Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 22, No. (1) : 2020 : 38-43

ISSN-0972-3005

NUCLEOTIDE DIVERSITY AMONG *TRICHODERMA* SPP. COLLECTED FROM DIFFERENT LOCATIONS AND DETECTION OF WITHIN-SPECIES DIVERSITY

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(Received 23 October, 2019; accepted 28 November, 2019)

Key words : *Trichoderma, Nucleotide diversity, Cluster analysis, Saudi Arabia, Egypt.*

Abstract – The diversity of *Trichoderma* (Hypocreales, Ascomycota) species from Egypt and Saudi Arabia was achieved, and DNA barcoding of 21 strains based on the internal transcribed spacers 1 and 4 (ITS1 and 4) of the ribosomal RNA gene cluster and the partial sequence of the ITS1 and ITS4 gene revealed that the diversity of *Trichoderma* according to molecular findings there were two main species predominated in these isolates. *T. harzianum* consisted about 33.4 % of the isolates and *T. longibarchiatum* consisted about 19.1% of the isolates. Consequently, variation among individuals of the same species was apparent. The GC% in *T. viride* was 55%, in Egyptian isolates. Also, within Saudi Arabia isolates, the *T. longibrachiatum* varied in GC% was between 56% and 57%. Cluster analysis of the Egyptian isolates showed two groups, the first group subdivided into two subgroups. The first subgroup included *T. harzianum* isolates, the second sub group harboured *T. virdie* and *T. longibrachiatum*. The second group included *T. asperellum*, and *T. koninigigiopsis*. Also, the cluster analysis of Saudi Arabia isolates showed two groups. The first group subdivided into two subgroups; the first subgroup included *T. harzianum* isolates and *H. lixii*, the second subgroup harboured *T. longibrachiatum* isolates and *H. koningii*2. The second group included *T. atrovirdie*, *T. saturnisporum*, and *H. koningii1*. The Saudi Arabia isolates showed higher nucleotide diversity compared to its Egyptian counterpart. Number of haplotypes identified in Saudi Arabia isolates was two-fold higher than that of Egyptian isolates.

INTRODUCTION

Trichoderma commonly available in soil and root ecosystems has gained immense importance since last few decades due to its biological control ability against several plant pathogens. Some strains of *Trichoderma* like *T.harzianum*, *T. longibrachiatum*, *T. atroviride*, *T. viride*, *T. virens* and *T. koningii* are efficient as a biocontrol agent which have the ability to inhibit pathogen growth in the soil (Hassan *et al.,* 2013; Kredics *et al*., 2014; Parmar *et al.,* 2015), hence improving the overall health of the plant. Antagonistic microorganism, such as *Trichoderma* reduces. growth, survival of pathogen by different mechanism like competition antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. Such microorganisms are now available commercially and are used in crop management and practices (Parmar *et al.,* 2015). Although morphological identification of *Trichoderma* species is carried out initially soon after isolation but it becomes necessary to identify the species at molecular level (Hassan *et al.,* 2014). Molecular identification and characterization of fungal genomes is generally performed with a universal set of ITS markers that amplify the specified 18S rRNA fragment of the fungal DNA. Internal Transcribed Spacer (ITS) regions of nuclear DNA currently selected as the standard marker for fungal DNA barcoding and in analyzing the fungal diversity (Bellemain *et al.,* 2010; Schoch *et al.,* 2012; Hassan, 2014). Molecular identification through DNA barcoding has gained success where the International Union of Microbiological Societies has hosted an official website of International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (http://isth.info/). This website is designed as an open platform to gather the most

