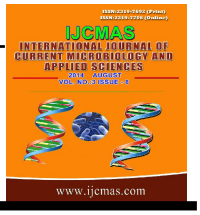
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 9 (2014) pp. 1059-1071**
http://www.ijcmas.com



Original Research Article

Molecular Characterization of *Trichoderma* Populations Isolated from Soil of Taif City, Saudi Arabia

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A B S T R A C T

Ninety isolates of *Trichoderma* (Teleomorph: *Hypocrea*) species and one isolate of *Gliocladium viride* (Tel. *Hypocrea lutea*) were isolated from soil samples collected from different locations in Taif city. Two soil samples cultivated with *Purica granatum* showed high incidences of isolates (13 and 12 isolates, respectively). Regions of nuclear rDNA, containing 18S ribosomal RNA gene (partial sequence); internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (complete sequence); and 28S ribosomal RNA gene (partial sequence) were amplified to identify the collected isolates. The sequencing results indicated that 78 isolates of the population were identified as *Trichoderma harzianum* (Tel. *Hypocrea lixii*). Also, two isolates were Identified as *T. longibrachiatum* (Tel. *H. orientalis*) and one isolate as *Gliocladium viride* (Tel. *H. lutea*). The remaining 10 isolates were postulated as three new species according to their phylogenic tree. *T. harzianum* isolates collected during this work showed high degrees of variability which supported that it is a "species complex".

K e y w o r d s

Soil
Trichoderma harzianum,
T. longi - brachiatum
Gliocladium viride
Hypocrea lixii
DNA sequence

Introduction

Genus *Trichoderma* is the most promising biocontrol agent against plant pathogenic fungi. *Trichoderma* is widely distributed fungus all over the world and occurs nearly in all soil (Kacprzak *et al.*, 2014). Also, it was isolated previously from soil in different regions of Saudi Arabia and used as biocontrol agents (Molan, 2009 and Hussein and Youssef, 2011).

Trichoderma species are difficult to distinguish morphologically, so molecular methods including DNA sequencing and genealogical concordance phylogenetic species recognition using several unliked genes are needed to give accurate identification of *Trichoderma* spp. (Druzhinina *et al*., 2006). *Trichoderma* is monophyletic (Kullnig-Gradinger *et al*., 2002), with teleomorphs in the genus *Hypocrea*. The taxonomy and identification of *Trichoderma* has remained problematic, Rifai (1969) distinguished nine "species aggregates". The introduction of molecular

methods and cladistic analysis of DNA sequences in the evolutionary mycology in the early 1990's opened a new era for fungal systematic and has already resulted in the dramatic taxonomic changes (Druzhinina *et al.,* 2010). The development of molecular tools has enabled the positive identification of any strain and the development of a phylogenetic tree. Several investigations could distinguish between different *Trichoderma* strains by randomly amplified polymorphic DNA (RAPD – PCR and sequence analysis of ribosomal DNA (Siameto *et al*., 2011). Kindermann *et al*. (1998) attempted a first phylogenetic analysis of the whole genus, using sequence analysis of the ITS1 region of the rDNA. Phylogenetic studies of 88 species showed that *Trichoderma* and *Hypocrea* form a single holomorph genus, within which two major clades can be distinguished (Chaverri *et al*., 2004). Also, the importance of phylogenetic analysis using molecular characters for the classification of *Trichoderma* was reviewed by Samuels and Seifert (1995).

Universally primered PCR (UP-PCR) fingerprinting combined with ITS1 ribotyping were used to study the genetic relatedness of strains of *Trichoderma* and *Gliocladium* to evaluate the ability of the methods to discriminate closely related strains and to identify markers for development of specific detection assays for selected strains (Bulat *et al.*, 1998). These methods grouped *Trichoderma* species into 15 genetic entities Over the past 35 years, the number of named *Trichoderma* species has increased from nine aggregate species (Rifai, 1969) to about 80 phylogenetic species (Samuels *et al*., 2002). The application of genealogical concordance between unlinked DNA loci (phylogenetic species concept) resulted in 100 phylogenetic species (Druzhinina *et al*., 2006) and this number is growing quickly (Druzhinina *et al*., 2012).

Many studies based on a single gene have been cast into doubt. Identification based only on ITS sequences sometimes fails to distinguish between closely related taxa because more than one species may share the same ITS genotype (Druzhinina *et al*., 2005). Nowadays, many molecular phylogenetic studies are basing their taxonomic proposals on analyses of multiple genes (Mukherjee *et al*., 2013 and Shahid, *et al*. 2014). Fragments of genes including the 18S rDNA, the 28S rDNA, the small mitochondrial rDNA subunit (mtSSU), the endochitinase 42 (ech42), or the translation elongation factor 1 (tef1) gene have been described as useful tools for phylogenetic studies (Samuels *et al*., 2002).

