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Phylogenetic Relationships of *Trichoderma harzianum* Based on the Sequence Analysis of the Internal Transcribed Spacer Region -1 of the rDNA

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Abstract: The goal of this study is to determine whether sequence analysis of internal transcribed spacer -1 region of the rDNA can be used to detect species level of *Trichoderma harzianum*. Internal transcribed spacer- 1 region (ITS 1) of the ribosomal DNA was amplified by polymerase chain reaction (PCR). To test the selected universal primers (ITS1 and ITS2) and conditions of the PCR, thirty-six of Malaysian *Trichoderma* isolates were used. The results of PCR product were positively performed purification. The PCR purification products were proved possible to amplify the ITS 1 region of all *Trichoderma* strains. The amplified DNA was sequenced and aligned against using ex-type strains sequencings from *TrichoBLAST* /GenBank and established *Trichoderma* taxonomy. Thirty-six isolates were positively identified as *Trichoderma harzianum* (32 strains) *Trichoderma virens* (3 strains) and *Trichoderma longibrachiatum* (1 strain) formed clearly defining phylogenetic analysis. *T. virens* and *T. longibrachiatum* which were used as an outgroup in these analyses. To this end, thus are proposed that the ITS-1 region sequences are used as the reference's sequence for future study involving the identification and taxonomy of *Trichoderma harzianum*. Amplification of ITS 1 region of the rDNA has showed potential as a rapid technique for identifying *Trichoderma harzianum* successfully fungi in all cases.

Keywords: Trichoderma; Internal Transcribed Spacer; Sequencing; Polymorphic Chain Reactions (PCR)

INTRODUCTION

The fungal genus *Trichoderma* (Ascomycetes, Hyprocreales) contains species that are of vast economic importance owing to their production of industrial enzymes (cellulose and hemi-cellulose), antibiotics,[1,2] and their ability to act biological control agents against plant pathogens since 1920s[3].

Taxonomy of *Trichoderma* is currently based largely on morphological character[4,5] such as conidial form, size, color and ornamentation, branching pattern with short side branches, short inflated phialides and the formation of sterile or fertile hyphal elongations from conidiophores. *T. harzianum* is an aggregate species, divided in three, four or five subspecific groups, depending on the strains. However, most species descriptions are based on examination of a limited number of strains where the morphological differences are clear but these differences become less clear as more strains are studied. This result suggests that there are not enough morphological and cultural characters to reliable define species level.

Identification of *Trichoderma* isolates at the species level has proved difficult, due to the degree of morphological similarities. Rifai[4] adopted the concept of "species aggregate" and distinguished nine aggregates, some of which comprised two or more morphologically indistinguishable species. Gams and Bissett[6], in an attempt to differentiate phenotypically similar species, proposed 'sections' based on morphology, to accommodate similar forms within the species concept of Rifai[4]. Current studies find that morphological analysis was highly prone to error and roughly 50% of the *Trichoderma* spp. obtained by morphological analysis alone was wrongly identified[7].

Molecular methods have recently been introduced into *Trichoderma* taxonomy with revision of sects *Longibrachiatum* and *Trichoderma*, respectively and related teleomorphs[8]. In these techniques have been proven to be valuable tools in fungal taxonomy and their application has led to the reconsideration of several genera[9]. In *Trichoderma*, isoenzyme analysis[10,11], RAMS[12,13] and rDNA sequencing[14,15] have been used to distinguish species within specific groups of strains.

Kindermann *et al.*[16] attempted a first phylogenetic analysis of the genus *Trichoderma*, using the sequence analysis of the ITS 1 region of the rDNA. Nevertheless, the use of phylogenies based on single gene sequences is now normally discredited, especially as regards the use of ITS1 and/ or ITS2, as some fungi and plants have been shown to contain paralogous copies[17]. Taylor, *et al.*[18] proposed basing phylogenetic species concepts between five or more gene trees. These results demonstrated molecular techniques indicating interrelations among species and, while

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combined with phenotypic characters, can lead to a reliable taxonomy that is reflective of phylogenetic relationships.

Druzhinina *et al*.,[19] was able to identify 70 out of a total of 77 investigated the genus of *Trichoderma*. Kubicek *et al*.,[20] demonstrated that seventy-eight isolates of *Trichoderma*, 37 strains were positively identified as *T. harzianum*, other were *T. virens* (16 strains), *T. spirale* (8 strains), *T. koningii* (3 strains), *T. aureoviride* (3 strains), *T. asperellum* (4 strains), *Hypocrea jecorina* (2 strains), *T. viride* (2 strains), *T. hamatum* (1 strain) and *T. ghanense* (1 strain). Ospina-Giraldo *et al*.,[21] showed that phylogentic analyses were closely related to an isolates of *T. harzianum* compared others *Trichoderma* isolates.

