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Identification of *Trichoderma* spp. by DNA Barcode and Screening for Cellulolytic Activity

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Abstract

Species identification of isolates of *Trichoderma* from different locations of Nile delta of Egypt was performed and their cellulolytic activities were analyzed. On the basis of morphological characters, 75% of isolates were identified to species level and they were divided into four aggregate groups. Morphological characterization alone was insufficient to precisely identify *Trichoderma* species because they have relatively few morphological characters and limited variation that cause overlapping and misidentification of the isolates. Therefore, there was a necessity to use molecular technique to compensate for the limitations of morphological characterization. DNA sequencing of 5.8S-ITS region was carried out using specific primers ITS1 and ITS4. By comparing the sequences of the 5.8S-ITS region to the sequences deposited in GenBank using BLAST program all isolates can be identified to species level with homology percentage of at least 99%. In addition, TrichOKEY search tool, was used to assess the reliability of Genbank and results were in 92% agreement with the BLAST results. Data indicated a narrow species diversity and there were two main species predominated namely; *T. longibarchiatum* and *T. harzianum*. Distribution of nucleotides as well as the (G+C) content in ITS region of isolates indicated a wide range of interspecies variation. Finally, isolates were assessed for their total cellulase activities using a cellulose-azur method, for exoglucanases activity using Avicel method and for endoglucanases activity using carboxymethyl cellulose (CMC) and acid swollen cellulose methods. Consequently, eleven isolates were selected to be the best isolates among the 28 isolates used for cellulolytic ability.

Keywords: Morphological identification; Molecular identification; ITS region; Endoglucanases; Exoglucanases

Introduction

Trichoderma species are cosmopolitan fungi, frequently present in all types of soil, manure and decaying plant tissues. Their dominance in soil may be attributed to their diverse metabolic capability and aggressive competitive nature [1]. The economic importance of *Trichoderma* is due to their production of extracellular industrial enzymes, such as cellulolytic enzymes [2]. These enzymes are extensively used in industry such as degradation of cellulose materials which are used in textile and paper industry, in wastewater treatment and in biodegradation of plant lignocellulosic materials [3]. Cellulases are enzymatic complex, that comprises exo-β-1,4-glucanases (EC 3.2.1.91), endo- β -1,4-glucanases (EC 3.2.1.4) and β -1,4-glucanases (EC 3.2.1.21), that act synergistically in the hydrolysis of β-1,4-glycosidic bonds present in cellulose polymers for review see [4]. Therefore, many methods were developed to screen and select highly cellulases producing *Trichoderma*. The cellulose azure method is one of the choices where cellulose azure assay using dyed cellulose, a method of measuring primarily cellobiohydrolase activity by dye release [5]. The cellulose azure method is the most reliable qualitative assay for cellulolysis. This method also tests for simultaneous action of all cellulase enzymes. Degradation of cellulose results in the release of a bound dye, the vertical migration of which can be observed and the intensity of blue dye indicates the activity of cellulase [6]. Another assay was developed for screening of highly producing exoglucanase isolates using microcrystalline cellulose (Avicel) as substrate crystalline pure cellulose [7]. Also, cellulase can only degrade a specific substrate, therefore, screening of cellulase-producing *Trichoderma* can be performed on agar plates using a cellulosic substrate such as Avicel or carboxymethylcellulose (CMC) as carbon source for *Trichoderma* growth [8]. At the same time, quantitative assay of endoglucanase activity can be detected using carboxymethyl cellulose (CMC) by detection of clear zone around the colony using the Congo red stain [9].

Finally, another method for selection of hypercellulolytic *Trichoderma* spp. was using Petri plate with Walseth-cellulose as a sole source of Carbone [10].

Due to the diverse economical applications of *Trichoderma*, the correct species identification of *Trichoderma* is vital. Morphological characterization of *Trichoderma* isolates to species is currently based largely on many criteria such as conidial form, size, color and ornamentation, branching pattern, side branches, phialides and the formation of hyphal and elongation from conidiophores [11]. However, incorrect species identification using morphological characters is very common even for experts because of the high similarity of morphological characters [12]. However, recently many molecular methods and identification tools were developed, which are based on DNA sequence analysis. Therefore, it is now possible to identify every *Trichoderma* isolate to its species [13].

There are several molecular methods to characterize fungi species. Sequence analysis of the ITS region is the most famous method among molecular characterization methods. In eukaryotic cells, there are two internal transcribed spacers flanking the 5.8S gene. The two spacers, together with the 5.8S gene, are normally referred to as the ITS region [14]. The rRNA genes are universally conserved, while the ITS region

and intergenic spacer (IGS) are highly variable [15]. The ITS region is one the fastest evolving region and they may vary among species within a genus. Thus, the sequences of these regions can be used for identification of closely related species [16]. Sequence analysis of the ITS region have been used successfully to generate specific primers capable of differentiating closely related fungal species [17]. It has typically been most useful for molecular systematic study at species level, and even within species [18].

Finally, the use of ITS sequence analysis to identify an isolate at the species level involves submission of sequences to NCBI BLAST web site and identification of respective species on the basis of the degree of sequence similarity (e.g., >98%). Also, the International Subcommission on *Trichoderma* and Hypocrea Taxonomy has developed a method namely *Trich*OKEY 2. It is a program for molecular identification of *Trichoderma* on species levels based on an oligonucleotide ITS DNA BarCode (http://www.isth.info). Therefore, the objectives of the present study were: 1) species identification of some unknown isolates from different locations of Nile delta of Egypt and 2) documentation of their cellulolytic activity.

