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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(1):161-170. **OPEN ACCESS**

Separation of different *Trichoderma* species based on partial TEF-1α and RPB2 protein coding genes sequences against ITS regions

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The genus *Trichoderma* includes many different species with varied phonotypic characteristics, although some species of *Trichoderma* have economical values in industry, the phylogeny and discrimination between several species within this genus have stayed challenging problem to solve based on traditional methods of studying morphological characteristics.DNA Sequencing for ITS regions (internal transcribed spacer) of rDNA, Translation elongation factor 1α (TEF-1α) and second largest subunit of RNA polymerase II (RPB2) have become a valuable tool in the identification and discrimination between fungi species. This study was conducted to evaluate efficacy of ITS, TEF-1α, RPB2 genes sequences retrieved from GenBank database to separate between many different *Trichoderma* species using two phylogenetic analyses including Neighbor –joining and maximum likelihood. Phylogenetic sequence analysis of the ITS regions resulting in poor resolution of species discrimination between the investigated *Trichoderma* taxa indicating to low sequence variations within ITS regions. On the other hand, phylogenetic analysis conducted based on the partial sequence of TEF-1α and RPB2 genes resulted indistinct species –specific clades of phylogenetic trees indicating that both TEF-1α and RPB2 genes sequences have sufficient variations to discriminate between different species of this genus, In fact, RPB2 had a higher resolution than TEF-1α for discrimination between different *Trichoderma* species. This study propose RPB2 gene as a potential DNA barcode for *Trichoderma* species discrimination.

Keywords: *Trichoderma,* RPB2, TEF-1α, ITS

INTRODUCTION

Several members belonging to fungi kingdom have important medical and industrial uses, among them *Trichoderma*, which is a genus of fungi in the phylum Ascomycota, Most species within the genus *Trichoderma*exist in soils, and are non pathogenic to plants and can be isolated and cultured in the laboratory (Harman et al., 2004). This fungus can grow properly in cultures in temperatures 25–30 °C; however, some members of this genus have shown some growths at higher temperatures around 45 °C.

*Trichoderma*mycelium appears white and transparent at early growth on media, and turns to green and yellow with time. Most species of this genus has shown unique morphological characteristics including conidia, Conidiophores, and Chlamydospores. It has been known that some species of *Trichoderma* are industrial important as they are used to produce commercial quantities of enzymes such as chitinase which is produced by *T. harzianum* and also xylonite that is produced by *T. longibrachiatum* (Felse and Panda 1999; Azin et al., 2007). Moreover, Some

Trichoderma species have been proved to be used in biological control against many fungal pathogens of plants such as *T. harzianum* (Harman,2006) and some *Trichoderma* species have medical uses by producing immunosuppressant agents (Chong et al. 2009).

The genus *Trichoderma* was characterized for first time by Persoon in 1794, since then, mycologists has studied this genus and its taxonomy was considered hard and complex. However, manystudies considered *Trichoderma* holding just one species for while (Druzhinina et al., 2005).

The usage of DNA nucleotide sequences in the mycological identification of species resulting in solving many challenges of employing conventional methods, thereby, having fast identification and the ability to discriminate between species of morphologically similar within a genus (Sun & Guo 2012).With the advent of DNA Barcoding and sequencing, reports confirmed presence of over 80 species in this genus, and Hypocrea is considered the sexual stage (Teleomorph) of this genus (Samuels &Gary 2006).

Among DNA barcoding genes for fungi identification, The ITS region (internal transcribed spacer) of rDNA is the preferred short DNA sequence for fungi identification (Schoch et al., 2012). Although ITS is currently considered a potential genetic marker for species discrimination of fungi. However, fungi ITS regions did not seem to have enough sequence variations in some fungi groups, therefore, It would not be the right choice as genetic marker or a potential DNA sequence candidate for species separation of some fungi and even forgenus *Trichoderma*, (IWEN et al 2002).However, the taxonomy of this genus have remained difficult problem to solve by ITS sequences analysis as species of *Trichoderma*do not contain enough sequence differences within ITS regions to be separated (Mukherjee et al., 2013).

