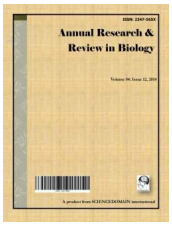

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Variability and Genetic Structure of a Natural Population of *Trichoderma* spp. Isolated from Different Substrates in Morocco

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Ten species of *Trichoderma* antagonists have been collected from different ecosystems (forest soils, compost and strawberry plants) and characterized according to their molecular tools. Molecular characterization was performed by amplifying and analyzing the sequences of the internal transcribed separator coding 1 and 4 (ITS). *Trichoderma* isolates were identified as *Trichoderma asperellum*, more than 90% similarity, except the SDLA2 strain gave *Trichoderma harzianum* species (The isolate SDLA2 was closely related at the nucleotide level to the China's isolate gi|748053410|gb|KM277992.1| *Trichoderma harzianum*, 100% of similarity). The dissimilarity matrix based on ITS analysis showed that the *Trichoderma* isolates: SELM4, SMAA6, SMAA8 and SDLA27 were the less dissimilar (1.3% to 1.6%), while the isolate SDLA2 with the others isolates, except with the isolate gi|748053410|gb|KM277992.1 (1.1%) are the most dissimilar (9.9% to 16.3%).

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1. INTRODUCTION

Species of the filamentous fungal genus *Trichoderma* are belonging to the *Hypocreales* order of the *Ascomycota* division. The genus involves representatives with excellent antagonistic abilities against a series of plant pathogenic fungi, being therefore promising candidates for the biological control of fungal pests in agriculture. Modes of action with proposed roles in biocontrol capabilities of *Trichoderma* strains include mycoparasitism, antibiosis by the production of antifungal metabolites, competition for nutrients and space, induction of defense responses in the plant as well as plant growth promotion [1,2,3,4]. They are often the predominant components of the mycoflora in soils of various ecosystems, such as agricultural fields, meadow, forest, salt marshes and deserts, in all climatic zones [5]. Several studies reported about a series of new isolates as well as new phylogenetic species of *Trichoderma* in a series of natural ecosystems [6,7]. *Trichoderma* species are also able to degrade domestic waste relatively quickly without emitting bad odors [8]. Also the hypocreomycetidae genus *Trichoderma* was known for their rapid growth, capability of utilizing diverse substrates and resistance to noxious chemicals [9]. Use of *Trichoderma* to control plant diseases is not harmful to the environment unlike chemical pesticides [10,11]. There is *Trichoderma* species have been demonstrated to produce protease and chitinase that degrade the cell-wall during the parasitic interaction [12,13]. Proteases produced by pathogenic insects possess similar properties to those from *Trichoderma,* augmenting the possibility that proteases are involved in entomopathogenicity. Previous report regarding larvicidal activity of *Trichoderma harzianum* against the cotton leaf worm has suggested that this species is pathogenic towards the insect [14]. A knowledge concerning the behavior of these fungi as antagonists is essential for their effective use since they can act against target organisms in several ways [15]. Therefore it is important to investigate the diversity of *Trichoderma* in the soil since such information can lead to the isolation of *Trichoderma* species having higher antagonistic efficiency and development of better biological control methods to manage plant pathogenic fungi [16,17].

