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Comparative molecular genetic diversity between *Trichoderma* spp. from Egypt and Saudi Arabia

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Abstract

Background: The phylogeny and phylogenetic relationships of *Trichoderma* species were investigated by many methods including maximum parsimony and distance analysis of DNA sequences from multiple genetic loci. *5.8S rDNA* sequence analysis is a powerful tool in evolution studies.

Main body: The aim of this study was to identify and determine the genetic distance between 36 *Trichoderma* isolates from Egyptian and Saudi Arabian soils based on their *5.8S rDNA* sequences. The genetic diversity of *Trichoderma* (Hypocreales, Ascomycota) species from Egypt and Saudi Arabia was investigated. The DNA barcoding of 36 isolates, based on the internal transcribed spacers 1 and 4 (ITS1 and 4) of the ribosomal RNA gene cluster and the partial sequence of this gene, revealed the diversity of these isolates. BLAST query showed that two species, namely *T. longibarchiatum* (42.7% of the isolates) and *T. lentiforme* (33.3% of the isolates), pre-dominated the isolates. Variation among specimens of the same species was detected. Cluster analysis of the Egyptian isolates showed two groups; the first group was subdivided into two subgroups, the first of which included *T. lentiforme* and *T. crassum* isolates, and the second subgroup harbored *T. longibrachiatum*, *T. pseudokoningii*, and *T. bissettii*. The second group included *T. neokoningii* and *T. yunnanens*. The cluster analysis of isolates from Saudi Arabia also consisted of two groups. The first group was subdivided into two subgroups; the first included *T. longibrachiatum* and *T. pseudokoningii*. The second subgroup harbored *T. simmonsii* and *T. lentiforme*. Also, no correlation between genetic and geographic distance was detected.

Conclusion: The results of the present study indicated that the Saudi Arabian isolates showed greater nucleotide diversity compared to Egyptian isolates. Also, these findings will assist in future studies while assessing *Trichoderma* genetic diversity.

Keywords: *Trichoderma*, Nucleotide diversity, Cluster analysis, Saudi Arabia, Egypt

Background

One of the main goals of the agricultural development is to secure enough food to the growing population worldwide, where the use of biotechnology is essential in this context (Mazrou et al. 2020). The most significant agricultural-related problems in Egypt is the decrease in edible crops in the presence of an increasing population, the erosion of agricultural lands, and the loss of crops associated with diseases and pathogens (Hassan 2014 and Mazrou et al. 2020). One promising resolution to tackle these problems depends on the use of biological control. The term biological control means the use of biological materials to control plant-causing biological diseases and increase crop productivity (Hassan et al. 2019). *Trichoderma* spp. are of great benefit to Egyptian and Saudi Arabian agriculture. *Trichoderma* has many beneficial properties such as being environmental-friendly fungi, a biological control agent, and a promoter

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of plant growth. Several *Trichoderma* species (e.g., *T. bissettii*, *T. crassum*, *T. lentiforme*, *T. longibrachiatum*, *T. neokoningii*, *T. pseudokoningii*, and *T. yunnanens*) are effective biocontrol agents due to their ability to inhibit pathogen growth in soil (Atanasova et al. 2013; Kredics et al. 2014; Parmar et al. 2015). These species help to the overall of the plant antagonistic microorganisms. *Trichoderma* spp. reduce the growth and survival of pathogens through various mechanisms such as competition antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion. Such microorganisms are now available commercially and are used in crop management and practices (Atanasova et al. 2013; Parmar et al. 2015). There are many difficulties in the morphological identification of *Trichoderma* due to the homoplasy of morphological and phenetic characters, particularly among the *Trichoderma* anamorph forms; hence, the molecular methods offer a dependable way to identify *Trichoderma* species (Hassan et al. 2014; Lakhani et al. 2016). Molecular identification and characterization of fungal genomes are generally performed with a universal set of internal transcribed spacer (ITS) markers that amplify the specified *5.8S rRNA* fragment of the fungal DNA. The ITS region of nuclear DNA is currently selected as the standard marker for fungal DNA barcoding and for analyzing fungal diversity (Schoch et al. 2012; Hassan 2014). Hassan et al. (2019) improved the use of sequence-characterized amplified region (SCAR) markers as a simple marker system that enables rapid and inexpensive species identification which contributed to overcome *Trichoderma* identification difficulties. Molecular identification of fungal species using molecular techniques such as DNA barcoding has gained considerable attention; therefore, the International Union of Microbiological Societies hosts an official website, namely the International Sub-commission on *Trichoderma* and *Hypocrea* Taxonomy (http://isth.info/). This website is designed as an open resource to host the most recent data on *Hypocrea*/*Trichoderma* taxonomy and evolution. Additionally, this website presents a collection of user-friendly tools for the rapid molecular identification of *Hypocrea*/*Trichoderma* based on DNA barcode. The *TrichOKEY* and *TrichoBLAST* databases are available on the website, which can assist in the quick identification of *Trichoderma*/*Hypocrea* species, using ITS anchors (Srivastava et al. 2014; Fahmi et al. 2016).