Materials and Methods

Samples collection

Different types of samples from soil, and decomposed organic matters, such as wheat straw and rice straw were collected from six governorates of Egypt. The samples were taken from a 15 cm depth and collected in sterile polyethylene bags, which were transported to the laboratory and stored at 4 °C until use [19].

Isolation of *Trichoderma* sp.

A serial dilution technique [20] was followed and a 10^3 dilution of each sample was prepared. 250 µL of each solution was pipetted onto a Potato Dextrose Agar (PDA) amended with 1 g/L streptomycin (Merck) plate and incubated at 28 °C for one week [21]. The culture plates were examined daily, individual colonies were isolated and uncommon colonies were reisolated onto a PDA plate [22]. Morphological characteristics were observed for identification and the plates were stored at 4°C [23]. In addition, four isolates were kindly provided by Prof. Dr Medhat Aldenary, Faculty of Agriculture, Tanta University and three isolates from our laboratory were included in this investigation.

Identification of *Trichoderma* isolates

Morphological identification

Two techniques, visual observation on petri dishes and micro-morphological studies in slide culture, were adopted for identification of *Trichoderma* species. For visual observation, the isolates were grown on PDA agar for 3-5 days. The mode of mycelia growth, color, odor and changes of medium color for each isolate were examined every day. For micromorphological studies, a slide culture technique was used. Examination of the shape, size, arrangement and development of conidiophores or phialides provided a tentative identification of *Trichoderma* spp. [24]. Further verified and confirmed identification was performed at Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt.

Molecular identification

Molecular identification with sequences analysis of the internal transcribed spacer (ITS) 1 and 4 of ribosomal DNA (rDNA) was carried.

a) DNA extraction and purification: Isolates were inoculated onto Potato Dextrose Broth and maintained on a rotary shaker at 25°C for five days. Fungal cells were collected by filtration and homogenized in liquid nitrogen. Genomic DNA was extracted from mycelia of *Trichoderma* isolates using EZ-10 SPIN COLUMN GENOMIC DNA MINIPREPS KIT (BIO BASIC INC.) according to the manufacturer's instructions. Both the purity and quantity of DNA were checked by agarose gel electrophoresis.

b) PCR was used to amplify the internal transcribed spacer regions 1 and 4 (ITS1 and 4) of the rRNA gene cluster using the following primer pair amplified as designed by Hermosa et al., [25] with modifications (Table 1).

PCR amplifications were performed in a total volume of 50 µl by mixing 200 ng of the template DNA with 0.6 µM of each primer, 25 µl of 2x MyTaq ™ Red Mix. DNA thermal cycles was used with the following PCR profile: an initial denaturation of 5 min at 95°C, followed by 35 cycles (1.0 min at 94°C, 2 min at 55°C and 2 min at 72 °C) with a final extension of 5 min at 72°C. Aliquots (5 µl) were analyzed by electrophoresis in 1.2% (wt/vol) agarose gel in 1× TBE buffer (0.045 M Tris-borate and 1 mM EDTA, pH 8.2) and stained with ethidium bromide (final concentration 0.2 mg ml⁻¹).

c) DNA sequencing: purified and sequencing of the PCR products (amplified DNA) were performed by Macrogen Inc. (South Korea) using state-of-the-art robotics and instrumentation.

d) Sequence submission: Sequences were submitted to GenBank through Bankit (a World Wide Web sequence submission server available at NCBI home page). The sequences are available on line (http://www.ncbi.nlm.nih.gov) and can be located by accession numbers: KT964120, KT964121, KT964122, KT964123, KT964124, KT964125, KT964126, KT964127, KT964128, KT964129, KT964130, KT964131, KT964132, KT964133, KT964134, KT964135, KT964136, KT964137, KT964138, KT964139, KT964141, KT964143, KT964144, KT964140, KT964142, KT964145, KT964146, and KT964147

e) Sequence Analysis: The sequences of ITS1-5.8S-ITS4 region of all isolates were analyzed using Molecular Evolutionary Genetics Analysis (MEGA4 version 5.10.). The sequencing data were compared against the Gene Bank 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 on the Gene Bank database. Besides, the 5.8S-ITS sequences were compared to a specific database for *Trichoderma* using *Trich*OKEY 2 program, which available online from the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH, www.isth.info) [13].

Screening of Trichoderma isolates for cellulolytic activity

Total cellulases (Cellulose azure agar): This method carried

Primer Name	Sequence(5'- 3')	Mer	GC%
ITS-1	5' - TCC GTA GGT GAA CCT GCG G - 3'	19	63.16
ITS-4	5' - TCC TCC GCT TAT TGA TAT GC - 3'	20	45

Table 1: Molecular identification with sequences analysis of the internal transcribed spacer.

out as described by Pointing SB et al., [6]. Cellulolysis was assessed by monitoring release of azure dye from cellulose-dye complex and diffusion into clear agar not containing cellulose-azure. As follow: Cellulolysis basal medium (CBM) 0.5 % (w/v) Diammonium tartrate (C4HI2N2O6), 0.01% (w/v) yeast extract , 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0. 1% (w/v) KH_2PO_4 0.001% (w/v) $CaCl_2.2H_2O$ supplemented with 1.6% w/v agar was transferred to 25mL glass culture bottles, autoclaved, allowed to solidify. Then carefully aliquot of 1 ml of CBM medium supplemented with 1% w/v cellulose azure (azure I dye, sigma C.I.52010) aseptically was loaded on the surface of the solidified agar as an overlay. Media were inoculated with 5×10^5 spores of *Trichoderma* isolates and incubated at 28°C in darkness. Migration of dye into the clear lower layer indicated the presence of Cellulases. Cellulolysis was assessed by monitoring release of azure dye from cellulose-dye complex and diffusion into clear agar. The relative cellolytic activity of each isolate was scored by comparing the intensity of blue color of the medium with standard blue color scale of 1 to 10 (maximum) over an incubation period of 15 days [26].

