Australia (MK870340), showed 99% similarity (Fig 5 and 6).

Analysis of the nucleotide sequences, illustrated in Fig 7 and 8, confirmed that isolates 4 and 7 belong to the



These isolates were recovered from soil and roots of healthy tomato plants. M represents the molecular-weight size marker (100 bp ladder), with fragment sizes labeled on the left side of the fig. NC refers to the negative control, where no DNA was added to the PCR reaction mix.

Fig 1: PCR-amplified DNA products obtained using the ITS1 and ITS4 primers from *Trichoderma* spp. isolates in this study.

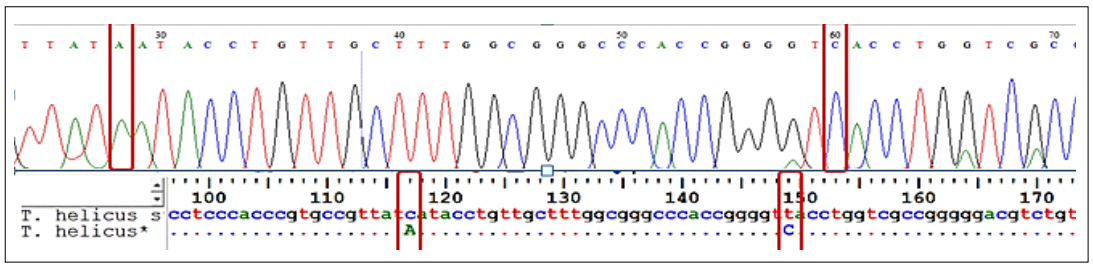


Fig 2: Variations in specific nucleotide sequence positions between *T. harzianum* isolates (2 and 5) identified in this study.

fungus *Trichoderma reesei*. They exhibited 100% similarity to each other and to the *T. reesei* isolate (MW789354) registered at the NCBI. However, the similarity of isolates 4 and 7 with other isolates registered at the NCBI was lower, reaching a minimum of 96%. These less similar isolates included those identified in China (FJ481035) and Russia (OR435208). Isolates 4 and 7 showed 97-99% similarity with the remaining isolates registered at the NCBI.

The results also identified *T. viride* (isolate 3) among the *Trichoderma* spp. fungi identified in this study. This isolate showed 100% similarity with the *T. viride* isolate (HQ259987) identified in India and 99% similarity with other

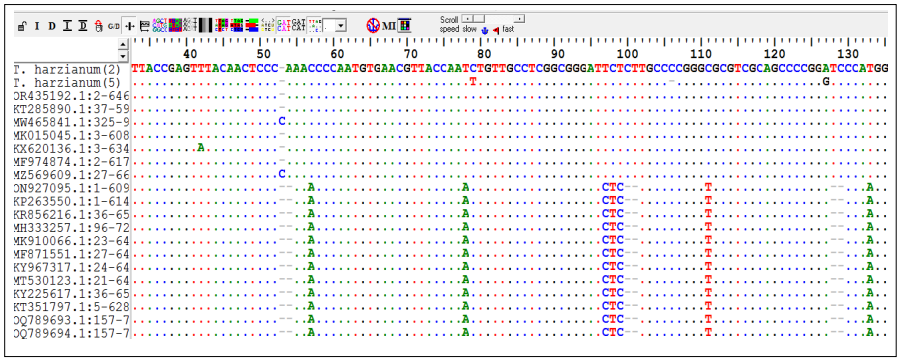


Fig 3: Similarities and differences in specific nucleotide sequence regions (sequence alignments) of PCR-amplified DNA products from *T. harzianum* isolates (2 and 5) identified in this study, compared to nucleotide sequences of other *T. harzianum* isolates previously recorded in the National Center for Biotechnology Information (NCBI).