To date, a limited number of *ITS* gene sequence are used for the molecular identification of the fungal genus *Trichoderma*. However, the use of these gene sequences is limited to the successful and accurate identification of only a few species (Hassan 2014; Fahmi et al. 2016; Hassan et al. 2019). Moreover, few studies dealt with the genetic diversity of *Trichoderma* in Egypt and Saudi Arabia.

The present study is intended to provide novel combinations of ITS primers that are both species- and strain-specific. Also, *ITS1* and *ITS4* region sequences will be utilized to identify*Trichoderma* species, assess its genetic diversity, and determine if the geographic locations have a probable role in shaping *Trichoderma*'s genetic diversity.

Materials and methods

A total of 36 *Trichoderma* isolates were collected from Egypt and Saudi Arabia across various geographic locations (Fig. 1). Potato dextrose agar (PDA) medium was used to culture the *Trichoderma* isolates, and it is containing 4 g potato extract, 20 g dextrose, and 15 g agar.

DNA extraction, polymerase chain reaction, and electrophoresis

Genomic DNA isolation

Trichoderma isolates were cultured on Czapek Dox broth at 28 °C for 5 days. Czapek Dox broth media contain 30 g sucrose, 3 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, and 0.01 g ferrous sulfate, and pH was adjusted to 7.3. The total genomic DNA for each strain was extracted, using the Norgen Plant/Fungi DNA Isolation Kit (Sigma, Thorold, Canada) as previously described in Hassan et al. (2019). The genomic DNA was subsequently used for sequencing.

PCR amplification of ITS region

ITS1 and ITS4 regions at the *5.8S rDNA* gene from all specimens were amplified, using the primer pair ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) as suggested by Hermosa et al. (2000) and Hassan (2014). PCR was performed as described in Hassan et al. (2019), in PCR tubes containing 1 μl (10 ng) of genomic DNA, 12.5 μl of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 1 μl of each primer (20 pmol), and deionized distilled water (up to a total volume of 25 μl). For DNA amplification, the tube contents were heated at 94 °C for 10 min in a C1000 Touch™ Thermal Cycler (Bio-Rad, Munich, Germany). Subsequently, Taq polymerase was added, followed by 35 cycles of 1 min at 94 °C, 45 s at 60 °C, and 1 min at 72 °C, with a final 7-min extension at 72 °C.

DNA sequence analysis

The nucleotide sequences of the *5.8S-ITS* region were determined, using the Gene analyzer 3121 sequencing service (Macrogen Co., Seoul, South Korea). The deduced 36 sequences were subjected to the National Center for Biotechnology Information (NCBI) BLAST search tool (http://blast.ncbi.nlm.nih.gov) to detect sequence similarity. Each sequence was individually BLAST searched, where BLAST results with the lowest expected