recent knowledge on *Hypocrea/Trichoderma* taxonomy and evolution. Additionally, it presents a collection of accessible tools for quick molecular identification of *Hypocrea/Trichoderma* based on DNA barcode. The Trich OKEY and TrichoBLAST available on the website assist in the quick identification of *Trichoderma/Hypocrea* species by locating the various combinations of ITS anchors in the user-entered nucleotide sequence (Druzhinina *et al.,* 2005; Srivastava *et al.,* 2014; Fahmi *et al.,* 2016). Bioinformatics is now commonly used in scientific research, speeding it up through the use of computers and information technology with high level of accuracy. In agriculture, bioinformatics has aided in decoding plant genomes and in studying the expression of various genes involved in a particular mechanism (Fahmi *et al.,* 2016). Use of bioinformatics in agriculture has led to the identification of genes and their evolution, diversification, function, mutation, expression in different environmental conditions and how a gene (or genome) codes for a protein (or proteome). Genomics and proteomics analysis can help in accelerating the scientific discoveries in agriculture that can be used to improve crop production, plant protection, disease diagnosis, etc. (Xue *et al.,* 2008; Srivastava *et al.,* 2014). *Trichoderma* is considered as a great benefit to Egyptian agriculture, it incorporates various factors such as being an environment friendly fungus, a biocontrol agent and plant growth promoter. In such research, bioinformatics has been applied together with the biotechnological aspect of fungal dissemination as it is cost-effective, time saving and produces better results to achieve the desired goals. A finite number of ITS sequences are being used for the molecular identification of the fungal genus *Trichoderma* that are limited to the successful and accurate identification of a few species only (Hassan, 2014; Fahmi *et al.,* 2016). So, an attempt has been made, using bioinformatics approach and tools, to generate new combinations of ITS primers that are both species and strain specific. The aim of this study was to utilize sequences of ITS1 and ITS2 region to analyze the genetic diversity of some *Trichoderma* isolates which collected from Egypt and Saudi Arabia across different geographic locations.

MATERIALS AND METHODS

A total of 21 *Trichoderma* isolates were collected from KSA and Egypt across different geographic locations.

DNA extraction, polymerase chain reaction and electrophoresis

Genomic DNA isolation

Fungal mycelia of the selected fungus strains were inoculated onto PDA broth for five days. Genomic DNA for each *Trichoderma* strain was extracted using DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions.

PCR amplification of 5.8S-ITS region

ITS1 and ITS2 regions together with the 5.8S gene in rRNA from both species were amplified using the primer pair of ITS-1 (52-TCC GTA GGT GAA CCT GCG G-32) and ITS-4 (52-TCC TCC GCT TAT TGA TAT GC-32) as designed by Hermosa *et al.* (2000) and Hassan, (2014). with some modifications. PCR amplification was performed in 25 µL reaction mixture containing 1X PCR buffer (DreamTaq™), 2 mmol/L MgCl2, 0.08 mmol/L of each primer, 160 mmol/L of each deoxynucleotide triphosphate, 1.25 U of Taq DNA polymerase (DreamTaq™DNA Polymerase) and about 10 ng of genomic DNA. PCR amplification was carried out in the C1000™ Thermo Cycler Bio-Rad, Germany, programmed as follows: an initial denaturation for 5 minutes at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing of primers at 56 °C for 45 seconds, and extension at 72 °C for 30 seconds, and the amplification was completed with one cycle of final extension at 72 °C for 5 minutes.

Sequence analysis of 5.8S-ITS region

The nucleotide sequences of 5.8S-ITS region were determined using the sequencer (Gene analyzer 3121) at Scientific Research Center, Biotechnology and Genetic Engineering Unit, Taif University, Saudi Arabia. The deduced sequence was aligned using BioEdit (Hall, 1999). The forward and reverse sequences were checked and edited manually when needed. Then, a consensus sequence was generated from each alignment. The sequencing data were compared against the GenBank database (http://www.ncbi. nlm.nih.gov/BLAST/), where a nucleotide blast program was chosen to identify the homology between the PCR fragments and the sequences in the GeneBank database.

Statistical analysis

Cluster analysis

Prior to statistical analysis, *Trichoderma* sequences collected from Egypt and Saudi Arabia were

individually aligned and manually edited using BioEdit (Hall 1999). To determine the genetic relationship among the isolates, the number of base substitution per site among all sequences was obtained based on the Maximum Composite Likelihood method (Tamura *et al.,* 2004) as implemented in MEGA4 (Tamura *et al.,* 2007). The phylogenetic tree was reconstructed based on Nei and Gojobori method (Nei and Gojobori 1986) using unweighted pair group method with arithmetic mean (UPGMA) as implemented in MEGA4. The relative robustness of individual branches was estimated by bootstrapping (Felesenstein 1985), in which 1000 bootstrapped trees were generated from the resampled data.