Trichoderma harzianum comprises multiple phenotypically indistinguishable evolutionary lines. Multiple morphological, physiological and molecular characters revealed high infraspecific variation in *T. harzianum* (Samuels *et al*., 2002; Chaverri *et al*., 2003). Kullnig-Gradinger *et al*. (2002) established a species phylogeny of the genus *Trichoderma*, based on the sequence analysis of multiple independent loci, by investigating all the *Trichoderma* species described by Bissett (1992). PCR-based markers with primer M13 and ITS sequences of rDNA were used to confirm the identity of two *Trichoderma* species: *T. harzianum* (*H. lixii*) and *T. longibrachiatum* (*H. orientalis*) (Abd-Elsalam *et al*., 2010).

In the present study, 18S ribosomal RNA gene, ITS1, 5.8S ribosomal RNA gene, ITS2, and 28S ribosomal RNA gene sequences were used for the species identification of *Trichoderma* isolated from cultivated soil of Taif city, Saudi Arabia.

Materials and Methods

Isolation of *Trichoderma* from soil samples

Eighteen cultivated soil samples were collected from different localities in Taif city. Moisture contents, pH values, total soluble salts and organic matter contents of these samples were determined (Jackson, 1958). The dilution-plate method was used to isolate *Trichoderma* from soil as described by Moubasher and Abdel-Hafez (1978) using *Trichoderma* medium E (TME, Papavizas *et al.*, 1982). The medium contains V8 juice, 200 ml; glucose, 1 g and completed to 1000 ml distilled water, 6 ml of 1N NaOH and the fungicide pentachloronitrobenzene (PCNB, 100 µg/ml) were added after autoclaving.

DNA extraction: The collected isolates of *Trichoderma* (91 isolates) were cultured in 100 ml Erlenmeyer flasks containing 20 ml PDA medium, after 5 days incubation, mycelium was collected by vacuum filtration and ground to a fine powder in liquid N2. Fifty milligrams of the powder was transferred to a 1.5 ml Eppendorf tube and mixed with 0.7 ml 2x cetyl trimethyl ammonium bromide (CTAB) buffer. The Eppendorf tubes were incubated at 65°C for 30 min, then, 0.7 ml of chloroform was added and mixed briefly. After centrifugation at 15,000 rpm for 30 min, the supernatant was transferred into a new tube mixed with 0.6 ml isopropanol chilled to 20°C, followed by centrifugation for 5 min at maximum speed. The supernatant was discarded and the remaining pellet was washed twice with 1 ml of 70% ethanol, followed by drying under vacuum and, thereafter, dissolved in 1 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The DNA concentration was evaluated by agarose gel electrophoresis (Moeller *et al*., 1992).

ITS regions sequencing: A region of nuclear rDNA, containing 18S ribosomal RNA gene (partial sequence); internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (complete sequence); and 28S ribosomal RNA gene (partial sequence) was amplified by PCR using the primer combinations ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and LR3R (5'-GGTCCGTGTTTCAAGAC-3') in 50 µl volumes (White *et al*., 1990), in an automated temperature-cycling device (Biotron, Biometra, Goettingen), using the following parameters: 1 min initial denaturation at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 50°C, 90 s extension at 74°C, and a final extension period of 7 min at 74°C.

Sequence analysis: DNA sequences were aligned first with Clustal X 1.81. Geneious software (Meintjes, *et al.*, 2012) was used to construct UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree according to the UPGMA algorithm (Sneath and Sokal, 1973), distance was clustered using Jukes-Cantor model (Jukes and Cantor, 1969). Also, the results were subjected through the computer-assisted analysis, and the produced matrices were analyzed by TREECON for Windows (version 1.3b, 1998) wherein the evolutionary tree dendrogram was constructed and produced (Van de Peer and Wachter, 1994).

Results and Discussion

The results revealed that, there is no correlation between soil properties (moisture contents, pH values, total soluble salts and organic matters) and incidence of *Trichoderma* (Table 1). Abou-Zaid and Abd El-Fattah (2007) indicate that the soil moisture content is low in all habitats of Taif

area. TME medium enhances the population and counts of *Trichoderma* by restricting the growth of other fungi (Shaban and El-Komy, 2000).

Ninety strains of *Trichoderma* and one of *Gliocladium viride* (as identified molecularly) were isolated in the present investigation from 18 soil samples collected from different localities in Taif city (Table 1). Soil samples nos. 11 and 9 showed high incidences of isolates (13 and 12 isolates, respectively), which were cultivated with *Purica granatum*. However soil samples cultivated with *Beta vulgaris*, *Coriandrum sativum* and *Cyperus* sp. were represented each by only one strain (TUT11, TUT5 and TUT101, respectively) (Table 1).