Exoglucanases

A method by Bose RG et al., with some modifications was carried out to screen for high cellulase-producing isolates [27]. The basic medium consisted of a Mandel's mineral solution supplemented with trace elements and 2% agar [28]. Avicel PH-101 NF was used as the sole source of carbon at a concentration of 1%. 0.01% Triton-X 100 was added to limit the colony size and to facilitate the screening of isolates. The cellulose agar plates were seeded with 5×10^5 spores in 20μl and incubated at 28°C for 21 days until clear zones around fungal colonies were observed. Then the diameter of the clear zone was measured.

Endoglucanases

Dye staining of carboxymethylcellulose agar (CMC agar): The cellulolytic activity of fungal strains was determined by their ability to grow and form cleared zones around colonies on Mandel's agar medium (MAM) supplemented with 0.5% w/v low viscosity carboxymethyl cellulose (CMC) [29]. The medium was autoclaved, dispensed into Petri dishes, allowed to solidify and inoculated with 5×10^5 spores in 20 μl *Trichoderma* isolates and incubated at 28°C. After growth for 5 days, the plates were flooded with 1% aqueous Congo red and allowed to stain for 15 minutes. The stain was washed off from the agar surface with distilled water and the plates were then flooded with 1 M NaCl to destain for 15 minutes. The NaCl solution was then removed. CMC degradation around the colonies appears as a yellow-opaque area against a red color for undegraded CMC. The diameter of the clear zone was measured [6].

Walseth cellulose Plate-clearing assay

A method by Khiyami et al., with some modifications was carried out to screen for high cellulase-producing isolates [26]. The basic medium consisted of a Mandel's mineral solution supplemented with trace elements and 2% agar. Phosphoric acid-swollen cellulose (Walseth cellulose) was used as the sole source of carbon at a concentration of 1%. 0.01% Triton-X 100 was added to limit the colony size and to facilitate the screening of isolates [30]. The plates were seeded with 5×10^5 spores in 20 μl and incubated at 28°C for 6 days until clear zones around fungal colonies were observed. Then the diameter of the clear zone was measured.

Statistical analysis

One-way ANOVA followed by Duncan's multiple range test

(DMRT) was used to assess the statistical significance of changes in all indices with the level of significant difference set at $p<0.05$. Statistical analysis software (SPSS 16.0.0 release; SPSS Inc., Chicago, IL) was used for all analyses.

Results

A total of 28 fungal isolates were analyzed in this investigation. 21 isolates were collected samples from six governorates of Nile delta of Egypt namely; Menufia, Gharbia, Kafr el Sheikh, Sharqia, Dakahlia and Ismalia (Figure 1). In addition, four isolates were given from Tanta university collection and three isolates were from our laboratory collection. Preliminary screening was carried out for all isolates which showed that these fungal isolates were *Trichoderma* species. The type of samples and isolate codes were shown in Table 2.

Morphological identification of the 28 *Trichoderma* isolates was performed and resulted in identification of all 28 isolates as *Trichoderma* genus (Table 3). However, as for species identification, seven isolates were not identified to species level (25%). The rest of the isolates (75%) were species identified and they divided into four groups. Seven isolates were identified as *Trichoderma harzianum*, six isolates as *Trichoderma hamatum*, five as *Trichoderma viride* and three as *Trichoderma koningii*.

As for molecular identification, genomic DNA of all *Trichoderma* isolates was extracted. PCR Amplification of 5.8S-ITS Region was conducted using specific primers ITS1 and ITS4. 5.8S-ITS DNA fragments were amplified from all *Trichoderma* isolates and PCR products were sequenced. Then, BLAST program was used to determine the species identity of *Trichoderma* isolates. The BLAST results were presented in Table 4. According to the BLAST results, 15 isolates were identified as *T. longibarchiatum* (54%) and seven isolates were identified as *T. harzianum* (25%), while five isolates were identified as *T. album, T. virens, T.viride, T. asperellum* and *T. saturnisporum*. Only, one isolate MNF-MSH-*Trich*23 could not be identified to species level. Also, *Trich*OKEY search was also used to assess the reliability of BLAST results. Three isolates were not identified to species level namely, MNF-MSH-*Trich*4, MNF-MSH-*Trich*22, MNF-MSH-*Trich*23
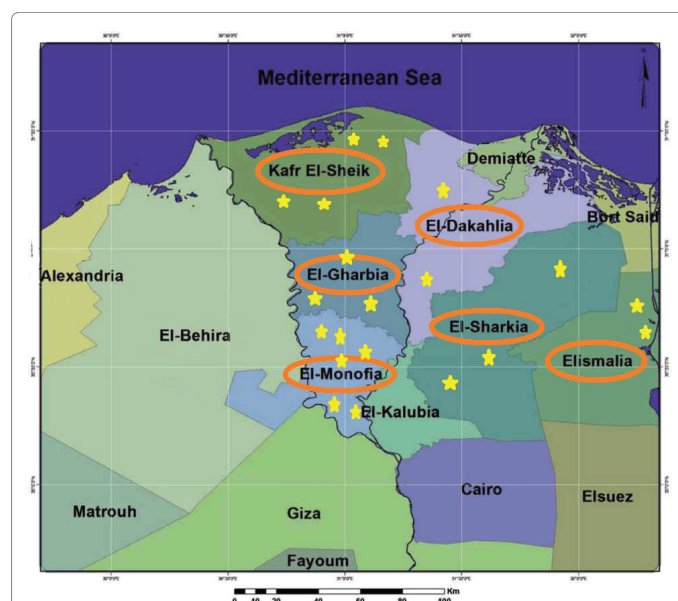