Nucleotide diversity

To determine the level of sequence variability, the following common genetic parameters were estimated for Egypt and Saudi Arabia sequences; 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.

Analysis of molecular variance (AMOVA)

To determine the level of genetic differentiation for the studied sequences, the isolates were dived into two groups; Egypt and Saudi Arabia. A hierarchical analysis of molecular variance (AMOVA) was performed using Arlequin 3.5 (Excoffier and Lischer 2010). The significance of the fixation index (Φ_{ST}, Excoffier *et al.* 1992) was tested by 1000 permutations of the data set.

Isolation-by-distance

The degree of isolation-by-distance was assessed by testing the association between geographic and genetic distances for all pairs of isolates within each group of sequences. Pairwise geographical distances between 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 refers to the idea that isolates may be spatially distributed across some regions. This term seeks to determine if there is a statically significant relationship between (1) genetic distance and geographic distance, and (2) the strength of this relationship. The main use for plots of genetic distances by geographic distance is to assess whether more distant genotype pairs are more different genetically (Bohonak, 2002). 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 tested based on a Mantel test (Mantel, 1967) with 1000 permutations, using program IBD (Jensen *et al.,* 2005).

RESULTS

Oligonucleotide barcode is a powerful tool for the identification of *Trichoderma* species and should be used as an alternative or as a complement to morphological methods. Therefore, the molecular data of ITS sequence is more trusted in characterization and identification of isolates under study. Consequently, according to molecular findings there were two main species predominated in these isolates. *T. longibarchiatum* represented 36% of the isolates while *T. harzianum* represented 64 % of the isolates. Most of these two species came from the isolates of the collected materials. 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 variation on sequence and length among different isolates, and there was a genetic differentiation in a various degrees. Consequently, variation among individuals of the same species was noticed. For the GC% in *T. viride* was 55%, in Egyptian isolates while the ITS length was about 600 bp. Also, within Saudi Arabian isolates, the *T. longibrachiatum* varied in length between 629 and 719 nucleotides and in GC% between 56% and 57%. This indicated a wide range of interspecies variations which is consistent with the idea of haplotype presence among species.

Cluster analysis of the Egyptian isolates showed two groups (Figure 1), the first group subdivided into two subgroups. The first subgroup included *T. harzianum* 1, 2, 3, and 4, the second subgroup harboured *T. virdie* and *T.longibrachiatum*. The second group included *T. asperellum 1*, *T. asperellum 9*, *T. koninigigiopsis 1*, and *T. koningiopsis 2*. Also, the cluster analysis of KSA isolates showed two groups (Figure 2). The first group subdivided into two subgroups; the first subgroup included *T. harzianum* 1, 2, 3, *H. lixii,* while the second subgroup harboured *T. longibrachiatum1, 2, 3* and *H. koningii2*. The second subgroup harboured *T.atrovirdie*, *T.saturnisporum*, and *H. koningii* 1.

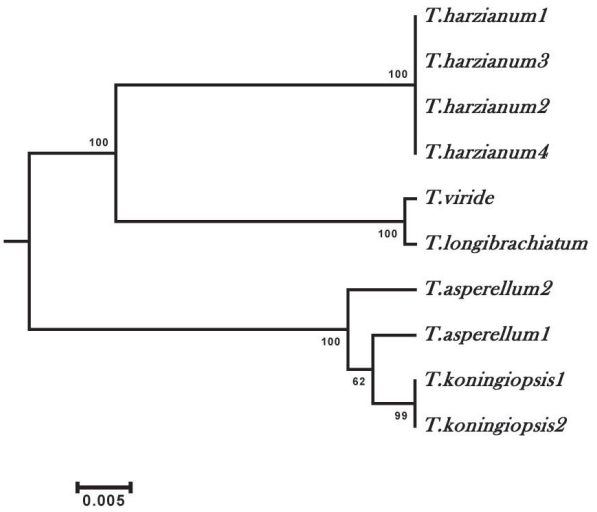

Fig. 1. Phylogenetic relationship of 10 *Trichoderma* isolates from Egypt using UPGMA method. Number next to the branches represents bootstrap values.