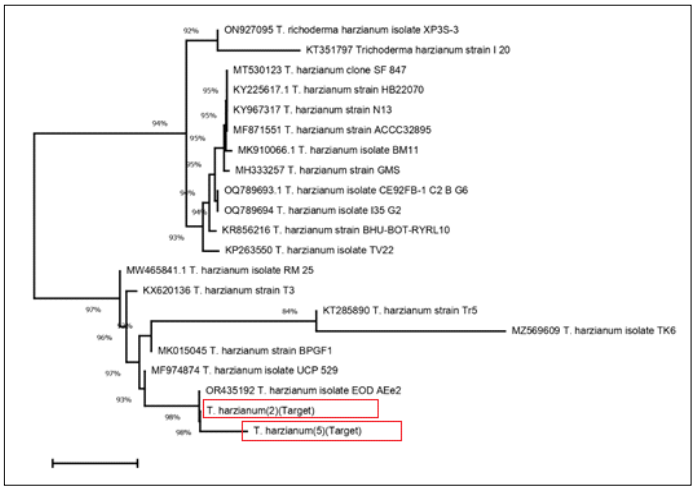


Fig 4: Neighbor-Joining phylogenetic tree showing the genetic relationship between *T. harzianum* isolates (2 and 5) identified in this study and other *T. harzianum* isolates previously recorded in the National Center for Biotechnology Information (NCBI).

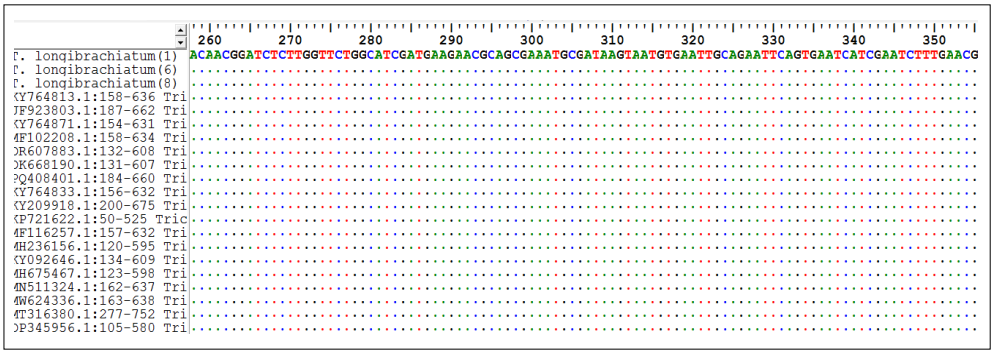


Fig 5: Similarity in nucleotide sequence alignments of PCR-amplified DNA products from *T. longibrachiatum* isolates (1, 6 and 8) identified in this study, compared to nucleotide sequences of other *T. longibrachiatum* isolates previously recorded in the National Center for Biotechnology Information (NCBI).

isolates, including those identified in India (OP480797 and HQ259984) and the United States (KJ406563) (Fig 9).

Based on the results of the genetic analysis, it was confirmed that there are two new isolates of *T. harzianum* (5) and *T. reesei* (4) that are genetically distinct and not previously registered at the National Center for Biotechnology Information (NCBI). Therefore, they were registered under the accession numbers PV112458 and