Figure 1: Map of the isolation sites in six governorates in the North Nile Delta in Egypt.

tSample type	Source	Isolates code
Decomposed Wheat straw	Menoufia	MNF-MSH-*Trich*1
Field soil	Menoufia	MNF-MSH-*Trich*2
Decomposed rice straw	Gharbiya	MNF-MSH-*Trich*3
Decomposed rice straw	Kafr el Sheikh	MNF-MSH-*Trich*4
Field soil	Sharqiya	MNF-MSH-*Trich* 5
-	Genetics department collection	MNF-MSH-*Trich*6
Decomposed rice straw	Sharqiya	MNF-MSH-*Trich*7
Decomposed Wheat straw	Menoufia	MNF-MSH-*Trich*8
Decomposed rice straw	Gharbiya	MNF-MSH-*Trich*9
Decomposed rice straw	Kafr el Sheikh	MNF-MSH-*Trich*10
Decomposed tree leaves	Menoufia	MNF-MSH-*Trich*11
-	Genetics department collection	MNF-MSH-*Trich*12
-	Genetics department collection	MNF-MSH-*Trich*13
Decomposed rice straw	Dakahlia	MNF-MSH-*Trich*14
Decomposed rice straw	Dakahlia	MNF-MSH-*Trich*15
Decomposed tree leaves	Ismaillia	MNF-MSH-*Trich*16
-	Tanta university collection	MNF-MSH-*Trich*17
-	Tanta university collection	MNF-MSH-*Trich*18
-	Tanta university collection	MNF-MSH-*Trich*19
-	Tanta university collection	MNF-MSH-*Trich*20
Field soil	Kafr el Sheikh	MNF-MSH-*Trich*21
Decomposed rice straw	Kafr el Sheikh	MNF-MSH-*Trich*22
Decomposed Wheat straw	Sharqiya	MNF-MSH-*Trich*23
Garden soil	Menoufia	MNF-MSH-*Trich*24
Decomposed rice straw	Gharbiya	MNF-MSH-*Trich*25
Garden soil	Ismaillia	MNF-MSH-*Trich*26
Decomposed Wheat straw	Menoufia	MNF-MSH-*Trich*27
Decomposed Wheat straw	Menoufia	MNF-MSH-*Trich*28

Table 2: *Trichoderma* isolate codes, isolate origins and types of sample used in this study.

Species name	Isolates code
Trichoderma harzianum	MNF-MSH-*Trich*1
Trichoderma viride	MNF-MSH-*Trich*2
Trichoderma hamatum	MNF-MSH-*Trich*3
Trichoderma Sp.	MNF-MSH-*Trich*4
Trichoderma hamatum	MNF-MSH-*Trich* 5
Trichoderma hamatum	MNF-MSH-*Trich*6
Trichoderma koningii	MNF-MSH-*Trich*7
Trichoderma Sp.	MNF-MSH-*Trich*8
Trichoderma hamatum	MNF-MSH-*Trich*9
Trichoderma koningii	MNF-MSH-*Trich*10
Trichoderma harzianum	MNF-MSH-*Trich*11
Trichoderma harzianum	MNF-MSH-*Trich*12
Trichoderma Sp.	MNF-MSH-*Trich*13
Trichoderma viride	MNF-MSH-*Trich*14
Trichoderma viride	MNF-MSH-*Trich*15
Trichoderma viride	MNF-MSH-*Trich*16
Trichoderma viride	MNF-MSH-*Trich*17
Trichoderma harzianum	MNF-MSH-*Trich*18
Trichoderma harzianum	MNF-MSH-*Trich*19
Trichoderma koningii	MNF-MSH-*Trich*20
Trichoderma Sp.	MNF-MSH-*Trich*21
Trichoderma harzianum	MNF-MSH-*Trich*22
Trichoderma Sp.	MNF-MSH-*Trich*23
Trichoderma harzianum	MNF-MSH-*Trich*24
Trichoderma Sp.	MNF-MSH-*Trich*25
Trichoderma Sp.	MNF-MSH-*Trich*26
Trichoderma hamatum	MNF-MSH-*Trich*27
Trichoderma hamatum	MNF-MSH-*Trich*28

Table 3: The identities of *Trichoderma* isolates identified morphologically used in this study.

and MNF-MSH-*Trich*27. Similarly, MNF-MSH-*Trich*23 isolate was only identified until genus level. However, 24 isolates were identified to species level (Table 5). They were divided into two main groups namely; *T. longibarchiatum* (14 isolates) and *T. harzianum* (seven isolates). In addition to three individual groups namely; *T. virens*, *T. asperellum* and *T. koningiopsis*. Based on the *Trich*OKEY results obtained, 22 out of 44 (92%) isolates were in agreement with the results obtained from GenBank database. The disagreement was in MNF-MSH-*Trich*15 and MNF-MSH-*Trich*19 isolates.

Finally, Table 6 showed the distribution of nucleotides as well as the guanosine + cytosine (G+C) content in ITS1+5.8S+ITS2 complete sequences. A total length of ITS1-5.8S-ITS2 sequence region ranged from 546 bp to 1028 bp in all accessions. While, the G+C content ranged from 24.2% to 68.8%.

Total cellulase, endoglucanase, and exoglucanase activities were determined using cellulose azure agar method, carboxymethyl cellulose (CMC), and microcrystalline cellulose (SIG) as substrates, respectively, according to standard conditions described by [5] (Table 7).