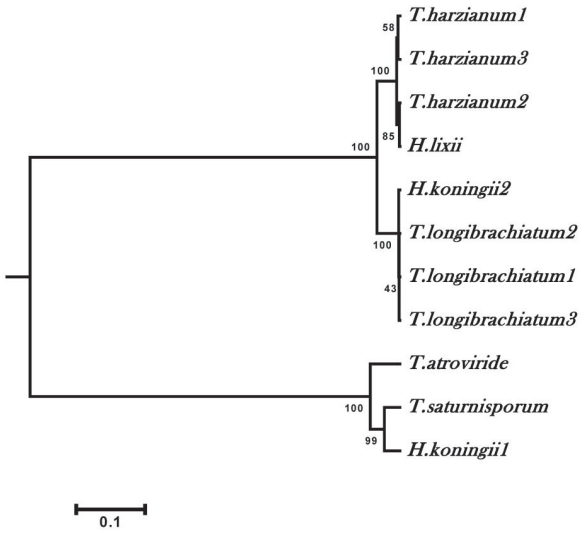

Fig. 2. Phylogenetic relationship of 11 *Trichoderma* isolates from Saudi Arabia using UPGMA method. Number next to the branches represents bootstrap values.

The Saudi Arabian isolates showed higher nucleotide diversity compared to its Egyptian counterpart (Table 1). Number of haplotypes identified in Saudi Arabia isolates was two-fold higher than that of Egyptian isolates (11 and 6, respectively). Also, the Saudi Arabia isolates showed 95% haplotype diversity compared to 80% in Egyptian isolates. Moreover, nucleotide diversity estimates showed 8 to 6-fold higher in Saudi Arabia compared to Egyptian isolates (16% vs. 2% and 24% vs. 4%, for Φ_w and π_T; respectively). AMOVA showed significant differentiation among Egyptian and Saudi Arabian isolates with most of genetic variance within rather than among groups. AMOVA showed significant but low genetic differentiation among the two groups ($\Phi_{ST} \approx 0.22$). However, most of genetic variation attributed to within group differences 78.18%. Analysis of log genetic distance and log geographic distance was insignificant in

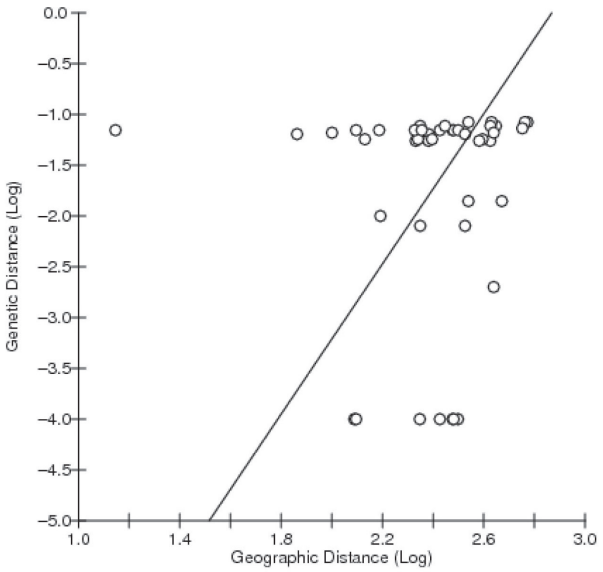

Fig. 3. Isolation by distance in 10 *Trichoderma* isolates obtained from Egypt. Genetic distance ($\ddot{O}_{ST}$, Jukes-cantor as evolutionary model) vs. geographical distance (Log geographical distance in km), showing the regression line.

Table 1. Nucleotide diversity estimates in *Trichoderma* isolates from Egypt and Saudi Arabia, standard error between brackets.