Proteins –coding genes have commonly been used in fungi identification and taxonomy study (Geiser et al., 2004). Of protein coding genes utilized are translation elongation factor 1α (TEF-1α) (Geiser et al. 2007)and second largest subunit of RNA polymerase II (RPB2) (Ertz et al., 2002).Promising studies proposed TEF-1α and (RPB2) genes as potential genetic markers in phylogenetic studies as two genes have sufficient sequences variability among fungi genera and even within fungi species. Moreover, The TEF-1α has been used and is preferred for discrimination among *Aspergillus* species (Geiser et al., 2007) and itis a useful genetic marker infungi taxonomy (Zhao et al., 2011). Additionally TEF-1α gene sequence was used to study relatedness among over 160 strains of dermatophytes species suggesting TEF-1α is a candidate DNA barcoding for separating strains of a species within a genus (Mirhendi et al., 2015). Meanwhile, the TEF-1α and RPB2 genes sequences were used asa supplement for ITS data to construct phylogenetic studies for fungi genera (Lopes et al., 2014).

Although the genus *Trichoderma*are being medically and commercially valuable, its taxonomy still unresolved, therefore a necessary need requiring a study to report the proper DNA barcoding gens for resolving *Trichoderma* species taxonomy. The objective of this paper to asses sufficiency variation within ITS, TEF-1α, RPB2 genes sequences retrieved from GenBank database to construct phylogenetic trees through both neighbor –joining and maximum likelihood analysis to discriminate between many different *Trichoderma* species

MATERIALS AND METHODS

DNA sequences of partial ITS, TEF-1α and RPB2 genes

In this study, gene sequences of rDNA ITS, TEF-1α and RPB2 genes were retrieved from NCBI-GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and saved in fasta file format. The sequences retrieved represent 28 species of genus *Trichoderma,* which belongs to phylum Ascomycota (Table 1).

Among accessions retrieved, three accessions were considered as out groups in phylogenetic analysis including *Rhizoctoniasolani* (KC833678.1),*Rhizopus azygosporus* (AF157285.1), and *Rhizoctonia endophytica* (KP171658.1) (Table 1).This study considered *Rhizoctonia* species and *Rhizopus azygosporus*as out groups as they belong to Basidiomycota phylum while *Rhizopusazygosporus* belongs to Zygomycota phylum.

DNA analysis and phylogenetic analyses

Gene sequences of ITS, TEF-1α and RPB2 genes retrieved were aligned using Clustal Wthrough MEGA 7 software (Kumar et al., 2016), The Clustal W sequences pairwise alignment were conducted under values 15 and 6.6 for the gap opening penalty and extension penalties respectively.