The purpose of the present study was to establish a species of *Trichoderma harzianum* gene sequence in Malaysian isolates based on the sequence analysis of ITS-1 regions of the rDNA gene.

MATERIALS AND METHODS

***Trichoderma* Strains:** A total of 32 *T. harzianum* were used; 18 isolates were from the Mycology laboratory, Department of Biology, UPM and 14 isolates were of cultured, isolated and identified by morphological based this author. As out group samples one culture of *T. longibrachiatum*, three of *T. virens* etc. were used and thus were all obtained in Table 1.

DNA Extraction Methods: Cultures were maintained on potato dextrose agar (PDA) (Difco) and mycelia for DNA extraction were grown in liquid cultures at 28±2°C in potato dextrose broth (PDB) (Difco) for 7 days. Mycelia were firstly collected by filtering through a double layered of muslin cloth, washed with distilled water and frozen at -20°C. Fungal genomic DNA was extracted using the Phenol- Chloroform Method[22]. From 50 mg of frozen mycelium was added 500 µl extraction buffer (consisted of 1M Tris HCl pH 8.5, 1M NaCl pH 8.5, 1M EDTA pH 8.0 and 10% Sodium dodecyl sulphate) for 8 h at 40 ^{0}C, after that adding 350 µl buffered phenol and 150 µl chloroform with frequently mixing. The mixture was centrifuged at 13,000 rpm for 10 min at 4°C. After centrifugation, the upper aqueous layer was transferred to a clean 1.5 ml centrifuge tube adding 3 µl RNAse solutions. The mixture was incubated at 40°C by water bath for 15 minutes. After incubation, equal volumes of chloroform were added with frequently mixing. Then the mixture was centrifuged again at 13,000 rpm for 10 minutes at 4°C. The upper aqueous phase again was transferred and the DNA was precipitated with 250 µl isopropanol for over night at -20°C. The tube was centrifuge at 13,000 rpm for 10 minutes at 4°C and the pellet was thoroughly washed twice with 500 µl of 70% ethanol, vacuum-dried and dissolved in ddH_2O. Final DNA concentration was about 50 to 100 ng/µl.

Polymerase Chain Reaction (PCR) Amplification of Internal Transcribed Spacer (ITS)-1 Region of rDNA: Two universal primers were used in this study, they were applied the primer ITS1 (5' TCCGTAGGTGAACCTGCGG 3') for forward and the primer ITS2 (5' GCTGCGTTCTTCTTCATCGATGC 3') for reverse, the primers were used to amplify of internal transcribed spacer-1 (ITS 1) region of rDNA. The sequences were commercially designed by Medigene Company (Figure 1).

A nuclear rDNA region, containing the internal transcribed spacer region-1 was amplified by polymerase chain reactions using the primer combinations ITS1 and ITS2[23]. The PCR amplifications were performed using a Peltier Thermal Cycler-200 with the following program: an initial denaturation of 5 minutes at 95°C, followed by 36 cycles of 1 minute at 94 ^{0}C, an annealing temperature at 55°C for 1 minute, extension at 72°C for 3 minutes with a final extension of 10 minutes at 72°C.

Aliquots of 10 µl from each PCR product were analyzed for presence of DNA by electrophoresis in a 1.5% agarose horizontal minigels for using 1 X TBE (0.045 M Tris-borate and 1 mM EDTA, pH 8.2) at 70V for 1 to 2 hours, depending on the size of the amplified fragments from each primer as running buffer. After electrophoresis, the gels were stained with ethidium bromide (0.5 µl/ml) for 15 to 30 minutes and were visualized under UV- light and photographed using the Alphamager 2200 version 5.5 apparatus. Comparisons of the banding patterns were detected using the 50 bp marker as a molecular size standard.

DNA Sequencing: Template DNA (50 µl) was directly prepared from PCR-products by purifying it with a commercial kit (QIAquick® PCR Purification Kit). The PCR purification products (30 µl) were sent to Medigene Company for the direct sequencing. DNA sequence for the internal transcribed spacer region-1 (ITS 1) of the rDNA was obtained. Amplification of nuclear rDNA, containing the ITS 1 region of rDNA gene was using as described previously Kindermann, *et al*.,[16].

After collected the sequences from Medigene SDN. BHD, all sequences obtained in this study were submitted to the NCBI GenBank/ or *TrichoBlAST* gene bank, their accession numbers were indicated in Table1. Previously published sequences used for phylogenetic analyses in this study were given by accession numbers.