First isolates were assessed for their total cellulase activities using Dye diffusion from a cellulose-dye complex (cellulose azure agar) method. Ten isolates recorded 10 in this scale means that they have good cellulolysis ability (Table 7). 12 isolates recorded ten namely; MNF-MSH-*Trich*2, MNF-MSH-*Trich*5, MNF-MSH-*Trich*7, MNF-MSH-*Trich*8, MNF-MSH-*Trich*9, MNF-MSH-*Trich*10, MNF-MSH-*Trich*14, MNF-MSH-*Trich*19, MNF-MSH-*Trich*21, MNF-MSH-*Trich*22, MNF-MSH-*Trich*26 and MNF-MSH-*Trich*27. Two isolates recorded seven

MNF-MSH-*Trich*16 and MNF-MSH-*Trich*17. The rest of the isolates recorded between eight and nine.

Secondly Avicel is used for measuring exoglucanase activity because it has a low degree of polymerisation of cellulose and it is relatively inaccessible to attack by endoglucanases despite some amorphous regions. Six isolates showed highly significant Avicellulases activity namely; MNF-MSH-*Trich*1, MNF-MSH-*Trich*7, MNF-MSH-*Trich*8, MNF-MSH-*Trich*14, MNF-MSH-*Trich*22 and MNF-MSH-*Trich*26 (Table 7).

Thirdly, Trichoderma isolates were identified for their endoglucanase activities on plate clearing assay by using carboxymethyl cellulose (CMC) and Congo red dye and by the formation of clear zone diameter. Based on measurements, six isolates gave clear zones of cellulase activity having diameter significantly larger than other isolates namely; MNF-MSH-*Trich*5, MNF-MSH-*Trich*7, MNF-MSH-*Trich*9, MNF-MSH-*Trich*10, MNF-MSH-*Trich*14 and MNF-MSH-*Trich*21. Carboxymethylcellulose (CMC) is a substrate for endoglucanase and so can be used as a test for endoglucanase and β-glucosidase activity (Table 7).

Another assay was conducted for endonuclease activities identification by using plate clearing assay of acid swollen cellulose as a substrate. Recording clearance of cellulose within the growth medium can be difficult to assess, particularly with dense or dark hyphal growth (Table 7). Six isolates showed high activities namely; MNF-MSH-*Trich*1, MNF-MSH-*Trich*8, MNF-MSH-*Trich*14, MNF-MSH-*Trich*22, MNF-MSH-*Trich*26 and MNF-MSH-*Trich*27.

Species name (Anamorph)	NCBI GenBank accession number	Max identification %	E value	Alignments Description	Isolates code
T. harzianum	KT964120	99	0.0	*Trichoderma harzianum* KC551815.1	MNF-MSH-*Trich*1
T. harzianum	KT964121	98	0.0	*Hypocrea lixii* HQ149775.1	MNF-MSH-*Trich*2
T. longibrachiatum	KT964122	99	0.0	*Trichoderma longibrachiatum* JX908726.1	MNF-MSH-*Trich*3
T. harzianum	KT964123	94	0.0	*Hypocrea lixii* EF596966.1	MNF-MSH-*Trich*4
T. harzianum	KT964124	93	0.0	*Hypocrea lixii* JX173856.1	MNF-MSH-*Trich*5
T. longibrachiatum	KT964125	99	0.0	*Trichoderma longibrachiatum* LN713973.1	MNF-MSH-*Trich*6
T. longibrachiatum	KT964126	98	0.0	*Trichoderma longibrachiatum* JX173872.1	MNF-MSH-*Trich*7
T. longibrachiatum	KT964127	99	0.0	*Trichoderma longibrachiatum* LN713973.1	MNF-MSH-*Trich*8
T. longibrachiatum	KT964128	98	0.0	*Trichoderma longibrachiatum* KP256797.1	MNF-MSH-*Trich*9
T. longibrachiatum	KT964129	99	0.0	*Trichoderma longibrachiatum* KM103341.1	MNF-MSH-*Trich*10
T. longibrachiatum	KT964130	99	0.0	*Trichoderma longibrachiatum* JX173872.1	MNF-MSH-*Trich*11
T. longibrachiatum	KT964131	100	0.0	*Trichoderma longibrachiatum* KP268994.1	MNF-MSH-*Trich*12
T. longibrachiatum	KT964132	99	0.0	*Trichoderma longibrachiatum* JX908727.1	MNF-MSH-*Trich*13
T. harzianum	KT964133	100	0.0	*Trichoderma harzianum* KT027929.1	MNF-MSH-*Trich*14
T. album	KT964134	97	0.0	*Trichoderma album* JF304318.1	MNF-MSH-*Trich*15
T. longibrachiatum	KT964135	99	0.0	*Trichoderma longibrachiatum* KM103341.1	MNF-MSH-*Trich*16
T. virens	KT964136	96	0.0	*Trichoderma virens* JX908730.1	MNF-MSH-*Trich*17
T. asperellum	KT964137	97	0.0	*Trichoderma asperellum* GU589849.1	MNF-MSH-*Trich*18
T. viride	KT964138	99	0.0	*Hypocrea rufa* JQ040401.1	MNF-MSH-*Trich*19
T. longibrachiatum	KT964139	99	0.0	*Trichoderma longibrachiatum* KF986656.1	MNF-MSH-*Trich*20
T. longibrachiatum	KT964141	99	0.0	*Trichoderma longibrachiatum* GU055306.1	MNF-MSH-*Trich*21
T. saturnisporum	KT964143	99	0.0	*Trichoderma saturnisporum* LM653121.1	MNF-MSH-*Trich*22
-	KT964144	-	-	-	MNF-MSH-*Trich*23
T. longibrachiatum	KT964140	95	0.0	*Trichoderma longibrachiatum* JN039070.1	MNF-MSH-*Trich*24
T. harzianum	KT964142	99	0.0	*Hypocrea lixii* GU589848.1	MNF-MSH-*Trich*25
T. harzianum	KT964145	99	0.0	*Trichoderma harzianum* KC551815.1	MNF-MSH-*Trich*26
T. longibrachiatum	KT964146	95	0.0	*Trichoderma longibrachiatum* KM203582.1	MNF-MSH-*Trich*27
T. longibrachiatum	KT964147	92	0.0	*Trichoderma longibrachiatum* KJ561614.1	MNF-MSH-*Trich*28