The soil fungi of Saudi Arabia have been investigated earlier (Youssef *et al.*, 1998), Although, *Trichoderma* was not detected in desert soil samples of Saudi Arabia studied by Abdel-Hafez (1982). The previous studies reported that *Trichoderma* is one of the basic constituents of fungi in soils and other sources (Moubasher, 1993; Gherbawy *et al*., 2004; Druzhinina and Kubicek, 2005; Hussein and Yousef, 2011). Two species of *Trichoderma* (*T. harzianum* and *Trichoderma* sp.) were also isolated from petroleum contaminated soil (Hussein and Yousef, 2011). Also, two *Trichoderma* complex species (*T. harzianumlH. lixii* and *T. longibrachiatumlH. orientalis*) were identified from soil collected from Rawdet Khuraim in Saudi Arabia using morphological criteria and DNA sequence analysis (Abd- Elsalam *et al.*, 2010).

Trichoderma sp. was the most abundant species in soil of Riyadh, Saudi Arabia (Molan, 2009). Gherbawy *et al.* (2004) identified molecularly only two taxa of *Trichoderma*/*Hypocrea* (*T. harzianum* and *H. orientalis*) in soils of the Nile valley, Egypt.

Molecular characterization of *Trichoderma* strains using ITS regions sequencing

Many reports have been published on isolation and identification of *Trichoderma* in Saudi Arabia (Molan, 2009; Hussein and Yousef, 2011). These reports data suffer from the fact that species identification had been performed morphologically following the key of Rifai (1969). So, in the current work molecular characterization for the collected isolates have been conducted.

Ninety-one isolates were sequenced, the length of the amplified fragments was 1200 bp. For the final analysis, a total of 110 sites were used. Table 2 showed all isolates collected in this study and their sequencing results. The sequencing results have been blasted against gene bank. Seventy- eight isolates showed similarity ranging from 98 to 100% with the sequence results of *Hypocrea lixii* and *H. nigricans*. Since *H. lixii* and *H. nigricans* are synonyms for the anamorphic form of *Trichoderma harzianum* (Chaverri, and Samuels, 2004), thus those 78 isolates were named as *T. harzianum* (Table 2). Druzhinina *et al.* (2010) analyzed *H. lixii/T. harzianum* species complex and resulted that, the evolutionary success of the *H. lixii/T. harzianum* species aggregate may be attributed to the very complex structures of the contemporary populations of these fungi.

This complexity comes from the fact that many of these species 'types' are overlapping and therefore, two closely related organisms become attributed to different species recognized based on incomparable criteria. Although, they considered the whole of the *H. lixii/T. harzianum* species complex might be as a single species because of its monophyletic origin.

Two strains (TUT50 and TUT65) showed 98% similarity with *Trichoderma longibrachiatum* HQ882796. So, these strains were identified as *T. longibrachiatum* (Table 2). One isolate (TUT73) was named as *Gliocladium viride* (Tel. *Hypocrea lutea*) because its similarity with *H. lutea* NJ 943361 was 98% (Table 2). The remaining 10 isolates (TUT5, 7, 30, 38 , 48, 59, 67, 68, 69 and 72) matched with some unidentified *Trichoderma* species deposited in the gene bank by less than 98%, 99% or 100% similarities. So, those isolates considered as candidates for new species.

Phylogenic Analysis

Neighbor – joining tree using Jukes-Cantor model (Jukes and Cantor, 1969) was used to study genetic diversity among 22 strains (only 9 was selected from 78 *T. harzianum* and all strains of other *Trichoderma* species) (Figure 1). The phylogenic tree revealed that, the strains could be categorized into 6 different clades each represent one species. First clade comprised 9 isolates (TUT1, 4, 9, 10, 12, 15, 22, 32 and 62) of *T. harzianum*, which clustered together with *H. lixii* NRRL54022=GQ328856 and *H. nigricans* NBRC31289 =JN943370. Second clade consists of 4 strains (TUT50, 59, 65 and 67) closely related to *T. longibrachiatum* NRRL54514=HQ882796. Third clade include strains TUT59 and 67, these two isolates can be considered as *Trichoderma* spp.. TUT48, 68, 69, 72 (*Trichoderma* spp.) and TUT73 (*Gliocladium viride*) clustered together with *H. lutea* B1A08568NA2CC477= JQ411366 in the fourth clade. From this cluster it is claimed that TUT48, 68, 69 and 72 are closely related to *H. lutea* (Anamorph: *Gliocladium viride*). Two isolates (TUT30 and 38) were represented by a single clade (fifth clade) with *Trichoderma* sp. BE8C869K=KC007207, and it is postulated that they are one species. The phylogenic tree showed that isolates TUT4 andTUT5 could be considered as related to one species different from *T. harzianum* (constitute separate clade). Strain no. TUT7 (unidentified *Trichoderma* species) could also be considered as separate species (Figure 1).