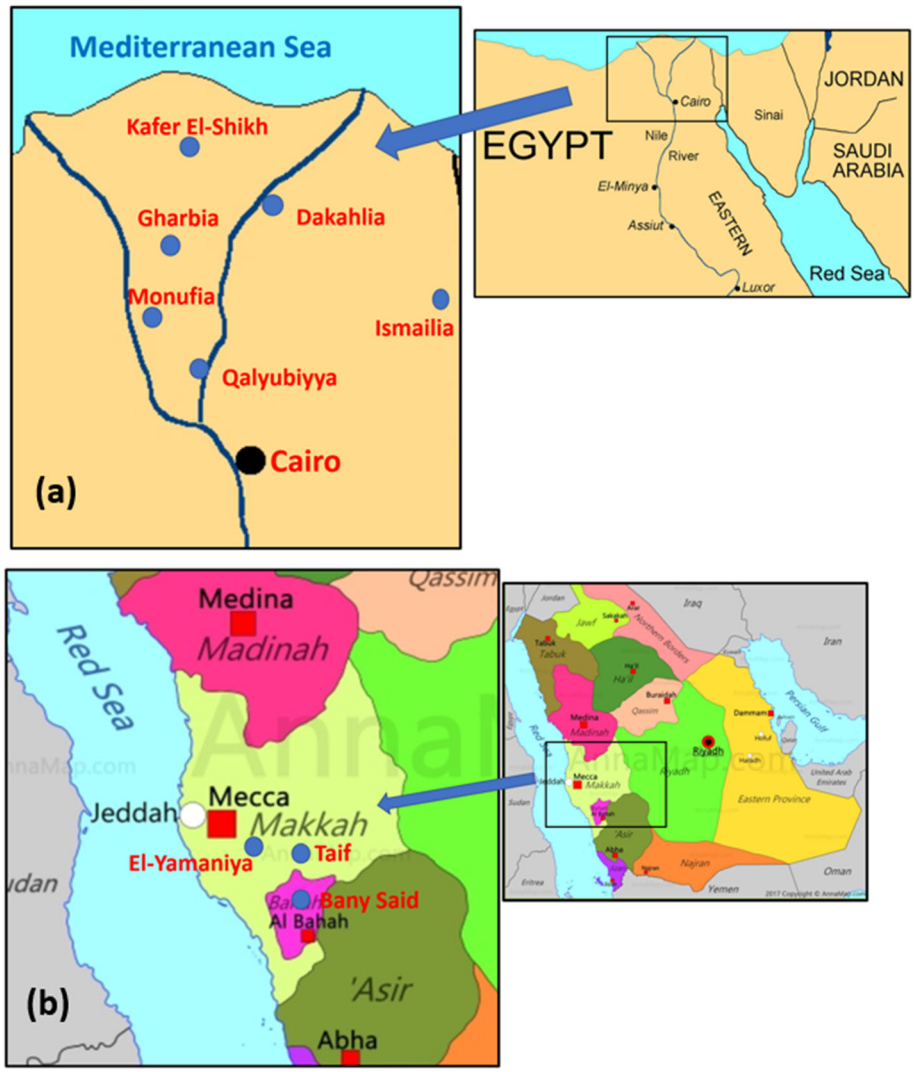

Fig. 1 A map showing the geographical locations where **a** Egyptian and **b** Saudi Arabian samples were collected

value (*e* value) which indicates the number of non-chanced alignments were selected. To ensure that BLAST outputs were governed by *e* values, BLAST algorithm parameters were decreased such that the expected threshold was set to a more stringent value of $1e^{-6}$. Alignment and editing of the 36 sequences were carried out using Clustal x version 2 (Larkin et al. 2007).

Phylogenetic analyses

Exploratory data and phylogenetic analyses were carried out using R Project for Statistical Computing (R Core Team 2017). Exploratory data analysis was performed using Seqinr (Charif and Lobry 2007) R package. Phylogenetic analyses were carried out by ape package (Paradis et al. 2004). Reconstruction of the phylogenetic tree was performed using the neighbor-joining method (Nei and Gojobori, 1986).

Nucleotide diversity

To determine the level of sequence variability, the following common genetic parameters were estimated for sequences from specimens originating from Egypt and Saudi Arabia: haplotype diversity (H_d), number of polymorphic sites (S), nucleotide diversity per site (θ_w), and nucleotide diversity (π_T). The DnaSP v5.10 (Librado and Rozas 2009) was used for the above computation.

Isolation-by-distance

The degree of isolation-by-distance (IBD) (Rousset 1997) was assessed by testing the association between geographic and genetic distances for all pairs of isolates within each group of sequences. Pairwise geographical distances among isolates were calculated using the GPS coordinates at the sampling localities in the Geographic

Distance Matrix Generator v. 1.2.3 (Ersts 2010). Geographic isolation-by-distance referred to the idea that isolates may be spatially distributed across regions. This term sought to determine if there is a statistically significant relationship between genetic distance and geographic distance and the strength of this relationship. Following the regression method of Rousset (1997), geographic distances were log-transformed and genetic distances were expressed as Φ_{ST}. The statistical significance of the associations was assessed based on a Mantel test (Mantel 1967) with 1000 permutations, using GenAlEx 6.5 (Peakall and Smouse 2012).

Results and discussion

Trichoderma species identification

The growth pattern of all 36 *Trichoderma* strains (24 and 12 isolates from Egypt and Saudi Arabia, respectively) cultured on PDA was monitored, and their proliferation pattern revealed significant changes (data not shown). Oligonucleotide barcode is a powerful tool for the identification of *Trichoderma* species and is recommended as an alternative or alongside morphological methods (Hassan et al. 2019). Molecular data from ITS sequences provided accurate characterization and identification of isolates. However, hereafter, isolates will be referred to as Saudi Arabian isolates or Egyptian isolates, regardless of the sampling location. Tables 1 and 2 showed the results obtained from NCBI BLAST queries for the 36 isolates.