The commercial use of *Trichoderma* must be preceded by precise identification, adequate formulation, and studies about the synergistic effects of their mechanisms of biocontrol. Prior to the utilization of molecular approaches, the identification of *Trichoderma* is historically based on the application of morphological species recognition concept [18]. However, accurate species identification based on morphology is difficult at best because of the paucity and similarity of useful morphological characters [19,20], and increasing numbers of morphologically cryptic species that can be distinguish only through their DNA characters are being described [21]. With the advent of molecular methods and identification tools, which are based on sequence analysis of multiple genes, it is now possible to identify every *Trichoderma* isolate and to recognize it as a putative new species [22]. The current diversity of the holomorphic genus *Hypocrea*/*Trichoderma* is reflected in approximately 160 species, whose majority has been recognized on the basis of DNA sequence analysis and molecular phylogeny of pure cultures and/or herbaria specimens [22]. Multiple genes have been demonstrated and translation elongation factor 1-a, internal transcribed spacer (ITS) and b-tubulin have successfully delimited between the closely related species in *Trichoderma* [23]. The species composition of the *Trichoderma* genus has been examined by molecular methods in a series of natural ecosystems, including a mid-European, primeval floodplain-forest [24], the Danube floodplain [25], Sardinia [26], soils from Russia, Nepal, Northern India [27], south-east Asia [28], China [29], North-Africa [30] and South America [31]. These studies have revealed a series of new genotypes as well as new phylogenetic species of *Trichoderma.*

Introduction of molecular approach enabled researchers to identify and delimit species of *Trichoderma* more reliably. The ITS regions of ribosomal DNA (rDNA) were the first studied gene [32]. The ITS spacer, approximately 600 to 1000 bp, is amplified by universal primers (ITS1 / ITS4), specific to fungi (ITS1f / ITS4) or specific to Basidiomycota (ITS1f / ITS4b) [33,34]. Nuclear rDNAs, and particularly the ITS regions, are a good target for phylogenetic analysis in fungi [35].

The present study was carried out to characterize and identify *Trichoderma* Species

isolated from different substrates in Morocco based on Molecular Identification by sequential analysis of ITS regions.

2. MATERIALS AND METHODS

2.1 Isolation of *Trichoderma spp.* Strains

The strains of *Trichoderma* spp. used in this study were collected from six localities of Morocco, Missour from Fés Boulmane region (East) and Elmnasra, Maamora, Dlalha, Gnanfa, Anabsa from Rabat-Kenitra region (Northwest). Ten isolates were collected from different ecosystems (forest soils, Compost, and strawberry plant) (Table 1). Other *Trichoderma* spp. nucleotides sequences used in our analyses were taken from the following GenBank entries: strains; gi|427379233|gb|JX422010.1| *Trichoderma asperellum*; gi|482514058|gb|KC569362.1| *Trichoderma asperellum*; gi|346721655|gb|JN004179.1| *Trichoderma asperellum* and gi|922664364|dbj|LC075713.1| *Trichoderma asperellum* from India; gi|168829589|gb|EU272534.1| *Trichoderma asperellum* from Colombia ; gi|212291366|gb|FJ412053.1| *Trichoderma asperellum* from Ethiopia. and gi|748053410|gb|KM277992.1| *Trichoderma harzianum* from China were used as reference sequence in this study.

2.2 Fungal Growth Conditions and DNA Extraction

The total protocol of the DNA extraction from mycelium of *Trichoderma* spp. is described by Murray and Thompson [36] and Doyle [37]. Preferably young mycelia promote the obtention of the better using DNA. The strains are subcultured on PSA (Potato: 200 g, Sucrose 20 g, Agar-agar 15 g, distilled water 1000 mL) and incubated at 28℃ in the dark for 4 days. The mycelia were lyophilized for 48 hours 0.1 g of mycelia were weighed from each sample and placed in 2 mL microtubes and ground using beads with the grinding apparatus for 5 min. 1 mL of preheated extraction buffer at 65 ℃ water bath was then quickly added. This extraction buffer was composed of 0.1 M Tris (pH 8); 5 M NaCl; 0.5 M EDTA; 2% CTAB; 0.2% mercaptoethanol. Microtubes or crushing material was then placed in a water bath at 65℃ for 1 hour with stirring (15 min each). After cooling in ice for 5 min, 800 µL of chloroform / isoamyl alcohol (24: 1) were added. The mixture was stirred gently for 20 minutes at room temperature. Centrifugation was performed at 13000 rpm for 15 min at 4℃. The supernatant was recovered and transferred into a sterile tube to which Eppendroff 750 µL of isopropanol cooled to -20℃ was added. The whole was gently mixed (formation of a precipitate in the form of filaments or turbidity), left for 1 hour at 4℃ or 30 min at -20℃ to precipitate the DNA. After centrifugation at 13000 rpm for 15 min, the supernatant was drained and the pellet was recovered, 1 mL of 75% ethanol cooled to -20℃ was added to the pellet. Then centrifugation at 13000 rpm for 15 min at 4℃ was performed. This DNA wash step was repeated a second time. The pellet of DNA was then dried under vacuum at room temperature. Then 200 µL TE buffer was added to dissolve the DNA. Everything is finally allowed to stand overnight or 24 hours at 4℃.