Another phylogenic tree was used to study genetic diversity among 78 isolates of *T. harzianum* isolated in this work (Figure 2). From this tree, *T. harzianum* isolates (78) constituted many sub clusters. These results indicated that this species comprised from several phylogenic species as previously reported by Chaverri *et al.* (2003). Many other molecular sequences data demonstrated that *Trichoderma harzianum* is a genetically variable complex, comprised by one morphological species and several phylogenic species (Gherbawy *et al*., 2004; Druzhinina and Kubicek, 2005 and Druzhinina *et al*., 2010). Also, figure 2 showed that clustering system of *T. harzianum* did not correlate with the habitat or location of the isolates. For example strains TUT91 (isolated from soil samples cultivated with *Psidium guajava* at Hawyah), TUT8 (cultivated with *Allium* sp. at Al-Qaim) and TUT80 (cultivated with *Purica granatum* at Hawyah) clustered together in one cluster with 75% bootstrap value. These results came in agreement with Gherbawy *et al.* (2004), during their study for *Trichoderma* populations from alkaline agricultural soil in the Nile valley. They reported that there is no correlation between RAPD pattern and the habitat.

Kullnig-Gradinger *et al.* (2002) using several genes, divided *Trichoderma* phylogeny into four clades. Clade A comprises species of Sect. *Trichoderma* and other species, clade C comprises species of Sect. *Longibrachiatum*, and clade D

contains only *T. aureoviride*. On the other hand, Clade B contains a large and taxonomically heterogeneous mixture of species, among which several subclades could be identified. The current trend in *Trichoderma* taxonomy is to describe species primarily on the basis of DNA sequence data (Hanada *et al.*, 2008). Buhariwalla *et al.* (2005) observed fourteen single nucleotide polymorphisms (SNPs) in the 28S rDNA region across the 38 isolates of *Trichoderma*. They reported that there is no variation of the 28S rDNA region among isolates of the same *Trichoderma* species, suggesting a high level of sequencing accuracy and sequence conservation within species in this region of the genome.

Table.1 Soil samples, strains of *Trichoderma* isolated from cultivated soil, locations, habitat and soil properties

Soil samples cultivated with (no.)	Strain numbers	No. of Isolates	Soil properties			
			MC%	pH	TSS	OM
Zea mays (1)	TUT1, TUT2	2	5.26	4.60	0.08	6.70
Imperata cylindrica (1)	TUT3, TUT4	2	9.89	4.56	0.15	4.52
Eurica sativa (1)	TUT20, TUT21	2	17.65	5.00	0.53	1.01
Allium sp. (1)	TUT7-TUT10, TUT17	5	25.00	4.94	0.73	5.36
Beta vulgaris (1)	TUT11	1	13.64	4.71	0.60	5.03
Coriandrum sativum (1)	TUT5	1	25.00	4.69	0.13	7.37
Phaseolus vulgaris (1)	TUT12-TUT16, TUT18-TUT19	7	23.46	5.00	0.18	1.17
Citrus limonum (2)	TUT47, TUT48, TUT99, TUT100	4	7.53-9.89	6.90-7.30	0.20-0.25	3.52-5.70
Purica granatum (1)	TUT49- TUT60	12	3.09	7.23	0.05	4.52
Rosa sp. (2)	TUT61- TUT63, TUT81- TUT90	13	7.53-20.48	6.80-7.02	0.18-0.48	5.03-5.70
Purica granatum (1)	TUT64- TUT80	13	3.09	6.87	0.08	2.68
Psidium guajava (1)	TUT91- TUT98, TUT104	9	12.36	6.65	0.25	5.36
Cyperus sp. (4)	TUT22, TUT23, TUT30-TUT46, TUT101	20	2.04-16.28	5.95-7.40	0.05-0.15	0.67-6.70
Total (18)		91	12.35*	6.08*	0.24*	4.45*

Precentage moisture content (MC%), pH values, total soluble salts (TSS), organic matter (OM), * = mean of soil properties

Table.2 *Trichoderma*/*Hypocrea* species, isolates code , accession numbers of isolates isolated during this study and their highest similarities with other gene bank isolates