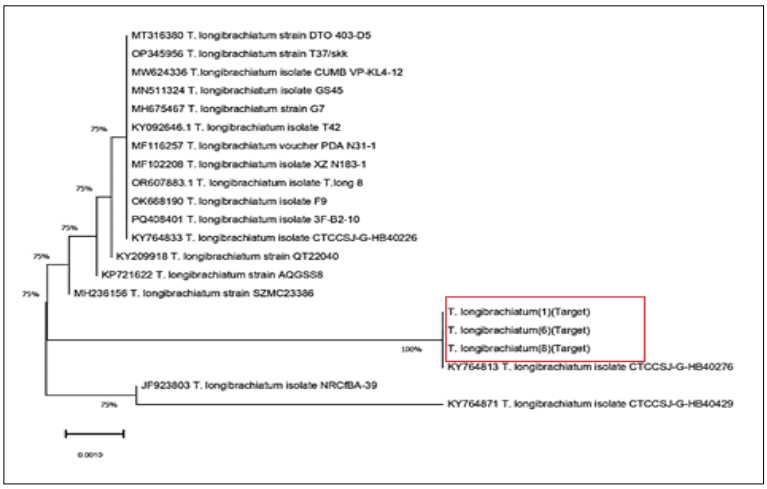


Fig 6: Neighbor-Joining phylogenetic tree showing the genetic relationship between *T. longibrachiatum* isolates (1, 6 and 8) identified in this study and other *T. longibrachiatum* isolates previously recorded in the National Center for Biotechnology Information (NCBI).

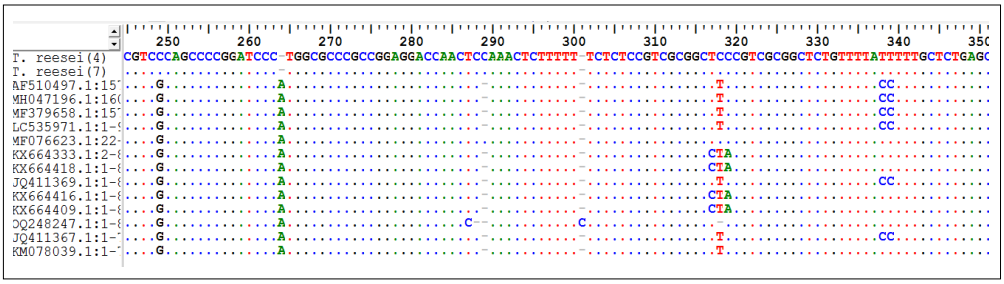


Fig 7: Similarities and differences in specific nucleotide sequence regions (sequence alignments) of PCR-amplified DNA products from *T. reesei* isolates (4 and 7) identified in this study, compared to nucleotide sequences of other *T. reesei* isolates previously recorded in the National Center for Biotechnology Information (NCBI).

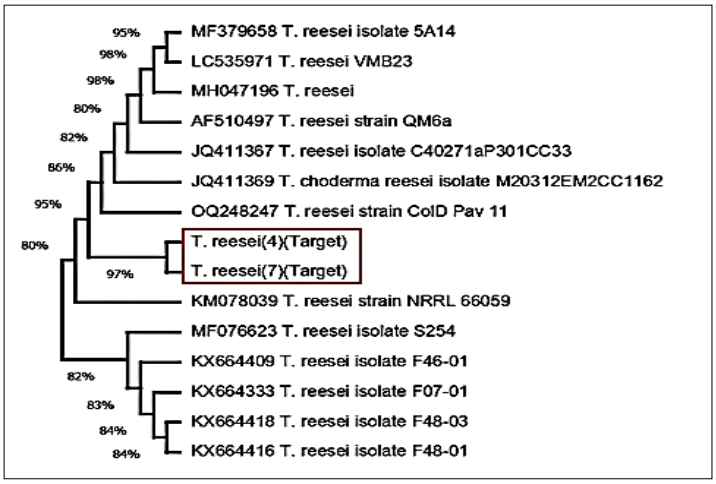


Fig 8: Neighbor-Joining phylogenetic tree showing the genetic relationship between *T. reesei* isolates (4 and 7) identified in this study and other *T. reesei* isolates previously recorded in the National Center for Biotechnology Information (NCBI).