2.3 Molecular Identification of the Isolated *Trichoderma spp*. Strains

ITS region (Internal Transcribed Spacer) of Ribosomal DNA was amplified with universal primers ITS1 and ITS4 [33]. ITS1: 5'-TCGGTAGGTGAACCTGCG G-3'; ITS4: 5'-TCCTCCGCTTATTGATATGC-3'.

The amplification reaction was performed in a total volume of 25 µL containing 5 µL of buffer 5X (Reagents : MyTaq DNA polymerase kit Bioline), 1 µL dNTP (20 mM), 1 µL of each of the primers (10 µM), 0,2 µL of Taq DNA polymerase (5 U μL^{-1}), 150 ng of the template DNA and Sterile double distilled water. These reactions were subjected to an initial denaturation of 1 min at 95℃, followed by 35 cycles of 15 s at 95℃, 20 s at 57℃, and 15 s at 72℃, with a final extension of 3 min at 72℃ in a « Verity » d'ABI thermal cycler. Electrophoresis performed for the PCR product by migration of the latter on an agarose gel at 1.5% in the presence of a molecular weight marker 100 bp.

2.4 Sequencing of the Amplified IST

The purified PCR products were sequenced on both strands in an automatic sequencer (Applied Biosystems 3130XL sequencer) by priming the sequencing reactions with the same oligonucleotides used for cDNA synthesis. Sequencing data associated with the 10 isolates analyzed in this study were deposited at the

NCBI Genbank database, accession numbers are listed in Table 1.

2.5 Sequence Analysis

Sequences were analyzed using the BioEdit program [38]. Multiple alignment was performed with the algorithm CLUSTALW program [39], implemented in the program BioEdit. Phylogenetic relationships were also determined with the maximum-likelihood (ML) algorithm of MEGA 6 [40].

3. RESULTS

This conducted molecular study has involved 10 strains collection. PCR amplification showed a single band of approximately 600 bp for all the *Trichoderma* spp*.* isolates (Fig. 1). After the analysis of sequences by comparison (BLAST) with data banks (GENBANK), these clips have more than 90% similarity gave the same species of fungus *Trichoderma asperellum* except strain BankIt1902509 SDLA2 KU987253 gave the *Trichoderma harzianum* (species) (Table 1).

A dendrogram was obtained by analysis of the databank sequences (Fig. 2). The phylogenetic relationships were deduced for 10 *Trichoderma* spp sequences, thus the 10 sequences available in GenBank. The 20 strains with partial sequences have reveled three divergent genetic groups. Moroccan isolates are grouped into two of these groups (I and III). The isolate of group III (SDLA2) was closely related at the nucleotide level to the China's isolate (gi|748053410|gb|KM277992.1| *Trichoderma harzianum*) (100% of similarity). The isolate of group I (SDLA33 and SMis1) showed up to 98% of similarity. Indian's isolates of *Trichoderma asperellum* (gi | 427379233 | gb | JX422010.1 |), (gi | 482514058 | gb | KC569362.1 |), (gi | 346721655 | gb | JC0075713 .1), Colombian isolate (gi | 168829589 | gb | EU272534.1 |), Ethiopian isolate (gi | 212291366 | gb | FJ412053.1|), India's isolate (gi|346721655|gb|JN004179.1|) fell into group II , as well as the isolate of Colombia and Ethiopia showed up to 95% similarity.