Isolates code	Accession Numbers	Gene bank strains	Identities	Given (Anamorph) names
TUT1	HE649403	*Hypocrea lixii* GQ328856	1098/1100 (99%)	*Trichoderma harzianum*
TUT2	HE649404	*H. lixii* GQ328856	1097/1102 (99%)	*T. harzianum*
TUT3	HE649405	*H. nigricans* JN943369	1090/1105 (99%)	*T. harzianum*
TUT4	HE649406	*H. nigricans* JN943369	1091/1117 (98%)	*T. harzianum*
TUT5	HE649407	*H. nigricans* JN943370	1081/1129 (96%)	*Trichoderma* sp.
TUT7	HE649408	*H. nigricans* JN943369	879/936 (94%)	*Trichoderma* sp.
TUT8	HE649409	*H. nigricans* JN943369	1100/1102 (99%)	*T. harzianum*
TUT9	HE649410	*H. nigricans* JN943368	1098/1101 (99%)	*T. harzianum*
TUT10	HE649411	*H. nigricans* JN943369	1085/1103 (98%)	*T. harzianum*
TUT11	HE649412	*H. nigricans* JN943369	1082/1103 (98%)	*T. harzianum*
TUT12	HE649413	*H. lixii* GQ328857	1081/1100 (98%)	*T. harzianum*
TUT13	HE649414	*H. lixii* GQ328857	1082/1104 (98%)	*T. harzianum*
TUT14	HE649415	*H. lixii* GQ328856	1093/1102 (99%)	*T. harzianum*
TUT15	HE649416	*H. lixii* GQ328858	1078/1100 (98%)	*T. harzianum*
TUT16	HE649417	*H. lixii* GQ328856	1096/1100 (99%)	*T. harzianum*
TUT17	HE649418	*H. nigricans* JN943369	1100/1103 (99%)	*T. harzianum*
TUT18	HE649419	*H. lixii* GQ328857	1094/1098 (99%)	*T. harzianum*
TUT19	HE649420	*H. lixii* GQ328857	1093/1099 (99%)	*T. harzianum*
TUT20	HE649421	*H. lixii* GQ328856	1098/1101 (99%)	*T. harzianum*
TUT21	HE649422	*H. lixii* GQ328857	1091/1099 (99%)	*T. harzianum*
TUT22	HE649423	*H. lixii* GQ328858	1050/1050 (100%)	*T. harzianum*
TUT23	HE649424	*H. lixii* GQ328857	1093/1098 (99%)	*T. harzianum*
TUT30	HE649425	*Hypocrea* sp. KC007207	1089/1095 (99%)	*Trichoderma* sp.
TUT31	HE649426	*H. nigricans* JN943369	1089/1101 (99%)	*T. harzianum*
TUT32	HE649427	*H. lixii*. HE649419	1082/1091 (99%)	*T. harzianum*
TUT33	HE649428	*H. nigricans* JN943369	1093/1109 (99%)	*T. harzianum*
TUT34	HE649429	*H. nigricans* JN943369	1067/1079 (99%)	*T. harzianum*
TUT35	HE649430	*H.* nigricans JN943369	1091/1105 (99%)	*T. harzianum*
TUT36	HE649431	*H. nigricans* JN943369	1093/1104 (99%)	*T. harzianum*
TUT37	HE649432	*H. nigricans* JN943369	1087/1104 (98%)	*T. harzianum*
TUT38	HE649433	*Hypocrea* sp. FJ434202	1070/1108 (97%)	*Trichoderma* sp.
TUT39	HE649434	*H. nigricans* JN943369	1092/1106 (99%)	*T. harzianum*
TUT40	HE649435	*H. nigricans* JN943370	1095/1102 (99%)	*T. harzianum*
TUT41	HE649436	*H. nigricans* JN943370	1097/1101 (99%)	*T. harzianum*
TUT42	HE649437	*H. nigricans* JN943370	1093/1102 (99%)	*T. harzianum*
TUT43	HE649438	*H. nigricans* JN943370	1087/1101 (99%)	*T. harzianum*
TUT44	HE649439	*H. nigricans* JN943370	1089/1100 (99%)	*T. harzianum*
TUT45	HE649440	*H. nigricans* JN943370	1095/1102 (99%)	*T. harzianum*
TUT46	HE649441	*H. nigricans* JN943370	1095/1101 (99%)	*T. harzianum*
TUT47	HE649442	*H. nigricans* JN943369	1097/1103 (99%)	*T. harzianum*