Table1: DNA sequences of ITS, TEF-1α and RPB2 genes retrieved form Genbank

Fungal species	GenBank #		
	ITS	TEF-1α	RPB2
T. cerinum	KP994910.1, MF408952.1 MF408952.1	AY278813.1	KP009174.1 KP009176.1
T. breve	NR 154574.1, KY687927.1	AY278814.1	KY687984.1, KY687983.1
T. simmonsii	MG132082.1, MG132084.1	KJ665714.1, KJ665718.1 KJ665716.1	KJ665337.1
T. harzianum	MF782820.1, MF782824.1 MG735708.1, MG735709.1	MG735712.1, MG735711.1 MF125298.1	MG917683.1, MG873465.1 MG917685.1
T. lixii	HQ608080.1, MG132080.1	FJ179573.1	
T. afroharzianum	NR 137304.1,	KP008787.1, KR911897.1	KT278940.1, FJ442691.1 KT278945.1, KP009149.1 KP009148.1
T. endophyticum	FJ884177.1	KX689257.1	
T. atrobrunneum	NR 137298.1, KJ665359.1, KJ665384.1	KJ665409.1, KJ665408.1 KJ665383.1, KJ665413.1 KJ665381.1, KJ665389.1 KX632629.1, AY364461.1 KJ665384.1	KX632572.1 , KJ665241.1 FJ442724.1, KX632571.1
T. spirale	MH633977.1, MH512965.1	FJ514779.1	KP009182.1
T. aggressivum	FJ442618.1, FJ442618.1 MF632118.1, MF952664.1	FJ514778.1	KP009170.1 KP009163.1
T. rossicum	MH633976.1, KY653726.1		KP009077.1, KP009080.1
T. virens	AF099008.1, AF099006.1		KP009091.1 KP009098.1 KP009100.1
T. tomentosum	KX619644.1, NR 134357.1		KP009177.1
T. velutinum	MH651384.1, MH651379.1		KP009178.1, KF134794.1
T. longifialidicum	NR 137309.1, KT278901.1	MF095879.1, MF095881.1 MF095880.1	
T. bannaense	NR 154570.1	KY688038.1, KY688037.1	KY688003.1 KY687979.1
T. solum	KY687932.1, KY687930.1	KY688051.1, KY688049.1	
T. ingratum	NR 154566.1, KY687917.1	KY688022.1	
T. pleuroticola.	MF871561.1, MF871554.1		KP009172.1
T. pseudodensum	NR 154562.1	KY688024.1, KY688023.1	
T. alni	NR 134375.1, EU518652.1		KX632578.1 KX632576.1 KX632574.1
T. liberatum	KY687914.1, KY687913.1	KY688026.1, KY688025.1	
T. brunneoviride	NR 134376.1	EU498318.1, EU498316.1	EU498358.1
T. pyramidale		KU051704.1, KX632627.1 KJ665698.1, KJ665697.1	KX632570.1, KJ665334.1 KU051702.1
T. italicum		KT149297.1	
T. guizhouense		KJ665506.1, KJ665505.1 KJ665507.1	KJ665273.1
T. crassum			KP009102.1
Rhizoctoniaendophytica			KP171658.1
Rhizoctoniasolani	KC833678.1		
Rhizopusazygosporus		AF157285.1	

Maximum likelihood analyses were performed on multiple alignments using MEGA 7 software (Kumar et al., 2016). The phylogenetic trees were constructed under Tamura Nei model and all positions containing missing data and gaps were eliminated. Neighbor –joining trees were conducted as well using MEGA 7 software (Kumar et al., 2016) under maximum likelihood composite model and all positions containing missing data and gaps were eliminated. In addition, the optimal tree with sum of branch length =0.47 was shown.

The trees constructed based on ITS and sequences was rooted with *Rhizoctonia solani*as an out group. While the trees constructed based on TEF-1α and RPB2 sequences were rooted with *Rhizopusazygosporus* and *Rhizoctoniaendophytica* respectively. Overall mean distance of sequences of ITS, TEF-1α and

RPB2 genes sequences were computed under maximum likelihood composite model through MEGA 7 software (Kumar et al., 2016) and Missing data and gaps were eliminated.

RESULTS

The two phylogenetic analyses including Neighbor –joining and Maximum likelihood were conducted on sequence data of ITS, TEF-1α and RPB2 (Figure1 2, 3). Using ITS *sequences* analysis, both Neighbor –joining and Maximum likelihood analyses generated trees of consistent results (Fig. 1). *Rhizoctoniasolani* was used in ITS phylogeny as an out group. The 23 Trichoderma species sequences were clustered into resolved 8 specific species clades as following

Species Clade l contains two*T. breve* isolates,

Species Clade 2 contains two*T. solum* isolates

Species Clade 3 contains two*T. aggressivum* isolates

Species clade 4 contains two *T. alni* strains

Species clade 5 contains two *T. liberatum* strains

Species clade 6 contains two *T. spirale* strains

Species clade 7 contains two *T. virens* strains

Species clade 8 contains two *T. longifialidicum* strains

Other 16*Trichoderma* species sequences involved in analysis were unresolved well (Fig. 1). In addition, it was clear that *Trichoderma* species strains of each *T. cerinum*, T*. simmonsii*, T*. harzianum*, *T. lixii*, *T. afroharzianum*, *T. endophyticum*, *T. atrobrunneum* forming monophyletic group. Moreover, other *Trichoderma* species strains were scattered separately on a tree instead of forming specific-species clades such as *T. rossicum* and *T. tomentosum* (Fig. 1).