Table 4: Molecular identification of *Trichoderma* isolates used in this study by NCBI BLASTN.

Discussion

The present study basically is a domestic assessment of *Trichoderma* representing six Egyptian governorates. Morphological characterization was conventionally used in the identification of *Trichoderma* species, and it remains as a potential method to identify *Trichoderma* species [31]. According to this investigation results, morphological and cultural characteristics could not distinguish *Trichoderma* isolates up to the species level efficiently. Similarly, most of the researchers were facing difficulty with regard to the identification of the *Trichoderma* species owing to the higher level of structural similarities [32]. Therefore, information from morphological study alone is insufficient to precisely identify a *Trichoderma* species because *Trichoderma* species have relatively few morphological characters and limited variation that may cause overlapping and misidentification of the isolates [31]. Besides that, morphological characteristics are influenced by culture conditions [33]. Therefore, there is a necessity to use molecular technique to compensate for the limitations of morphological characterization.

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 [34]. By comparing the sequences of the 5.8S-ITS region to the sequences deposited in GenBank, all of the *Trichoderma* isolates except MNF-MSH-*Trich*23 can be identified to species level with homology percentage of at least 99%. However, Druzhinina et al., mentioned that GenBank database contain many sequences of *Trichoderma*

isolates which may have been incorrectly identified and occurred under a false name [35]. Hence, *Trich*OKEY search tool, a program that specifically compare ITS1 and ITS2 sequences to a specific database for *Trichoderma* generated from only vouchered sequences were used to assess the reliability of BLAST results. *Trich*OKEY was used by many literatures and resulted in successful identification of *Trichoderma* isolates [31]. From the *Trich*OKEY results obtained all isolates except isolate MNF-MSH-*Trich*23 were identified. The results were in 92% agreement with the BLAST results. Isolate MNF-MSH-*Trich*23, however, was identified as an unknown *Trichoderma* species. Also, morphological data of MNF-MSH-*Trich*23 was insufficient to be identified. The main difference between the two data bases results were in two isolates MNF-MSH-*Trich*15 and MNF-MSH-*Trich*19. Results of this investigation confirmed the same difficulty facing other researchers where the morphological characterization is not reliable. The morphological identification agreed with molecular identification in only one isolate MNF-MSH-*Trich*1. It was concluded that, the morphological characterization is not reliable for identification of the isolates and oligonucleotide barcode is a powerful tool for the identification of *Trichoderma* species and should be useful 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* consisted about 50% of the isolates and *T. harzianum* consisted about 25% of the isolates. Most of these two species came from the isolates of the collected materials. This data indicted a

Identification reliability	Species identification	Clade	Section	Isolates code
high	*Trichoderma harzianum*	Harzianum	Pachybasium	MNF-MSH-*Trich*1
high	*Trichoderma harzianum*	Harzianum	Pachybasium	MNF-MSH-*Trich*2
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*3
-	The query sequence is incomplete			MNF-MSH-*Trich*4
high	*Trichoderma harzianum*	Harzianum	Pachybasium	MNF-MSH-*Trich*5
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*6
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*7
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*8
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*9
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*10
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*11
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*12
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*13
high	*Trichoderma harzianum*	Harzianum	Pachybasium	MNF-MSH-*Trich*14
high	*Trichoderma harzianum*	Harzianum	Pachybasium	MNF-MSH-*Trich*15
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*16
high	*Trichoderma virens*	Virens	Pachybasium	MNF-MSH-*Trich*17
high	*Trichoderma asperellum*	Pachybasium "A" or Hamatum	Trichoderma	MNF-MSH-*Trich*18
high	*Trichoderma koningiopsis*	Viride	Trichoderma	
standard	*Trichoderma ovalisporum*	Viride	Trichoderma	MNF-MSH-*Trich*19
high	*Trichoderma asperellum*	Pachybasium "A" or Hamatum	Trichoderma	
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*20
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*21
low	*Trichoderma sp. MA 3642*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*22
The sequence was not identified. ITS1 and ITS2 regions of Hypocrea/Trichoderma were not detected. The 5.8S RNA gene is found which is not diagnostic for species identification.				MNF-MSH-*Trich*23
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*24
high	*Trichoderma harzianum*	Harzianum	Pachybasium	MNF-MSH-*Trich*25
high	*Trichoderma harzianum*	Harzianum	Pachybasium	MNF-MSH-*Trich*26
The query sequence is incomplete				MNF-MSH-*Trich*27
high	*Trichoderma longibrachiatum*	Longibrachiatum	Longibrahiatum	MNF-MSH-*Trich*28

Table 5: Molecular identification of *Trichoderma* isolates used in this study by *Trich*OKEY2.

narrow species diversity of *Trichoderma* isolates in the middle Delta area of Egypt. Gherbawy et al., found only one species *T. harzianum* in the area of Delta of Egypt [36]. Other results from south of Egypt indicated that the two species *T. harzianum* and *T. longibarchiatum* were present [37]. The predominant *T. longibrachiatum* is the youngest clade of *Trichoderma* evolutionarily [38]. Also, it is a soil fungus which is found all over the world but mainly in warmer climates [39]. The second predominant species *T. harzianum* is the most commonly reported species in the genus, occurring in diverse ecosystems and ecological niches [40]. Therefore, this low degree of diversity may occur due to specific biotic or abiotic factors such as plant species, microbial competition, soil physical and chemical properties and application of pesticides or fertilizers in the geographical region [4].