Isolates code	Accession Numbers	Gene bank strains	Identities	Given (Anamorph) names
TUT48	HE649443	*H. lutea* JN943361	1069/1104 (97%)	*Trichoderma* sp.
TUT49	HE649444	*H. nigricans* JN943369	1091/1103 (99%)	*T. harzianum*
TUT50	HE649445	*T. longibrachiatum* HQ882796	1082/1102 (98%)	*T. longibrachiatum*
TUT51	HE649446	*H. nigricans* JN943369	1089/1104 (99%)	*T. harzianum*
TUT52	HE649447	*H. nigricans* JN943369	1091/1103 (99%)	*T. harzianum*
TUT53	HE649448	*H. nigricans* JN943369	1089/1103 (99%)	*T. harzianum*
TUT54	HE649449	*H. nigricans* JN943369	1092/1102 (99%)	*T. harzianum*
TUT55	HE649450	*H. nigricans* JN943369	1092/1103 (99%)	*T. harzianum*
TUT56	HE649451	*H. nigricans* JN943369	1093/1101 (99%)	*T. harzianum*
TUT57	HE649452	*H. nigricans* JN943369	1086/1104 (98%)	*T. harzianum*
TUT58	HE649453	*H. nigricans* JN943369	1094/1102 (99%)	*T. harzianum*
TUT59	HE649454	*H. nigricans* JN943369	691/786 (88%)	*Trichoderma* sp.
TUT60	HE649455	*H. nigricans* JN943369	1082/1102 (98%)	*T. harzianum*
TUT61	HE649456	*H. nigricans* JN943369	1098/1103 (99%)	*T. harzianum*
TUT62	HE649457	*H. lixii* GQ328856	1095/1101 (99%)	*T. harzianum*
TUT63	HE649458	*H. lixii* GQ328856	1099/1101 (99%)	*T. harzianum*
TUT64	HE649459	*H. nigricans* JN943369	1079/1101 (98%)	*T. harzianum*
TUT65	HE649460	*T. longibrachiatum* HQ882796	1090/1107 (98%)	*T. longibrachiatum*
TUT66	HE649461	*H. nigricans* JN943369	1074/1096 (98%)	*T. harzianum*
TUT67	HE649462	*H. lutea* JN943361	1066/1108 (96%)	*Trichoderma* sp.
TUT68	HE649463	*H. lutea* JN943361	1074/1110 (97%)	*Trichoderma* sp.
TUT69	HE649464	*H. lutea* JN943361	1072/1107 (97%)	*Trichoderma* sp.
TUT71	HE649465	*H. nigricans* JN943369	1087/1099 (99%)	*T. harzianum*
TUT72	HE649466	*H. lutea* JN943361	1068/1108 (96%)	*Trichoderma* sp.
TUT73	HE649467	*H. lutea* JQ411366	1076/1076 (100%)	*Gliocladium viride*
TUT75	HE649468	*H. nigricans* JN943369	1092/1102 (99%)	*T. harzianum*
TUT76	HE649469	*H. nigricans* JN943369	1084/1101 (98%)	*T. harzianum*
TUT79	HE649470	*H. nigricans* JN943369	1092/1100 (99%)	*T. harzianum*
TUT80	HE649471	*H. nigricans* JN943369	1098/1102 (99%)	*T. harzianum*
TUT81	HE649472	*H. nigricans* JN943370	1099/1100 (99%)	*T. harzianum*
TUT82	HE649473	*H. nigricans* JN943370	1096/1100 (99%)	*T. harzianum*
TUT83	HE649474	*H. nigricans* JN943370	1099/1103 (99%)	*T. harzianum*
TUT84	HE649475	*H. nigricans* JN943370	1090/1101 (99%)	*T. harzianum*
TUT85	HE649476	*H. nigricans* JN943370	1085/1093 (99%)	*T. harzianum*
TUT86	HE649477	*H. nigricans* JN943369	1086/1104 (98%)	*T. harzianum*
TUT87	HE649478	*H. nigricans* JN943370	1096/1101 (99%)	*T. harzianum*
TUT88	HE649479	*H. nigricans* JN943370	1091/1104 (99%)	*T. harzianum*
TUT89	HE649480	*H. nigricans* JN943370	1093/1103 (99%)	*T. harzianum*
TUT90	HE649481	*H. nigricans* JN943370	1079/1101 (98%)	*T. harzianum*
TUT91	HE649482	*H. nigricans* JN943369	1099/1104 (99%)	*T. harzianum*
TUT92	HE649483	*H. nigricans* JN943369	1095/1102 (99%)	*T. harzianum*

Isolates code	Accession Numbers	Gene bank strains	Identities	Given (Anamorph) names
TUT93	HE649484	*H. nigricans* JN943369	1097/1105 (99%)	*T. harzianum*
TUT94	HE649485	*H. nigricans* JN943369	1097/1106 (99%)	*T. harzianum*
TUT95	HE649486	*H. nigricans* JN943369	1094/1104 (99%)	*T. harzianum*
TUT96	HE649487	*H. nigricans* JN943369	1090/1100 (99%)	*T. harzianum*
TUT97	HE649488	*H. nigricans* JN943369	1096/1104 (99%)	*T. harzianum*
TUT98	HE649489	*H. nigricans* JN943370	1095/1101 (99%)	*T. harzianum*
TUT99	HE649490	*H. nigricans* JN943369	1095/1105 (99%)	*T. harzianum*
TUT100	HE649491	*H. nigricans* JN943369	1095/1104 (99%)	*T. harzianum*
TUT101	HE649492	*H. nigricans* JN943369	1096/1107 (99%)	*T. harzianum*
TUT104	HE649493	*H. nigricans* JN943369	1093/1103 (99%)	*T. harzianum*
Total isolates				91