According to TEF-1α*sequence* analysis, trees formed by Neighbor –joining and maximum likelihood analysis resulted in similar pattern of topology (Fig. 2). TEF-1α gene based phylogenetic tree was rooted with *Rhizopus azygosporus* which was used as out group (Fig. 2). TEF-1α gene based Neighbor –joining tree dividedthe analyzed *Trichoderma* species sequences into12 main clades or groups. The clade 1 contains two*. pseudodensum* isolates and two*T. liberatum* isolates are separated successfully to form clade 2. The *T. solum* sequences separated clearly to form clade 3. The *T. brunneoviride* strains were segregated to form clade 4.Analysis also placed*T. longifialidicum* and *T. bannaense*in clade 5 and 6 respectively discriminating between *T. longifialidicum* strains. The following fungal species *T. harzianum*, *T. simmonsii*, T*. pyramidale*, and *T. guizhouense strains* were separated on the tree to form clades 7, 8,9 and 10 respectively.

It was clear that clade 11 has a good resolution in*T. atrobrunneum* strains discrimination. Two *T. afroharzianum* isolates were clustered to form clade 12.However, the following fungi species including *T. harzianum* strain B129s, *T. harzianum* strain B1952 were clustered a way from other *T. harzianum* strains on tree and did *T. simmonsii* strain S547 as well. *T. lixii* strain CPK clustered with *T. atrobrunneum* strains in clade 11.On the basis of *RPB2* sequence analysis, trees constructed by Neighbor –joining and maximum likelihood had somehow similar topology (Fig. 3). However, Neighbor –joining tree had higher resolution in species grouping and separation. In the *RPB2*Tree, *Rhizoctoniaendophytica, which* belongs to basidiomycota considered as out group (Fig. 3). The resulting tree (Fig. 3) indicated that the analyzed RPB2 gene sequences had enough variations to separate between the investigated Trichoderma species or strains into species-specific groups or clades forming12 main clades. Within the clade 1, *T. afroharzianum* strains sequences clustered with each other forming a monophylogenetic group, *T. afroharzianum* strains are separated successfully in sub clade. Clade 2 contains *T. atrobrunneum* and its strains resolved clearly in sub-clade. The analysis placed *T. bannaense* sequences in clade 3. Clade 4 discriminated well between *T. harzianum* strains. *Trichoderma* species including each T*. pyramidale*, *T. aggressivum*, and *T. Brave* were clustered in clades 5, 6, 7 respectively.

The three *T. alni* strains were resolved in sub clade and placed in the clade 8. Moreover, it was clear separation of two *Trichoderma*species *T. cerinum* and T. velutinum in clades 9 and 10 and forming a monophyletic group. Both *T. virens* and*T. rossicum* were segregated into groups 11 and 12 respectively. *Trichodermavirens* strains were separated in the group 11 as a sister clades. *Trichoderma pyramidale* strain S73 and *T. atrobrunneum* strain T39, however, were separated individually away from their species on tree. Meanwhile, Computed overall mean distance of sequences for RPB2 gene was 0.1 and higher compared with zero value for both ITS, TEF-1α overall mean distance.