The criteria used to query sequences aimed to reduce the search space (database). For the 36 isolates queries, *E* values were 0, which indicated non-chanced alignments of the queries. For Egyptian isolates, the percentage identity with similar species ranged between 84 and 100%, while Saudi Arabian isolates showed similarities with similar species ranged between 97 and 99%. *Trichoderma* isolates were deposited in a gene bank with accession numbers MK680258–MK680293. Moreover, the NCBI BLAST query of Egyptian isolates resulted in 7 *Trichoderma* species, namely *T. bissettii*, *T. crassum*, *T. lentiforme*, *T. longibrachiatum*, *T. neokoningii*, *T. pseudokoningii*, and *T. yunnanens*. Of the 24 Egyptian isolates, 11 were identified as *T. lentiforme*, 7 were *T. longibrachiatum*, 2 were *T. neokoningii*, and only one isolate of each species (*T. bissettii*, *T. crassum*, *T. pseudokoningi*, and *T. yunnanense*) (Table 1). For the Saudi

Table 1 Location and BLAST search results for *Trichoderma* isolates from Egypt and their similarity with the most similar species

Isolate no.	Location	Species	Accession number	NCBI best match	Percent of identity	*E* value
1	Kafer El-Shikh	*T. bissettii*	MK680258	*T. bissettii*	99	0
2	Kafer El-Shikh	*T. crassum*	MK680259	*T. crassum*	99	0
3	Gharbia	*T. lentiforme*	MK680260	*T. lentiforme*	99	0
4	Gharbia	*T. lentiforme*	MK680261	*T. lentiforme*	99	0
5	Kafer El-Shikh	*T. lentiforme*	MK680262	*T. lentiforme*	99	0
6	Kafer El-Shikh	*T. lentiforme*	MK680263	*T. lentiforme*	99	0
7	Kafer El-Shikh	*T. lentiforme*	MK680264	*T. lentiforme*	84	6E−140
8	Kafer El-Shikh	*T. lentiforme*	MK680265	*T. lentiforme*	98	0
9	Dakahlia	*T. lentiforme*	MK680266	*T. lentiforme*	99	0
10	Dakahlia	*T. lentiform*	MK680267	*T. lentiforme*	99	0
11	Gharbia	*T. lentiforme*	MK680268	*T. lentiforme*	99	0
12	Gharbia	*T. lentiforme*	MK680269	*T. lentiforme*	100	0
13	Gharbia	*T. lentiforme*	MK680270	*T. lentiforme*	99	0
14	Kafer El-Shikh	*T. longibrachiatum*	MK680271	*T. longibrachiatum*	99	0
15	Kafer El-Shikh	*T. longibrachiatum*	MK680272	*T. longibrachiatum*	99	0
16	Gharbia	*T. longibrachiatum*	MK680273	*T. longibrachiatum*	99	0
17	Gharbia	*T. longibrachiatum*	MK680274	*T. longibrachiatum*	99	0
18	Monufia	*T. longibrachiatum*	MK680275	*T. longibrachiatum*	99	0
19	Monufia	*T. longibrachiatum*	MK680276	*T. longibrachiatum*	99	0
20	Monufia	*T. longibrachiatum*	MK680277	*T. longibrachiatum*	99	0
21	Dakahlia	*T. neokoningii*	MK680278	*T. neokoningii*	99	0
22	Dakahlia	*T. neokoningii*	MK680279	*T. neokoningii*	99	0
23	Ismailia	*T. pseudokoningi*	MK680280	*T. pseudokoningii*	99	0
24	Ismailia	*T. yunnanense*	MK680281	*T. yunnanens*	99	0

Table 2 Location and BLAST search results for *Trichoderma* isolates from Saudi Arabia and their similarity with the most similar species

Isolate no.	Location	Species	Accession number	NCBI best match	Percent of identity	*E* value
1	Taif government	*T. longibrachiatum*	MK680282	*T. longibrachiatum*	99	0
2	Taif government	*T. longibrachiatum*	MK680283	*T. longibrachiatum*	99	0
3	Taif government	*T. longibrachiatum*	MK680284	*T. longibrachiatum*	99	0
4	Taif government	*T. longibrachiatum*	MK680285	*T. longibrachiatum*	99	0
5	El-Yamaniya, Makkah	*T. longibrachiatum*	MK680286	*T. longibrachiatum*	99	0
6	El-Yamaniya, Makkah	*T. longibrachiatum*	MK680287	*T. longibrachiatum*	99	0
7	El-Yamaniya, Makkah	*T. longibrachiatum*	MK680288	*T. longibrachiatum*	99	0
8	El-Yamaniya, Makkah	*T. longibrachiatum*	MK680289	*T. longibrachiatum*	99	0
9	Taif government	*T. lentiforme*	MK680290	*T. lentiforme*	99	0
10	Bany Said, Al-Baha	*T. simmonsii*	MK680291	*T. simmonsii*	99	0
11	Bany Said, Al-Baha	*T. simmonsii*	MK680292	*T. simmonsii*	99	0
12	Bany Said, Al-Baha	*T. pseudokoningii*	MK680293	*T. pseudokoningii*	97	0