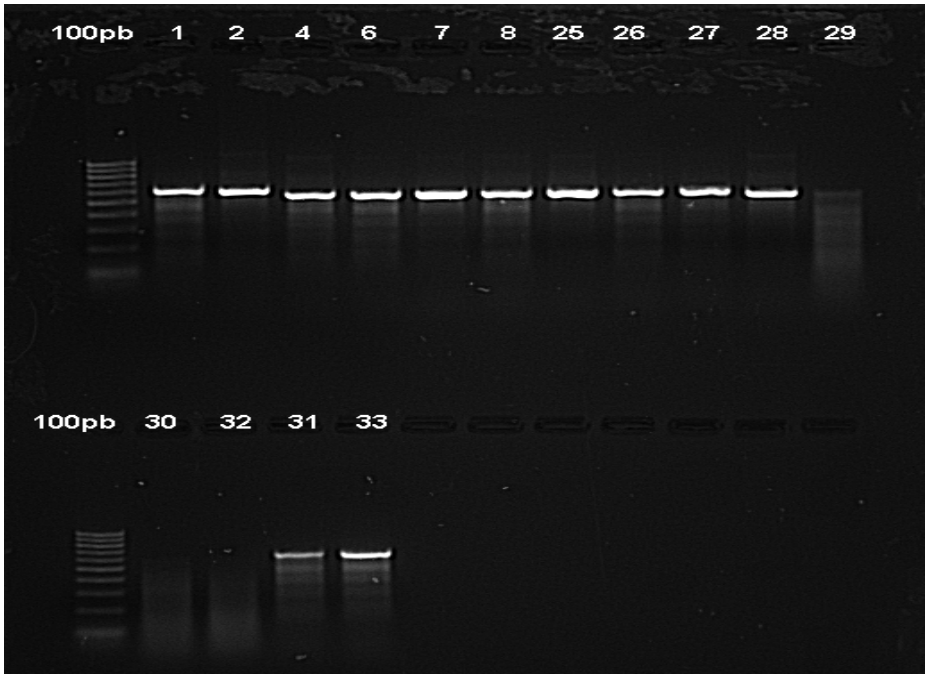

Fig. 1. Separation on agarose gel (1.5%) of PCR amplification products of the DNA of *Trichoderma* isolates using the ITS1 and ITS4 primers

Molecular weight marker 100 bp , 1-33 tracks :KU987252 SMis1- KU987253 SDLA2 - KU987244 SELM4 - KU987246 SMAA6- KU987247 SGNA7- KU987248 SMAA8 - KU987249 SDLA26- KU987250 SDLA27 - KU987251 SDLA28- KU987245 SDLA33. (Isolates 25, 29, 30, 31 and 32 were not amplified and will therefore be excluded from the result of the analysis process.)