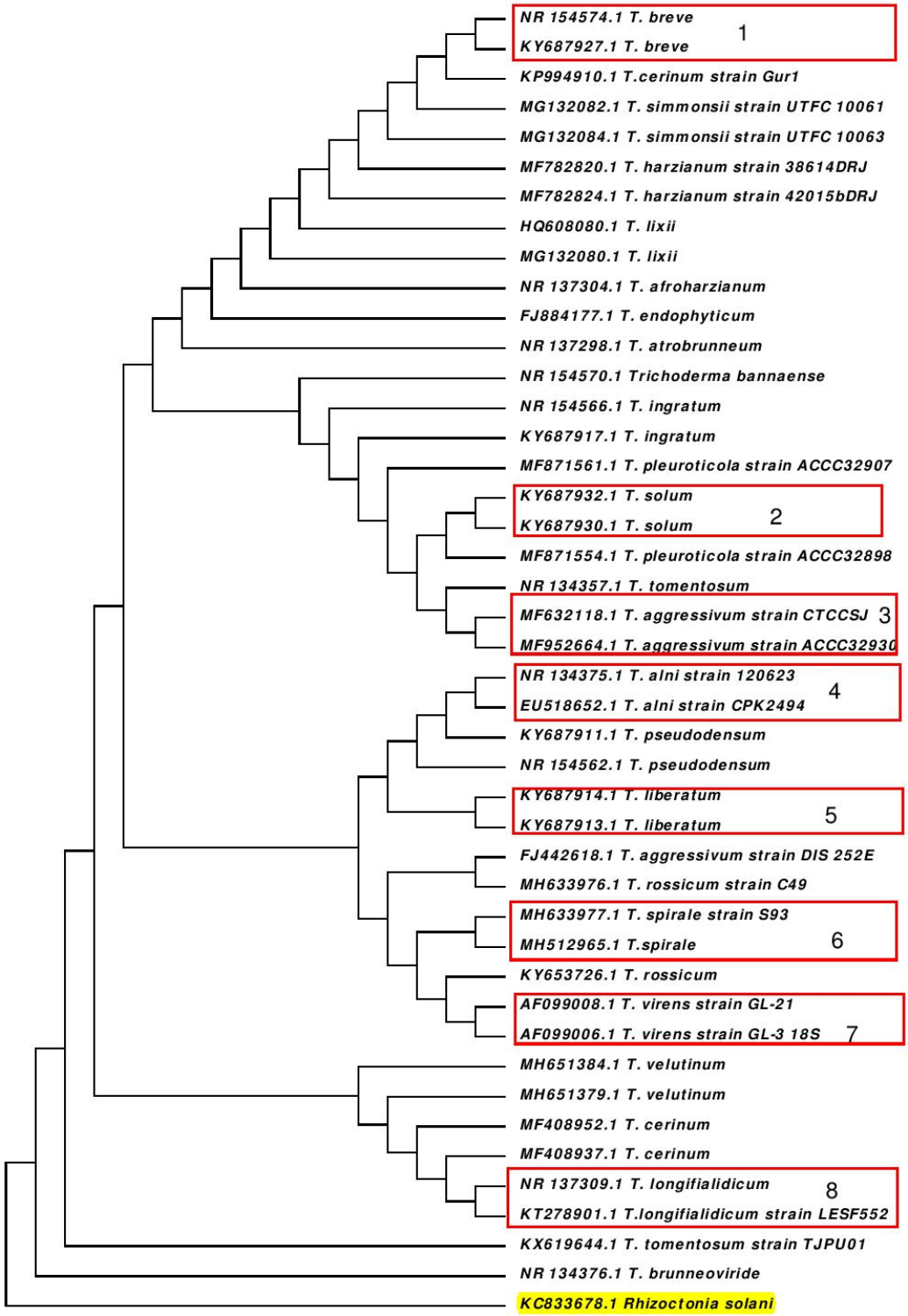

Figure 1.Neighbor –joining tree of 44 fungal isolates, representatives of 23 *Trichoderma* species, based on analysis of ITS sequence.