Figure.1 The phylogenic tree of *Trichoderma harzianum* (9 isolates), *T. longibrachiatum* (2), *Gliocladium viride* (*G. viride*, 1) and *Trichoderma* spp. (10) based on the sequence results of ITS1 region of the genomic rRNA gene using *Hypocrea lutea* B1A0856SNA2CC477=JQ411366, *T. longibrachiatum* NRRL 54514=HQ882796, *Trichoderma* sp. BESC869k=KC007207, *H. lixii* NRRL54022=GQ328856, *H. nigricans* NBRC31289=JN943370 from gene bank and *Trichoderma viride* ITV-VSL34=HQ833354 as out group

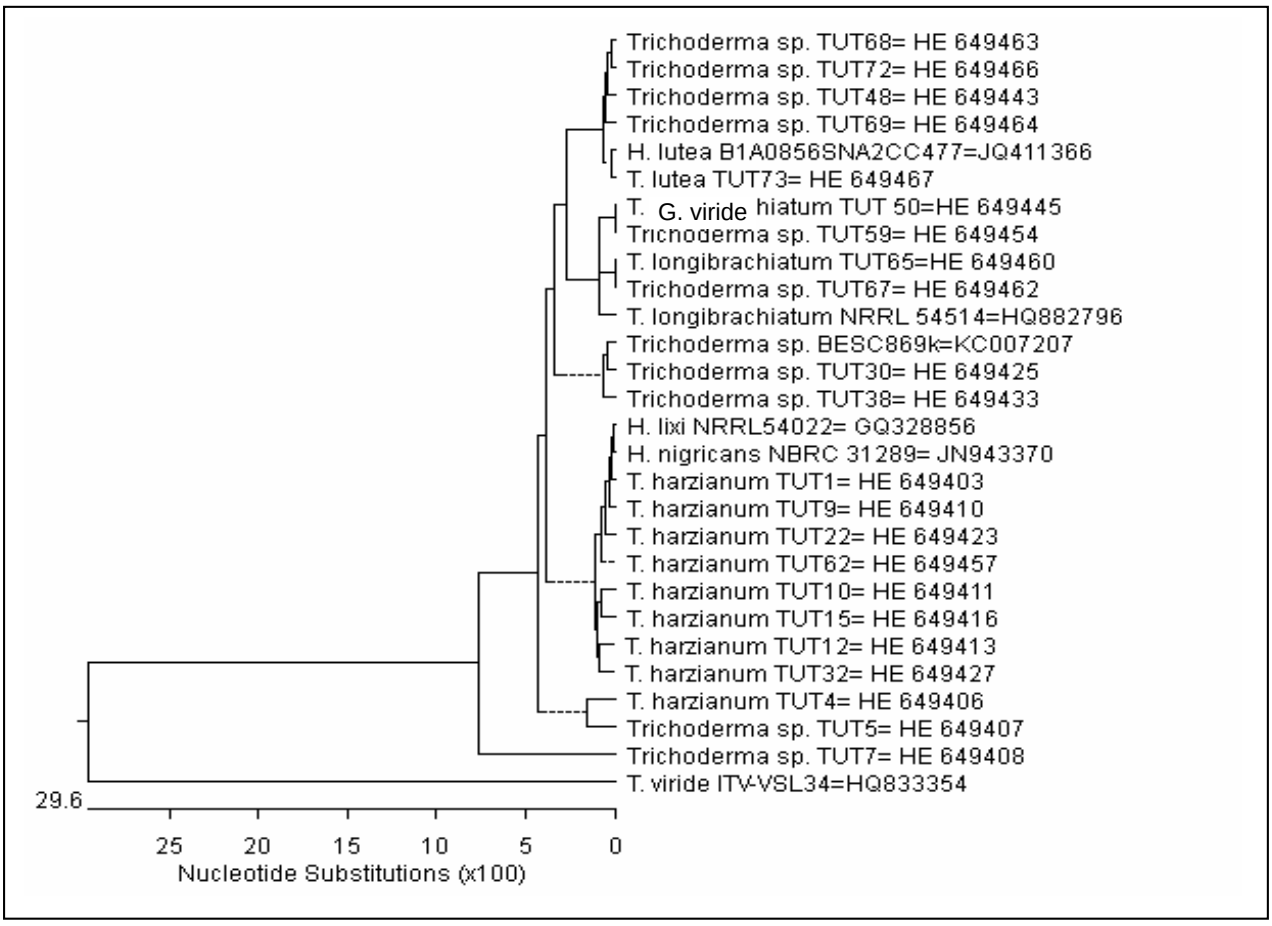