Arabian isolates, only 4 *Trichoderma* species were identified, *T. longibrachiatum*, *T. lentiforme*, *T. simmonsii*, and *T. pseudokoningii*. Of the 12 Saudi Arabian isolates, 8 belonged to *T. longibrachiatum*, 2 belonged to *T. simmonsii*, and only one belonged to each of *T. pseudokoningii* and *T. simmonsii* (Table 2). Comparing species found among Egyptian isolates with their counterparts in Saudi Arabia, *T. simmonsii* was found only among Saudi Arabian isolates, and *T. bissettii*, *T. crassum*, *T. neokoningii*, and *T. yunnanens* only appeared in Egyptian isolates. BLAST search at NCBI was used to match the nucleotide sequences of all eight *Trichoderma* species for the post-molecular identification validation. This confirmed the selected sequences as specific strains of *Trichoderma* species (Srivastava et al. 2014; Mazrou et al. 2020). A set of 5 oligonucleotide sequences present in all known *Hypocrea/Trichoderma* ITS1 - 5.8S RNA - ITS4 sequences, was used in combination to identify the species at a genetic level (Hassan 2014; Hassan et al. 2019). Using molecular markers, particularly those based on DNA sequenced data, was an essential technique to decipher the genetic variability of any species (Nagy et al. 2004; Bellemain et al. 2010). In addition, the interspecies identification of the species from different isolates was carried out in this study. The results showed that although the *rDNA ITS* sequence was very conservative, there were variations on sequence and length among different isolates, and there was a genetic differentiation at various levels. Therefore, the ITS sequence analysis clearly differentiated between Egyptian and Saudi Arabian species. Similarly, Kuhls et al. (1997) used sequence analysis to differentiate between *T. reesei* and *T. longibrachiatum*. Many other researchers have used ITS sequences to identify *Trichoderma* spp. (Sharma et al. 2009; Shahid et al. 2013). Consequently, variation among individuals of the same species was noticed (Fahmi et al. 2016; Hassan et al. 2019; Mazrou et al. 2020); therefore, it is of great interest to identify the species at the molecular level.

Sequence variations

Sequence length varied greatly among the isolates. For the Egyptian isolates, the shortest sequence length was attributable to *T. bissettii* of 430 bp, while *T. yunnanens* had the longest length of 874 bp. The percentage of GC content was consistent among species, with a similar trend observed for the Saudi Arabian isolates. In Egyptian isolates, the GC content ranged from 55% for *T. yunnanens* to 58% for *T. longibrachiatum*. Similarly, for the Saudi Arabian isolates, the GC content ranged from 55% in *T. simmonsii* to 59% in *T. pseudokoningii*. The base frequencies of Egyptian and Saudi Arabian isolates were presented in Fig. 2a, b. Only 2 species from the Egyptian isolates showed noticeable variation in nucleotide base proportions. Sequences from *T. neokoningii* and *T. yunnanens* revealed that the percent of thymine was higher than other bases, and no other noticeable differences were observed. In an earlier study, Abd-Elsalam et al. (2010) observed a slight variation between *T. harzianum* and *T. longibrachiatum* in Saudi Arabian isolates. This indicated a wide range of interspecies variations, which is consistent with the idea of haplotype presence among species (Gupta et al. 2014). Abd-Elhamid et al. (2017) also reported that molecular identification is very important to identify the *Trichoderma* species, so it is recommended to be used for confirming the morphological approaches in the identification of *Trichoderma* isolates obtained from Egyptian soils.