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 instance within *T. longibarchiatum* the GC% varied from 42.6% to 68.8%, while the ITS length was between 561 and 888 nucleotides. Also, within *T. harzianum* variation was in length between 546 and 1028 nucleotides

and in GC% was between 49.6% and 55.7%. This indicated a wide range of interspecies variatins which is consistent with the idea of haplotype presence among species [4].

As mentioned before, *Trichoderma* sp. exhibited the highest cellulose activity and consistency in producing cellulase when compared with other microorganisms [41]. Total cellulase activity was measured using cellulose azure agar method. It is highly recommended as it is the most reliable qualitative assay for cellulolysis [42]. The experiment for cellulose azure method was conducted for 15 days, the period of time will allow *Trichoderma* to degrade link of azure and cellulose. Fadel et al., reported that each microorganism have the different incubation time to do the enzymatic reaction to the substrate [43]. Further analysis was conducted to differentiate isolates into two categories either highly endonucleases or exonuleases producing isolates. Avicel has been used for measuring exoglucanase activities that cleave the accessible ends of cellulose modules to liberate glucose and cellobiose [44]. To identify highly producing endoglucanases producing isolates two methods were carried out namely; CMC and Walseth cellulose plate assay. CMC is chemically modified and used to resemble the cellulose and Congo red can only colorize the cellulose and the area that is decolorized indicated the endoglucanases enzyme activity [45]. Hydrolysis of cellulose, especially the endoglucanases enzyme, is crucial, as it initiates the next

Isolate code	T %	C %	A %	G %	G+C (mol %)	Length (nt) analyzed dataset
MNF-MSH-*Trich*1	26.7	23.6	23.7	26.0	49.6	1028.0
MNF-MSH-*Trich*2	22.0	24.8	24.4	28.8	53.6	833.0
MNF-MSH-*Trich*3	21.2	24.1	26.8	27.9	52.0	806.0
MNF-MSH-*Trich*4	25.2	32.7	19.7	22.4	55.1	630.0
MNF-MSH-*Trich* 5	24.8	25.4	25.5	24.3	49.7	678.0
MNF-MSH-*Trich*6	22.4	31.0	20.6	26.0	57.0	616.0
MNF-MSH-*Trich*7	29.0	25.3	22.3	23.4	48.7	766.0
MNF-MSH-*Trich*8	25.1	25.9	23.4	25.6	51.5	710.0
MNF-MSH-*Trich*9	21.6	24.1	31.4	22.9	47.0	730.0
MNF-MSH-*Trich*10	14.8	21.9	16.5	46.9	68.8	778.0
MNF-MSH-*Trich*11	29.6	32.6	19.0	18.8	51.4	709.0
MNF-MSH-*Trich*12	22.3	31.7	20.1	25.8	57.5	561.0
MNF-MSH-*Trich*13	26.3	27.2	21.6	24.9	52.1	662.0
MNF-MSH-*Trich*14	22.7	29.7	21.6	26.0	55.7	546.0
MNF-MSH-*Trich*15	25.4	25.4	20.4	28.8	54.2	760.0
MNF-MSH-*Trich*16	33.0	26.1	17.0	24.0	50.1	789.0
MNF-MSH-*Trich*17	21.6	25.9	27.1	25.4	50.3	874.0
MNF-MSH-*Trich*18	22.5	25.0	24.1	28.4	53.4	756.0
MNF-MSH-*Trich*19	23.3	29.3	22.2	25.2	54.5	662.0
MNF-MSH-*Trich*20	23.1	31.2	21.5	24.2	55.4	637.0
MNF-MSH-*Trich*21	22.9	26.9	22.7	27.5	54.4	673.0
MNF-MSH-*Trich*22	27.4	24.8	26.2	21.7	46.5	577.0
MNF-MSH-*Trich*23	32.5	24.3	25.2	17.9	42.2	658.0
MNF-MSH-*Trich*24	22.1	29.6	22.1	26.2	55.8	714.0
MNF-MSH-*Trich*25	20.8	22.0	26.8	30.4	52.4	998.0
MNF-MSH-*Trich*26	24.6	27.0	23.7	24.6	51.6	877.0
MNF-MSH-*Trich*27	31.5	24.4	26.0	18.2	42.6	731.0
MNF-MSH-*Trich*28	26.6	24.1	22.6	26.7	50.8	888.0

Table 6: Nucleotide Frequencies in Percent of the sequences of ITS1-5.8S-ITS2 regions analyzed using Molecular Evolutionary Genetics Analysis (MEGA4 version 5.20.) for 28 *Trichoderma* isolates.