Table 1. Origin and identity of *Trichoderma* isolates

Country	Isolation source	Organism	Isolate	Query length	Specimen voucher
Morocco: East, Missour	Compost	*Trichoderma asperellum*	BankIt1902509 SMis1 KU987252	427 bp	RAB 95369
Morocco: Gharb, Dlalha	Strawberry: Festival, Root	*Trichoderma harzianum*	BankIt1902509 SDLA2 KU987253	591 bp	RAB 95370
Morocco : Gharb, Elmnasra	Banana leaves	*Trichoderma asperellum*	BankIt1902509 SELM4 KU987244	573 bp	RAB 95371
Morocco: Kenitra, Maamora	Soil	*Trichoderma asperellum*	BankIt1902509 SMAA6 KU987246	507 bp	RAB 95372
Morocco : Gharb, Gnanfa	Strawberry: Sabrina, Root	*Trichoderma asperellum*	BankIt1902509 SGNA7 KU987247	574 bp	RAB 95373
Morocco: Kenitra, Maamora	Soil	*Trichoderma asperellum*	BankIt1902509 SMAA8 KU987248	547 bp	RAB 95374
Morocco: Gharb, Dlalha	Strawberry: Festival, Root	*Trichoderma asperellum*	BankIt1902509 SDLA26 KU987249	582 bp	RAB 95375
Morocco: Gharb, Dlalha	Strawberry: Festival, Root	*Trichoderma asperellum*	BankIt1902509 SDLA27 KU987250	569 bp	RAB 95376
Morocco: Gharb Dlalha,	Strawberry: Festival, Collar	*Trichoderma asperellum*	BankIt1902509 SDLA28 KU987251	572 bp	RAB 95377
Morocco: Gharb, Dlalha	Strawberry: Festival, Root	*Trichoderma asperellum*	BankIt1902509 SDLA33 KU987245	386 bp	RAB 95379

Table 2. Similarity matrix of *Trichoderma spp* strains based on ITS analysis

BankIt1902509 SELM4 KU987244																			
gi|482514058|gb|KC569362.1|	0.033																		
BankIt1902509 SDLA33 KU987245	0.073	0.087																	
gi|427379233|gb|JX422010.1|	0.030	0.022	0.076																
BankIt1902509 SMAA6 KU987246	0.013	0.033	0.058	0.024															
gi|482514058|gb|KC569362.1|	0.033	0.000	0.087	0.022	0.033														
BankIt1902509 SGNA7 KU987247	0.027	0.033	0.079	0.022	0.024	0.033													
gi|482514058|gb|KC569362.1|	0.033	0.000	0.087	0.022	0.033	0.000	0.033												
BankIt1902509 SMAA8 KU987248	0.016	0.030	0.058	0.022	0.005	0.030	0.024	0.030											
gi|346721655|gb|JN004179.1|	0.030	0.030	0.079	0.013	0.027	0.030	0.016	0.030	0.024										
BankIt1902509 SDLA26 KU987249	0.019	0.035	0.073	0.027	0.016	0.035	0.019	0.035	0.016	0.024									
gi|168829589|gb|EU272534.1|	0.041	0.008	0.097	0.030	0.041	0.008	0.041	0.008	0.038	0.038	0.044								
BankIt1902509 SDLA27 KU987250	0.016	0.035	0.070	0.024	0.013	0.035	0.013	0.035	0.011	0.019	0.011	0.044							
gi|922664364|dbj|LC075713.1|	0.033	0.000	0.087	0.022	0.033	0.000	0.033	0.000	0.030	0.030	0.035	0.008	0.035						
BankIt1902509 SDLA28 KU987251	0.030	0.027	0.073	0.022	0.022	0.027	0.016	0.027	0.016	0.022	0.027	0.035	0.024	0.027					
gi|212291366|gb|FJ412053.1|	0.041	0.008	0.097	0.030	0.041	0.008	0.041	0.008	0.038	0.038	0.044	0.000	0.044	0.008	0.035				
BankIt1902509 SMis1 KU987252	0.035	0.050	0.047	0.041	0.024	0.050	0.044	0.050	0.019	0.044	0.035	0.058	0.030	0.050	0.035	0.058			
gi|346721655|gb|JN004179.1|	0.030	0.030	0.079	0.013	0.027	0.030	0.016	0.030	0.024	0.000	0.024	0.038	0.019	0.030	0.022	0.038	0.044		
BankIt1902509 SDLA2 KU987253	0.105	0.118	0.163	0.108	0.115	0.118	0.096	0.118	0.112	0.099	0.105	0.127	0.108	0.118	0.102	0.127	0.134	0.099	
gi|748053410|gb|KM277992.1|	0.096	0.118	0.156	0.108	0.105	0.118	0.099	0.118	0.105	0.105	0.099	0.127	0.099	0.118	0.111	0.127	0.127	0.105	0.011