Phylogenetic analysis

Cluster analysis was carried out as a pre-processing step to glean an insight into the data distribution. The results of the cluster analysis were shown in Fig. 3. The studied 36 isolates formed two large clusters. The first

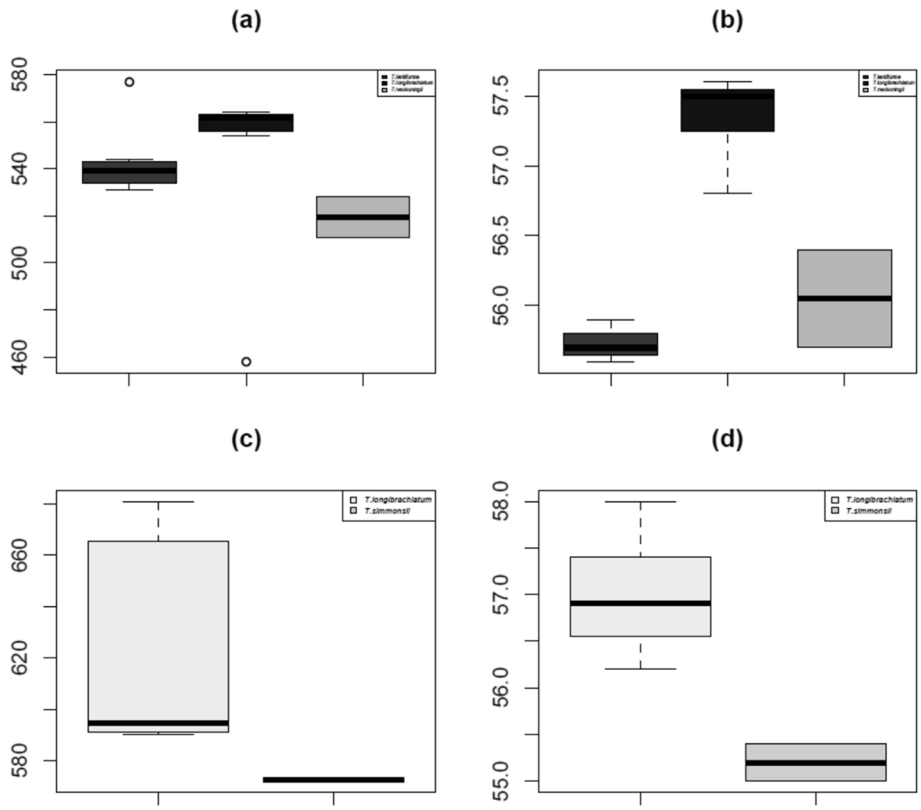

Fig. 2 Sequence length and percent of GC content of frequent species in Egyptian *Trichoderma* isolates (**a**, **b**) and Saudi Arabian *Trichoderma* isolates (**c**, **d**)

comprised all Saudi *Trichoderma* strains; this cluster was divided into two sub-clusters. One contained all 8 *T. longibrachiatum* and *T. pseudokoningii*, whereas the second comprised *T. lentiforme* and *T. simmonsii*. The second cluster comprised all the Egyptian *Trichoderma* species; this cluster incorporates three sub-clusters. The first comprised *T. yunnanens* and *T. neokoningii*. Although the isolates from Saudi Arabia and Egypt were grouped into different groups (Hassan et al. 2014; Fahmi et al. 2016), the Egyptian isolates showed more uniform patterns of clustering. *T. lentiforme* isolates from Egypt were grouped into one cluster; however, *T. crassum* was grouped with *T. lentiforme* Egyptian isolates. These results were similar to those obtained by Anees et al. (2010) in Pakistan, Abd-Elsalam et al. (2010) in Saudi Arabia, Hassan et al. (2014) in Egypt, and Lakhani et al. (2016) in India. Furthermore, *T. longibrachiatum* grouped with *T. pseudokoningii* in Saudi Arabian isolates. Besides, *T. lentiforme* and *T. crassum* grouped; however, they were subdivided into different subgroups. In an earlier study, Cázares-Garcia et al. (2013) and Hassan et al. (2014) observed a little variation between *T. lentiforme* and *T. longibrachiatum*. The low genetic differentiation among isolates from different regions is indicative of shred isolates between the two regions, nevertheless maintaining high genetic variability within each group.

Polymorphism and genetic diversity among species

The number of haplotypes identified among KSA isolates was higher than that of Egyptian isolates (11 and 8, respectively) (Table 3).

Also, the Saudi Arabian isolates showed 98% haplotype diversity compared to 76% among Egyptian isolates. However, the Saudi Arabian isolates showed lower nucleotide diversity (π_T) than their Egyptian counterparts.

AMOVA showed significant differentiation between Saudi Arabian and Egyptian isolates with the most genetic variance observed between rather than within regions. Also, it showed a significant genetic differentiation between the two groups (Φ_{ST} = 0.838, Table 4).