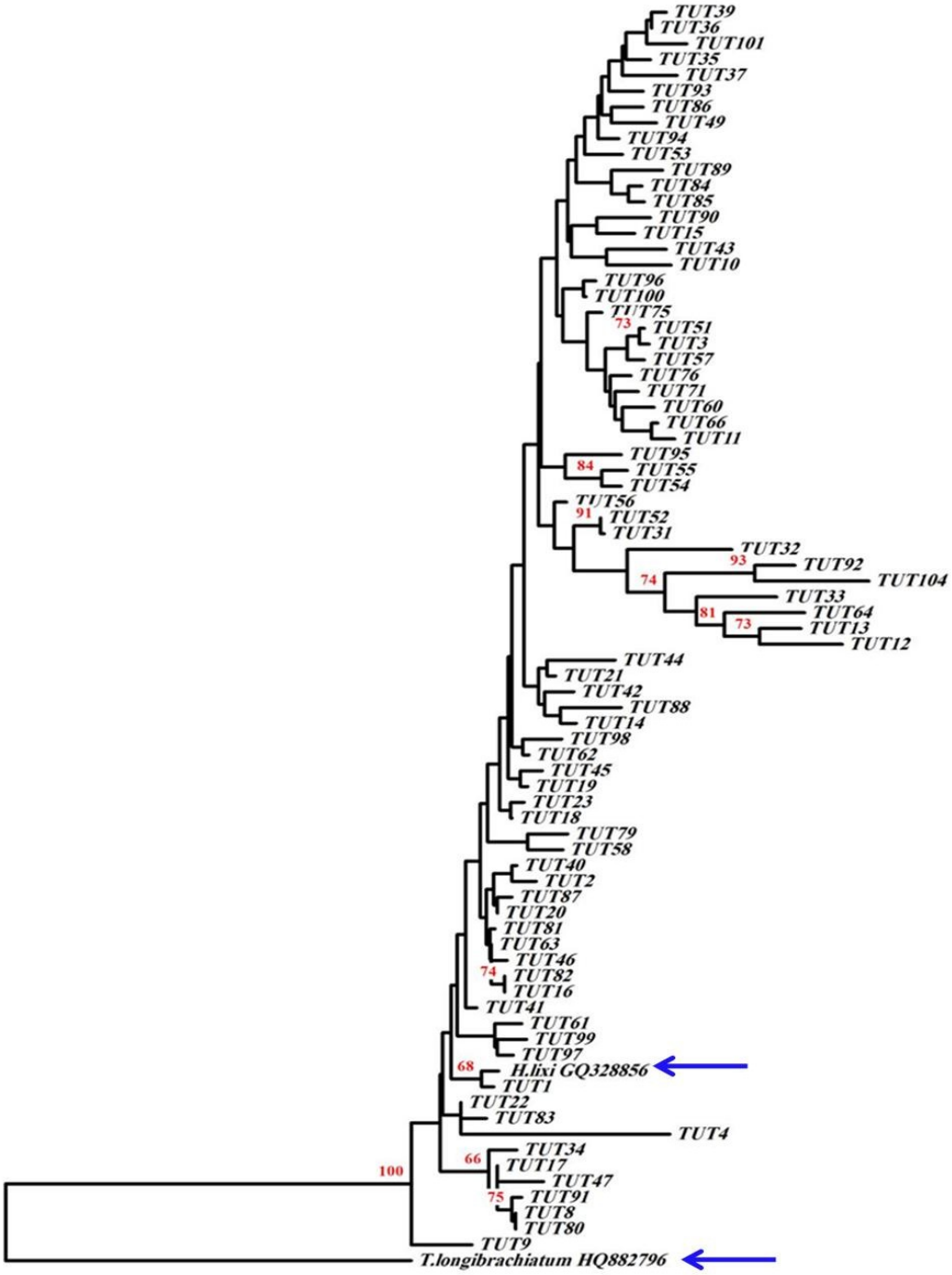


Figure.2 Neighbor- joining phylogenic tree of 78 isolates of *T. harzianum* resulting from the sequence results of ITS1 region of the genomic rRNA gene using *H. lixii* GQ328856 from gene bank and *Trichoderma longibrachiatum* HQ882796 as out group. Bootstrap values lower than 65 not shown.



Acknowledgment

Authors are especially grateful to professor Mady A. Ismail for his kind revision of the manuscript and his valuable guidelines.

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