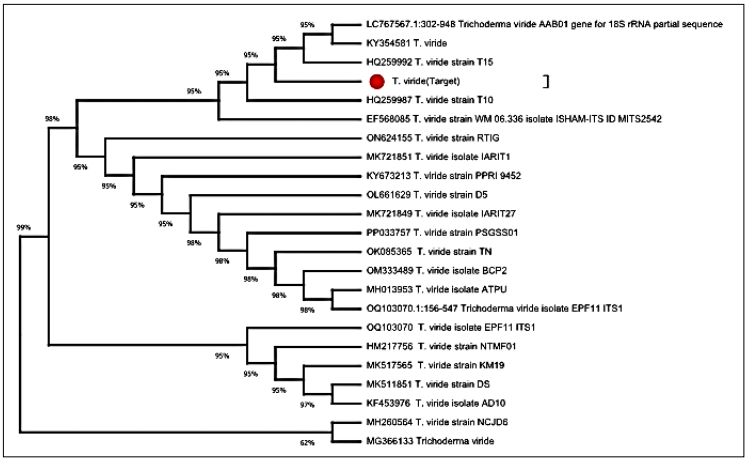


Fig 9: Neighbor-Joining phylogenetic tree showing the genetic relationship between *T. viride* isolate (3) identified in this study and other *T. viride* isolates previously recorded in the National Center for Biotechnology Information (NCBI).

PV114821, respectively. The remaining *Trichoderma* fungal species, represented by *T. longibrachiatum* (1), *T. harzianum* (2) and *T. viride* (3), were also registered at the NCBI under the accession numbers PV114839, PV113441 and PV114819, respectively.

The sequencing of PCR-amplified products using ITS1 and ITS4 primers has proven highly effective in identifying various fungal species, including *T. harzianum*, *T. longibrachiatum* and *T. viride*. Numerous studies have shown that the ITS region, which includes the ITS1 and ITS2 regions and the 5.8S ribosomal RNA gene, exhibits significant diversity among fungal species (Ruppavalli *et al.,* 2025). This diversity has made it a highly effective gene region for molecular identification of various eukaryotic organisms, including fungi (Aljuaifari *et al.,* 2019; Jubair *et al.,* 2020; AL-Abedy *et al.,* 2021a; Al-Shujairi *et al.,* 2022; Alhissnawi *et al.,* 2024).

In this study, the ITS region was utilized for the molecular identification of *Trichoderma spp.* fungi isolated in this study with high efficiency. This was achieved through the polymerase chain reaction (PCR) technique, known for its accuracy and speed in identifying various organisms, including insects and fungi (AL-Abedy *et al.,* 2018; Al-Fadhal *et al.,* 2018; Abdullah *et al.,* 2019; AL-Abedy *et al.,* 2021b; Mahmood and AL-Abedy, 2021).

Several factors contribute to the occurrence of variations or genetic diversity among species within the same genus of organisms, including fungal isolates. These factors include genetic mutations, genetic recombination and selective pressure, which lead to varying degrees of genetic diversity among fungal isolates. Such diversity is essential for adaptation, evolution and the ability of fungi to occupy diverse environments and interact with other living organisms (Gladieux *et al.,* 2014). Furthermore, understanding this genetic variation is crucial for studying fungal pathogens and exploiting beneficial fungi for agricultural and industrial purposes (Brunner *et al.,* 2013).

The genetic diversity of *Trichoderma* fungal species, especially the new isolates of *T. harzianum* (5) and *T. reesei* identified in this study, is of great importance because this genus includes various species that differ in their ability to control many plant pathogens.

CONCLUSION

Exact molecular profiling (PCR/ITS sequencing) of *Trichoderma* spp. isolates, identified in Iraqi soil, confirmed clear genetic difference, testifying to their adaptability to differ in conditions and highlighting the importance of characterization of such resources. The current research positively distinguished *T. harzianum*, *T. longibrachiatum* and *T. viride*. Besides, two new isolates (*T. reesei* and *T. harzianum*) previously not registered on NCBI were characterized, citing unique but uncharacterized strains occurring in the Iraqi environment and adding to regional diversity knowledge. Similar findings also validated the precision of ITS region use in *Trichoderma* species identification, suggesting its diagnostic validity. As *Trichoderma* possesses biocontrol potential, this study provides a platform for the further development of the potential of using these local isolates in sustainable agriculture. It immensely contributes to Iraqi *Trichoderma* genetic diversity, emphasizing the significance of local genetic material in the development of effective agricultural strategies.

Conflict of interest

The authors declare no conflict of interest.

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