	Egypt	Saudi Arabia
Number of isolates	10	11
Number of haplotypes (*H*)	6	11
Haplotype diversity (H_d)	0.80 (0.067)	0.95 (0.019)
Number of polymorphic sites (S)	50	306
Nucleotide diversity per site (θ_w)	0.0279 (0.01)	0.165 (0.05)
Nucleotide diversity (π_T)	0.0452 (0.003)	0.249 (0.05)

Table 2. Analysis of molecular variance (AMOVA) and fixation index Φ_{ST} among 21 *Trichoderma* isolates collected from Egypt and Saudi Arabia

Source of variation	df	Sum of squares	Variance component	% variation	Φ_{ST}
Among groups	1	491.50	43.87	21.82	0.218*
Within groups	19	2987.08	157.21	78.18	
Total	20	3478.57	201.09		

*P< 0.05

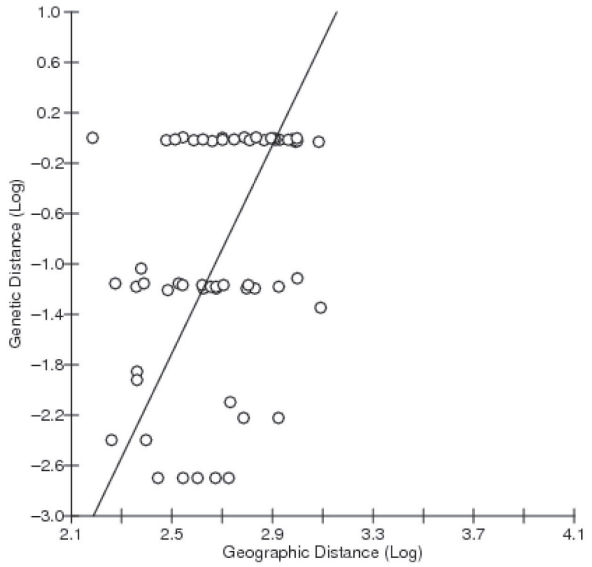

Fig. 4. Isolation by distance in 11 *Trichoderma*isolates obtained from Saudi Arabia. Genetic distance (Φ_{ST}, Jukes-cantor as evolutionary model) vs. geographical distance (Log geographical distance in km), showing the regression line.

Egyptian isolates, (r^2= 0.05, P> 0.05; Figure 3). However, significant correlation between log genetic distance and log geographic distance was observed in Saudi Arabian isolates (r^2 = 0.35, P<0.05; Figure 4).

DISCUSSION

Using molecular markers particularly those based on DNA sequence data are essential techniques to decipher the genetic variability of any species (Nagy *et al*., 2004). In the current study the genetic variability of 21 *Trichoderma* isolates from Egypt and Saudi Arabia collected from different geographic locations were assessed using sequence data. Although the isolates from Egypt and Saudi Arabia grouped into three groups (Figure 1 and 2, respectively), however, Egyptian isolates showed more uniform pattern of clustering. *T. harziannum* isolates from Egypt grouped in one cluster, however, *H. lixii* was grouped with *T. harziannum* in KSA isolates (Figure 2). Moreover, *T. longibrachiatum* grouped together with *H. koningii2* in Saudi Arabian isolates. In the current study *T.harzianuma* and *T. brachiatem* were in the same group, however, in different subgroups (Figure 1). The low genetic differentiation among isolates regions is indicative of shred isolates among the two regions; nevertheless, maintain high genetic variability within each group. Although AMOVA revealed that most of genetic differences are explained locally (within groups rather than among groups), it probably indicates presence of similar isolates among regions. In this study, DNA sequencing of the 5.8S-ITS region was carried out using specific primers 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). By comparing the sequences of the 5.8S-ITS region to the sequences deposited in GenBank, all of the *Trichoderma* isolates can be identified to species level with homology percentage of at least 99%. *Trich* OKEY search tool, a program that specifically compare ITS1 and ITS2 sequences to a specific database for *Trichoderma* generated from only vouchered sequences use to assess the reliability of BLAST results. *Trich* OKEY was used by many literatures and resulted in successful identification of *Trichoderma* isolates (Anees *et al*., 2010 and Hassan *et al*., 2014).

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