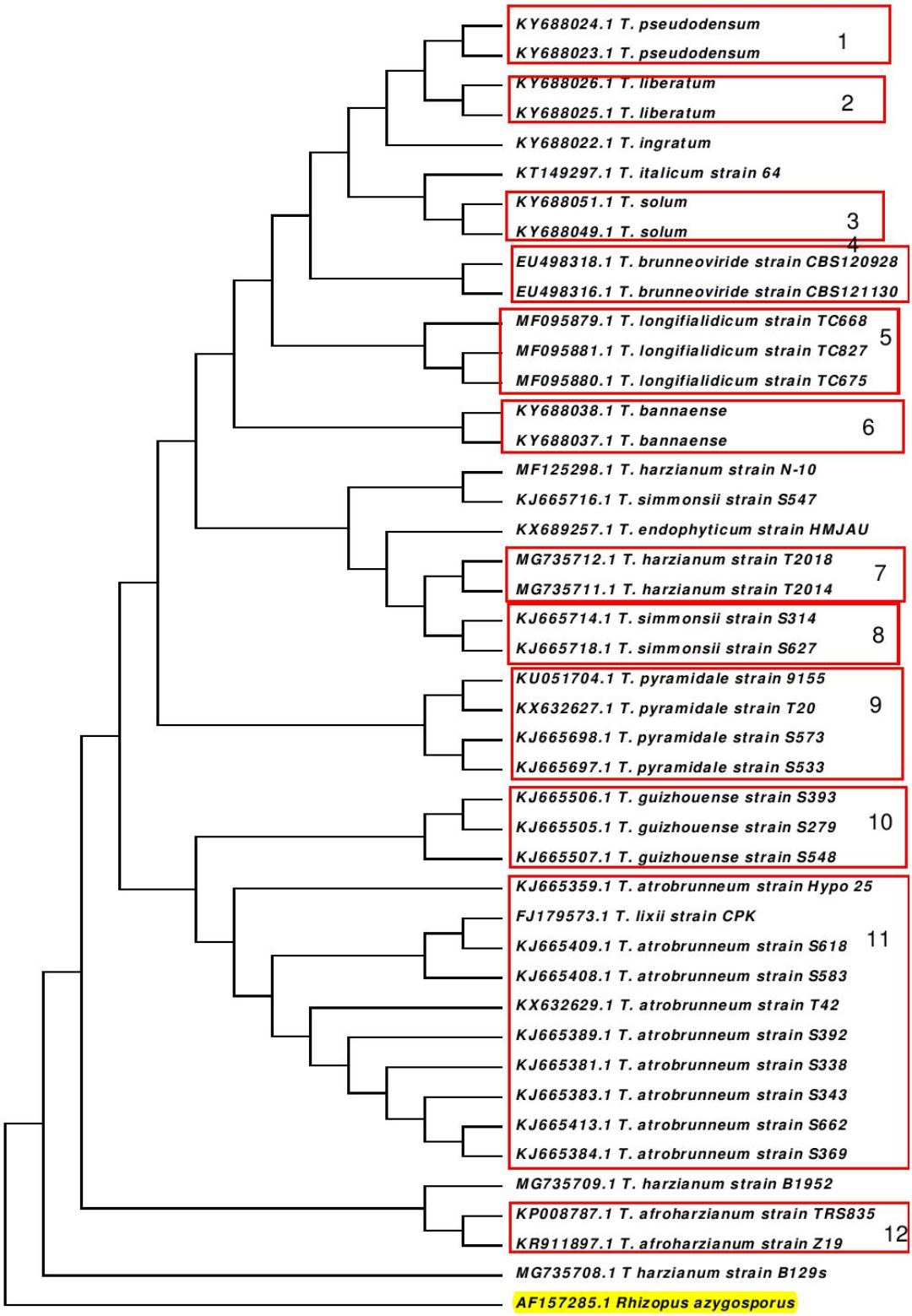

Figure 2.Neighbor –joining tree of 30 fungal isolates, representatives of 20 *Trichoderma* species, based on analysis of partial TEF-1α gene sequence.