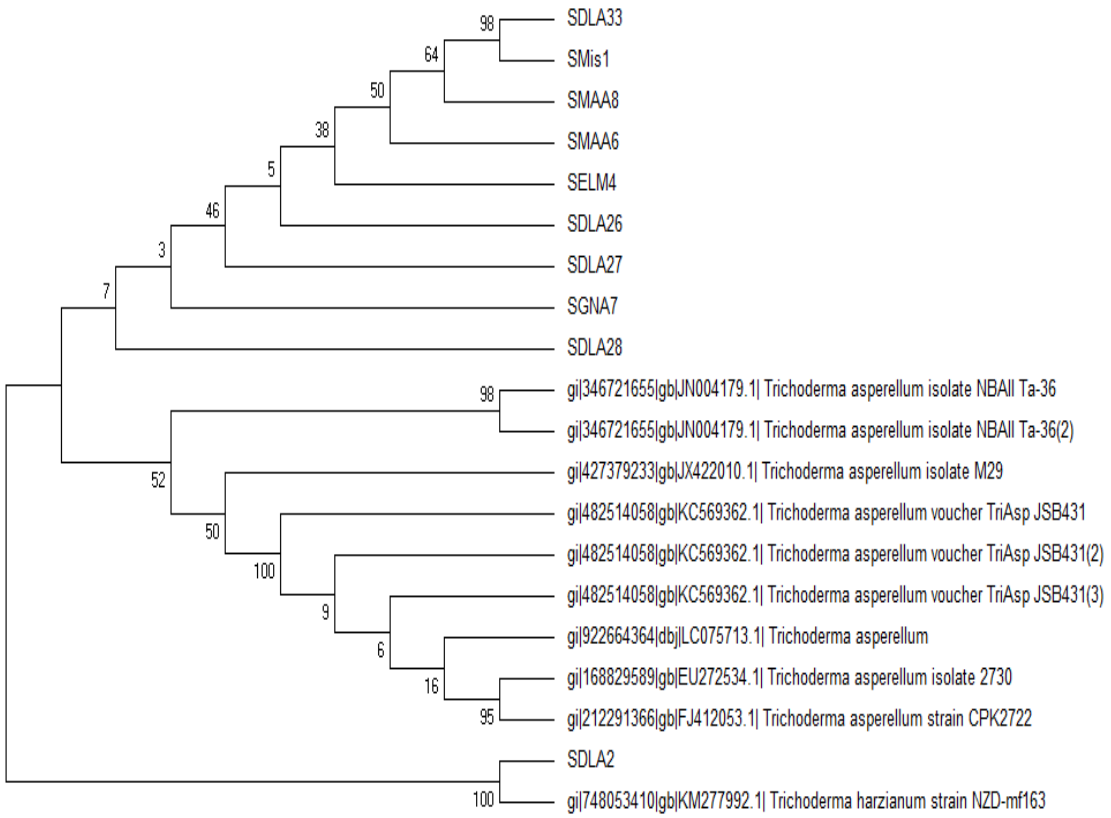

Fig. 2. Dendrogram representing the genetic relationships between the studied isolates generated by cluster analysis using the NJ method

The dissimilarity matrix based on ITS analyzes showed that the *Trichoderma* isolates: SELM4, SMAA6, SMAA8 and SDLA27 were the least dissimilar (1.3% to 1.6%), also gi | 482514058 | gb | KC569362.1 | with gi|168829589|gb|EU272534.1 ,gi|922664364|dbj|LC075713.1 and gi|212291366|gb|FJ412053.1 (0% to 0.8%). While the isolate SDLA2 with the others isolates, except with the isolate gi|748053410|gb|KM277992.1 (1.1%) are the most dissimilar (9.9% to 16.3%) (Table 2).