Most of the genetic variation was attributed to the between-group differences (83.77%). The highest genetic differentiation between isolates in the regions sampled was indicative of the presence of unique species in the two regions. This result was also supported by both cluster and phylogenetic analysis (Fig. 3). Additionally, the statistical analysis revealed further support of the presence of unique isolates where most of the genetic

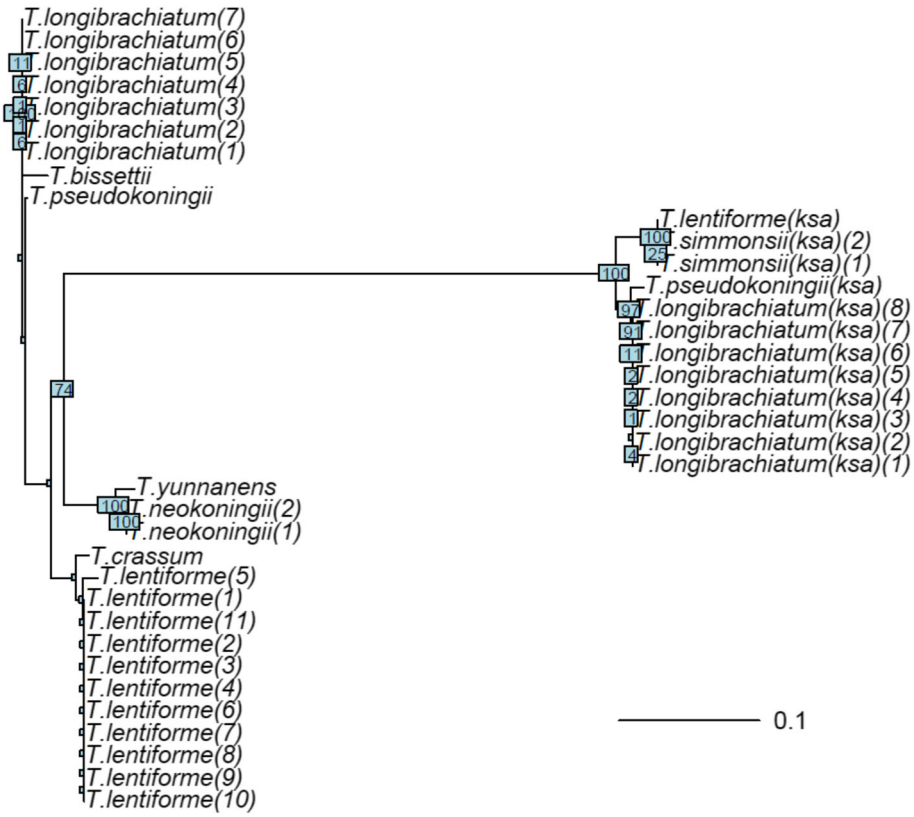

Fig. 3 Phylogenetic tree of Egyptian and Saudi Arabian *Trichoderma* isolates using the neighbor-joining method. Bootstrap values are shown in small boxes on each node

variance explained between the region's differences (Sharma et al. 2009; Shahid et al. 2013; Mazrou et al. 2020).

Isolation-by-distance

The Mantel test showed no correlation between the genetic and geographic distances in Egyptian and Saudi Arabian isolates ($r^2 = -0.38$, $r^2 = 0.23$, $P > 0.05$ for Egyptian and Saudi Arabian isolates, respectively) (Fig. 4a, b). The lack of correlation between genetic and geographic distances was indicative of the absence of isolation-by-distance pattern among isolates from the two regions. The observed pattern suggested the presence of high gene flow within each of the studied regions.

DNA sequencing of the 5.8S-ITS region was carried out, using specific primers within ITS1 and ITS4. The ITS region is one of the most reliable loci for the identification of a strain at the species level (Kullnig-Gradinger et al. 2002; Hassan 2014; Hassan et al. 2019). By comparing the sequences of the 5.8S-ITS region to the sequences deposited in GenBank, all the *Trichoderma* isolates can be identified to the species level with a homology percentage of at least 99%. The *TrichOKEY* search tool, a program specifically comparing ITS1 and ITS4 sequences to a specific database for *Trichoderma* generated from only vouchered sequences, was used to assess

Table 3 Nucleotide diversity estimates in *Trichoderma* isolates from Egypt and KSA, standard error between brackets

	Egypt	KSA
Number of isolates	24	12
Number of haplotypes (H)	8	11
Haplotype diversity (H_d)	0.757 (0.027)	0.985 (0.012)
Number of polymorphic sites (S)	51	35
Nucleotide diversity per site (θ_w)	0.0367 (0.002)	0.0259 (0.003)
Nucleotide diversity (π_T)	0.052 (0.002)	0.030 (0.002)