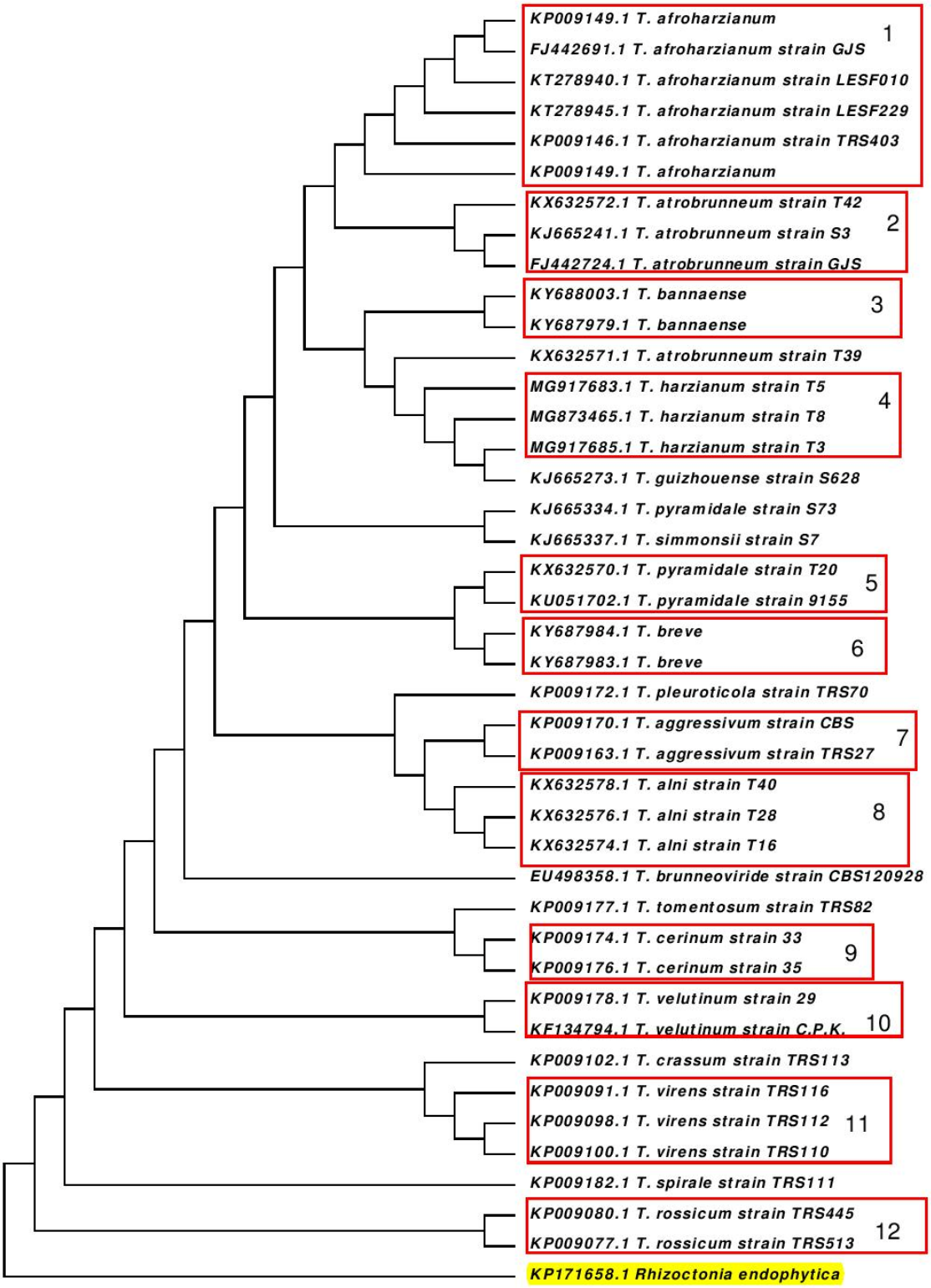

Figure 3.Neighbor –joining tree of 33 fungal isolates, representatives of 23 *Trichoderma* species, based on analysis of partial RPB2 gene sequence.

DISCUSSION

In this study, Phylogenetic analysis of 27fungal taxa belonging to the Trichoderma genus were conducted using ITS, TEF-1α and RPB2 nucleotide sequences retrieved from GenBank Database. The 23 different species of *Trichoderma* were involved in the ITS phylogeny analysis, the tree constructed by Maximum likelihood analysis resulted in a tree showed overall bad resolution of most analyzed species (Fig. 1).