4. DISCUSSION AND CONCLUSION

The results of this study bring out the importance of using molecular identification tools to describe *Trichoderma* spp. in a natural habitat. This corroborates even more Phylogenetics.

All isolates of *Trichoderma* spp were identified at the level of homology at least 90% with comparing the sequences of the STI region with the sequences deposited in Gen Bank. However, Druzhinina and Kubicek [25] have documented that Gen Databases contains many sequences of *Trichoderma* isolates that may have been misidentified and have a false name, but *Trichoderma* appears to be more complicated genus, since the phylogenetic relationships of several of these members are still unclear [41,42].

The results obtained from the phylogenetic analysis of the ITS sequences showed that the 10 isolates of *Trichoderma* spp. of Morocco used can be separated into two different species; *T. asperellum* represents the dominant group of *Trichoderma* spp. followed by a single strain of *T. harzianum*. In group III, the isolate BankIt1902509 SDLA2 KU987253 was identical in order to the *Trichoderma harzianum* strain of China (gi | 748053410 | gb | KM277992.1 |), and the other nine strains studied were almost identical in order to Indian isolates of *Trichoderma asperellum* (gi | 427379233 | gb | JX422010.1), (gi | 482514058 | gb | KC569362.1 |), (gi | 346721655 | gb | JN004179.1 |), (gi |

922664364 | dbj | LC075713. 1), Colombian isolate (gi | 168829589 | gb | EU272534.1 |), Ethiopian isolate (gi | 212291366 | gb | FJ412053.1 |), which fell in group II. On the other hand, the study of *Trichoderma's* biodiversity in South Asia, including Burma, Cambodia, Malaysia, Singapore, Taiwan, Thailand and Western Indonesia, revealed the presence of species with high metabolic diversity of *T. harzianum* [28]. But Hermosa et al. [43] showed from the Phylogenetic Sequence Analysis the ITS1 that the 17 strains of *Trichoderma* studied can be separated into five different species, and all are clearly distinct from *T. harzianum* Th2 and Th4, and Lieckfeldt et al. [44] found three strains of *Trichoderma* 3, 25, and ThVA, were grouped, with a high bootstrap value of 87%, with the *T. asperellum* TR 48 biocontrol isolate, and strain 3 had a sequence ITS1 Identical to that of the biological control strain T-203. Other authors found rDNA-ITS1 analysis of *Trichoderma* isolates obtained from rice soils in the Philippines included only *T. viride* and *T. harzianum* [45]. The distribution of *Trichoderma* species is influenced by genetic structure, biological niches, soil type and geographic regions. Xia et al. [46] found that the genetic diversity of *T. asperellum* and *T. virens* in the epiphyte is lower than that of the banana root epiphytes. In this study it was found that *T. asperellum* is the most dominant in the roots, the strawberry collar and the Maamora soil. The difference in agricultural fields influenced species composition and abundance of different *Trichoderma* species. Gherbawy et al. [47] found very low *Trichoderma* biodiversity in the agricultural soils of the Nile valley in Egypt, which contained only *T. harzianum* and the anamorph of *Hypocrea orientalis*.

The development of molecular techniques and the use of DNA Sequence analysis became the new systematic paradigm for *Trichoderma* [48] and determined the taxonomic placement of this genus [41,43].The development of molecular tools has also allowed the positive identification of any *Trichoderma* strain [48]. The domains of ITS and intergenic regions are much more variable and therefore more useful for phylogenetic studies of members of the same species or genus [33]. The use of divergence sequences to define species requires careful interpretation, especially because length polymorphisms and inversions can make comparison on a simple basis difficult [49,50].

In conclusion, this research demonstrates that the ITS regions of Ribosomal DNA gene complex are useful as target in molecular applications and offer a powerful tool for the identification and typing of *Trichoderma* spp. The benefits and precise consequences of the present results open several avenues for future research in the field of biotechnology.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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