Table 4 Analysis of molecular variance (AMOVA) and fixation index Φ_{ST} among 21 *Trichoderma* isolates collected from KSA and Egypt

Source of variation	df	Sum of squares	Variance component	% variation	Φ_{ST}
Between regions	1	2633.819	162.644	83.770	0.838*
Within regions	34	1071.458	31.513	16.230	
Total	35				

*$P < 0.05$

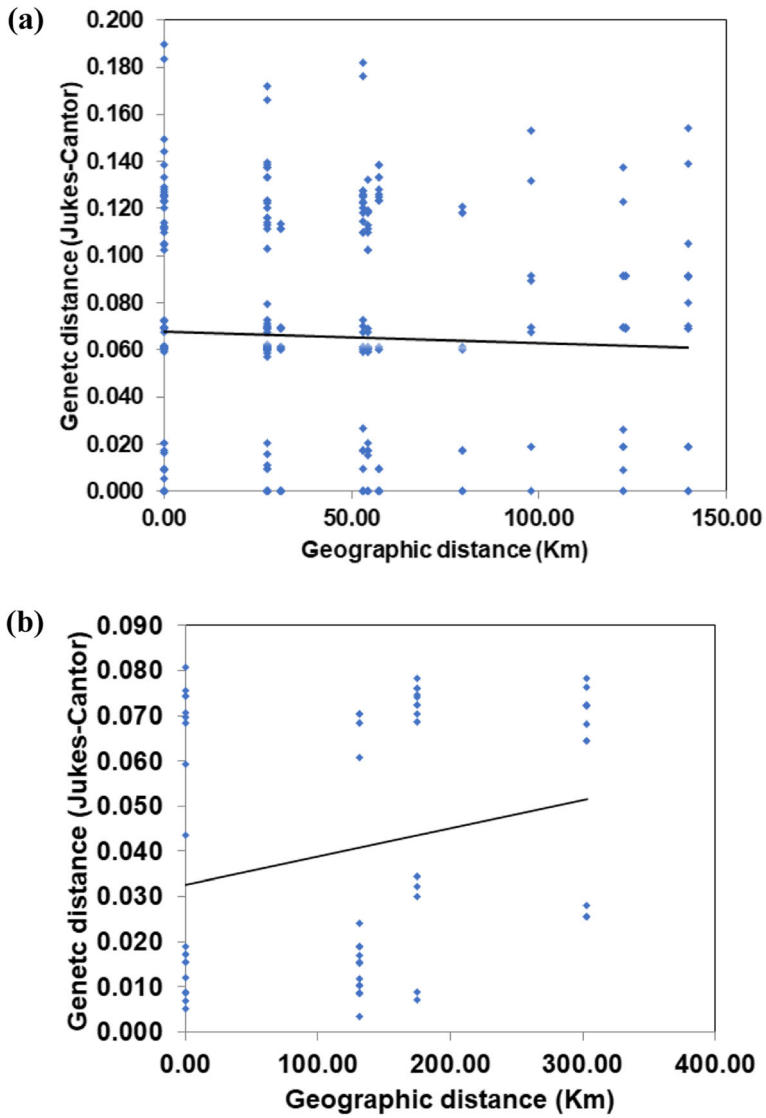

Fig. 4 Isolation-by-distance in *Trichoderma* isolates obtained from **a** Egypt and **b** Saudi Arabia. Genetic distance (Jukes-Cantor) vs. geographical distance (km), showing the regression line

the reliability of BLAST results. *TrichOKEY* has resulted in the successful identification of *Trichoderma* isolates (Anees et al. 2010; Hassan et al. 2014; Lakhani et al. 2016).

Conclusion

The obtained results have confirmed the efficiency and reproducibility of ITS as a powerful tool for the identification and genetic diversity assessment of *Trichoderma* spp. Egyptian isolates showed a more uniform phylogenetic pattern than Saudi Arabian isolates. Geographical differences have a significant impact on genetic differentiation of the local isolates where the most of genetic differences occurred among rather than within groups.

Abbreviations

ITS: Internal transcribed spacer; NCBI: National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; AMOVA: Analysis of molecular variance; IBD: Isolation-by-distance; PCR: Polymerase chain reaction

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Authors' contributions

All authors contributed significantly to this research and preparation of the manuscript. This study was conceived and designed by YM, AM, and MI. The laboratory work was carried out by MH, AB, and AH. Data were analyzed by YM, AS, and MI. The manuscript was written by AK, MI, and AS. The manuscript was revised by AB and AH. All authors have reviewed and agreed upon the final version of the manuscript.

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Availability of data and materials
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Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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