It is shown from ITS sequence based tree, only 8 of 23 Trichoderma species were separated to form distinct species specific-clades, Moreover, most ITS sequences were gathered under few monophyletic groups and many clades contain more than species meaning that most ITS branches or clades were not specific -species clades that indicates that ITS sequences did not have significant nucleotide variations resulting in unresolved species separation between analyzed *Trichoderma* species.

These ITS phylogeny results were supported by zero value for calculated overall mean distance of ITS sequences that indicated to low sequence variations between (*Trichoderma* species), which results in bad resolution for separation among *Trichoderma* species. Interestingly, such finding indicated that polymorphism in ITS regions sequences could be < 1% between fungus species. These results revealed a presence of low intraspecific variations between investigated ITS *Trichoderma* species sequences. Similar findings on other fungi genera proved that ITS was not the appropriate gene to be used to separate between different *Fusarium species* (Raja et al., 2017). Moreover, more reports supported these results in case of some fungi such as *Cladosporium* , and *Trichoderma*.

According to the TEF-1α maximum likelihood analysis (Fig. 2), 20 different species of *Trichoderma* were used in phylogenetic studies resulted in species -specific tree which showed12 distinct clades of specific species indicating that partial TEF-1α sequences have a significant variation leading to a better resolution of most species of *Trichoderma*. These findings were in agreement with results of many studies being EF-1α gene sequences could be a unique genetic marker and have a good sequence variation to be use in fungi taxonomy and species differentiation which is proven to be useful in studying fungi genera including *dermatophytes* (Mirhendi et al., 2015), *Trichoderma* and *Fusarium* (Zhao et al., 2011) and *Neofusicoccumkwambonambiense* (Lopes et al., 2014) and also in nectriaceous fungi (Zhao et al., 2011).

Some reports revealed that the TEF- 1α gene sequence was strongly suitable as a genetic marker to separate between species of *Fusarium* as the intron of TEF- 1α gene has enough sequence nucleotides variations among fungi species (O'Donnell et al., 1998).The phylogeny analysis of the partial RPB2 gene sequences was conducted for 21 different Trichoderma species in this study (Fig 3), partial RPB2 gene sequences showed sufficient sequence variations to separate between species of this genus. The RPB2 maximum likelihood analysis resulted in separating *Trichoderma* species into 12 well-resolved distinct clades.

These RPB2 gene sequences phylogeny results were supported by 0.1 value for calculated overall mean distance of RPB2 gene sequences, which indicated that variability in RPB2 gene sequence is around 10% between investigated *Trichoderma* species indicating the protein coding gene RPB2 could successfully be employed in fungi species identification and taxonomy, as its sequence revealed enough variation resulting in species discrimination. Previous findings proved these results and showed that RPB2 sequencing was used in identification of fungi genera *Neofusicoccum kwambonambiense* (Lopes et al., 2014) and *Trichoderma* species (Lu et al., 2004). Having sufficient sequence variation, mycologists used RPB2 gene as DNA barcoding inreclassifying of*Diaporthe* species (Udayanga et al., 2012a).

CONCLUSION

In summary, Phylogenetic analysis of different *Trichoderma* species using ITS, TEF-1α and RPB2 nucleotide sequences through both Neighbor –joining and Maximum likelihood analyses revealed that RPB2 gene sequence can be used as a potential genetic marker and DNA barcoding in *Trichoderma* species discrimination followed by TEF-1α and then by ITS. This study has confirmed that that Phylogeny analysis using protein coding genes could be a very valuable tool in identification and taxonomy of fungi in *Trichoderma* species and in cases where phylogenetic relationship between different species of a genus unresolved by ITS regions.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors express their deep appreciations for all participants and their guidance.

AUTHOR CONTRIBUTIONS

Alhawatema designed, collected, and performed the analysis experiments for collected data, and also wrote the manuscript. Both Alqudah and Al Tawaha shared in reading, editing, proofreading, and approved the final version.

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