Trichoderma isolate	Endonuclease*		Exoglucanases Avicel *	Total cellulase (Cellulose azure record)**
	Swollen cellulose	CMC		
MNF-MSH-*Trich*1	5.30 ᵃ	5.33 ᶠᵍʰ	5.43 ᵃ	9
MNF-MSH-*Trich*2	4.83 ᵇᶜᵈ	5.40 ᶠᵍ	4.63 ᶜᵈᵉᶠ	10
MNF-MSH-*Trich*3	4.20 ᵍ	4.97 ᵍʰⁱʲ	3.73 ⁱʲ	8
MNF-MSH-*Trich*4	2.06 ᵏ	5.97 ᵈᵉ	1.73 ˡᵐⁿ	9
MNF-MSH-*Trich* 5	4.77 ᶜᵈᵉ	6.67 ᵃᵇ	4.63 ᶜᵈᵉᶠ	10
MNF-MSH-*Trich*6	4.67 ᶜᵈᵉᶠ	4.17 ˡ	3.73 ⁱʲ	9
MNF-MSH-*Trich*7	3.73 ʰⁱ	6.50 ᵃᵇᶜ	5.03 ᵃᵇᶜ	10
MNF-MSH-*Trich*8	5.10 ᵃᵇᶜ	6.30 ᵇᶜᵈ	5.00 ᵃᵇᶜᵈ	10
MNF-MSH-*Trich*9	4.80 ᶜᵈᵉ	6.70 ᵃᵇ	4.73 ᶜᵈᵉᶠ	10
MNF-MSH-*Trich*10	4.23 ᶠᵍ	6.67 ᵃᵇ	4.47 ᵉᶠᵍ	10
MNF-MSH-*Trich*11	3.60 ʰⁱ	3.20 ᵐ	3.33 ʲ	8
MNF-MSH-*Trich*12	3.57 ⁱ	5.63 ᵉᶠ	3.97 ʰⁱ	8
MNF-MSH-*Trich*13	4.37 ᵉᶠᵍ	6.20 ᶜᵈ	4.50 ᵉᶠ	9
MNF-MSH-*Trich*14	5.40 ᵃ	6.53 ᵃᵇᶜ	5.45 ᵃ	10
MNF-MSH-*Trich*15	2.07 ᵏ	5.10 ᵍʰⁱ	1.73 ˡᵐⁿ	8
MNF-MSH-*Trich*16	4.20 ᵍ	6.17 ᶜᵈ	1.30 ⁿ	7
MNF-MSH-*Trich*17	2.00 ᵏ	5.20 ᶠᵍʰⁱ	1.97 ᵏˡ	7
MNF-MSH-*Trich*18	2.00 ᵏ	4.37 ᵏˡ	3.77 ⁱʲ	8
MNF-MSH-*Trich*19	4.43 ᵈᵉᶠᵍ	6.00 ᵈᵉ	4.65 ᵈᵉᶠ	10
MNF-MSH-*Trich*20	2.00 ᵏ	2.80 ᵐ	1.8 ˡᵐ	8
MNF-MSH-*Trich*21	4.03 ᵍʰ	6.77 ᵃ	4.03 ᵍʰⁱ	10
MNF-MSH-*Trich*22	5.10 ᵃᵇᶜ	4.93 ʰⁱʲ	5.23 ᵃᵇ	10
MNF-MSH-*Trich*23	3.00 ʲ	4.27 ˡ	1.37 ᵐⁿ	9
MNF-MSH-*Trich*24	1.36 ˡ	6.03 ᵈᵉ	2.27 ᵏ	7
MNF-MSH-*Trich*25	4.70 ᶜᵈᵉ	5.10 ᵍʰⁱ	4.33 ᶠᵍʰ	8
MNF-MSH-*Trich*26	5.27 ᵃᵇ	4.53 ʲᵏˡ	5.3 ᵃ	10
MNF-MSH-*Trich*27	5.00 ᵃᵇᶜ	4.77 ⁱʲᵏ	4.8 ᵇᶜᵈᵉ	10
MNF-MSH-*Trich*28	2.08 ᵏ	4.26 ˡ	3.98 ʰⁱ	8

* Clear zone diameter in cm, Values within a column followed by the same letter (s) are not significantly different at $p < 0.05$ level according to the Duncan's multiple range test (DMRT).
** Standard color scale of 1 (minimum) to 10 (maximum) [25].

Table 7: Total cellulase, endoglucanases and exoglucanases (cellobiohydrolases) enzymes activities of *Trichoderma* isolates.

synergistic actions involving β-glucosidase and cellobiohydrolases [46]. Walseth cellulose plate assay included converting the crystalline fraction of cellulose to the amorphous form by adding o-phosphoric acid to produce phosphoric acid swollen cellulose (PASC) [47]. None of screening methods reported in this research had sufficient precession to allow the selection of particular cellulase enzymes. This is may be due to the complexity of the celluletic system produced by *Trichoderma*. However, eleven isolates were selected to be the best isolates among the 28 isolates used. These are MNF-MSH-*Trich*1, MNF-MSH-*Trich*5, MNF-MSH-*Trich*7, MNF-MSH-*Trich*8, MNF-MSH-*Trich*9, MNF-MSH-*Trich*10, MNF-MSH-*Trich*14, MNF-MSH-*Trich*21, MNF-MSH-*Trich*22, MNF-MSH-*Trich*26 and MNF-MSH-*Trich*27. These findings were consistent with the result that most of the isolates were belonging to *T. longibarchiatum* and *T. harzianum*. These species have been adopted in various industries because of their ability to secrete large amounts of protein and metabolites [4].

Conclusion

According to this investigation results, the morphological characterization is not reliable for identification of the isolates and oligonucleotide barcode is a powerful tool for the identification of *Trichoderma* species and should be useful as an alternative or as a complement to morphological methods. According to molecular findings there were two main species predominated in the middle Delta area of Egypt namely; *T. longibarchiatum* and *T. harzianum*. Eleven isolates were selected to be the best isolates among the 28 isolates used for cellulolytic activity.

Acknowledgements

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