Sequence Alignment: Computer–aided alignment of the ITS 1 region sequence was performed using the computer software package CLUSTAL-W[24] in the

Table 1: List of species, code and locality for *Trichoderma* strains analyzed in this study

Species name	Code	Reference	Locality
T. harzianum	FA2, FA8, FA15, FA17, FA24, FA26, FA29, FA30, FA31, FA34, FA36, FA38, FA40, FA44	this author	Sedenak, Johor Baru
T. virens	FA35	this author	Sedenak, Johor Baru
T. harzianum	T32, T66, 71, T72, T80, T98, T100, T101, T102, T121, T124, T29, T45, T55, T58, T86, T106, T126	Illias, 2000	Negeri Sembilan
T. longibrachiatum	T28	Illias, 2000	Negeri Sembilan
T. virens	T49, T67,	Illias, 2000	Negeri Sembilan

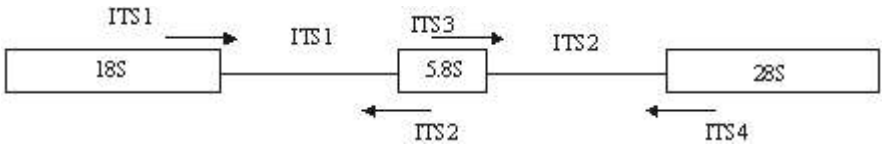

Fig. 1: Schematic representation of rDNA showing the internal transcribed spacer (ITS)-1 region and 5.8S gene with primers (primers: ITS1 and ITS2) amplifying different parts of the spacer. Primer sequences are given in the text above.

BioEdit Sequence Alignment Editor. The alignment was then optimized manually. Single gaps were treated as a fifth nucleotide (A, C, G, T and gaps). All gap positions within the alignment exceeding a single base in length were replaced by question marks. Sequence insertions without homology to any of the other sequences were deleted in the alignment and a single base was left over causing a minimal gap. In this way, gaps of all sizes were weighted equally corresponding to a hypothetical single evolutionary event. Therefore, finally, alignment adjustments were made manually in order to remove artificial gaps.

The aligned sequences were visually inspected and adjustments made to improve the alignment. *Rhizoctonia solani* (with gene bank accession number, D88538, deposited by Katsura *et al*.,[25], *Trichoderma harzianum* (Strain: THVA) (with gene bank accession number, AJ224021, deposited by Hermosa *et al*.,[26], *T. longibrachiatum* (Strain: CBS 816.68) (*TrichoBlAST* gene bank, accession number: Z31019/ATCC 18648, deposited by Kuhls *et al*.,[27] and *T. virens* (Strain: CBS 249.59) (with *TrichoBlAST* gene bank, accession number: AF222865, deposited by Kullnig and Kubicek[28] sequence were used as comparison of *Trichoderma* isolates sequence submitting to a *TrichoBLAST* search http://www.isth.info/tools/blast/index.php to give more accurate results[14].

2.6 Phylogenetic analyses: DNA Sequences were aligned visually with the CLUSTAL-X software[29], based on the algorithm of Waterman[30]. The interleaved NEXUS file was formatted using DNASP[31] computer software to be recognized by MEGA software package. Phylogenetic analyses were completed using the MEGA package.

Phylogenetic inference was performed by the unweighted pair-group method using arithmetic average (UPGMA)[32]. Bootstrap tests with 1,000 replications[33] were conducted to examine the reliability of the interior branches and the validity of the trees obtained. Distance was defined as the probability of nucleotide substitutions per site, based on the Kimura 2-parameter model (K2P)[34].

RESULTS AND DISCUSSIONS

Results:

Sequence Alignment: For direct sequencing of the PCR products, a total of 36 isolates of *Trichoderma* PCR products produced sequences that could be aligned and showed satisfactory homology with ex-type strain (THVA) of *T. harzianum* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS2 primers were determined in order to confirm that the sequences obtained corresponding to the actual ITS 1 region (Table 2).

A multiple sequence alignment was carried out that included the ITS 1 region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the ITS-1 region that were closely related and similar sequence indicated. Whether, 36 Malaysian *Trichoderma* isolates were used in the multiple sequence alignment. From the sequence alignment, variations were observed between *T. harzianum* isolates and others species of *Trichoderma* isolates.

Phylogenetic Analysis: The ITS 1 region sequence was used in these analysis because it had showed to be more informative and closest phylogenetic relative in the

Table 2: Identified *Trichoderma* spp and comparison with referred GeneBank

Isolates number	Identified as	GeneBank accession no	Strains and reference	(%) Identity
FA-2	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-8	*T. harzianum*	AJ224020	T3, Hermosa *et al*., 2000	100
FA-15	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-17	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-24	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-26	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-29	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-30	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-31	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-34	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-35	*T. virens*	AF222865,	CBS 249.59, Kullnig and Kubicek 2000	98
FA-36	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-38	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-40	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
FA-44	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-32	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T66	*T. harzianum*	AJ224020	T-3, Hermosa *et al*., 2000	100
T-71	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-72	*T. harzianum*	AJ224020	T-3, Hermosa *et al*., 2000	100
T-80	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-98	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-100	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-101	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-102	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-121	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-124	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-28	*T. longibrachiatum*	Z31019,	CBS 816.68, Kuhls *et al*., 1996	100
T-29	*T. harzianum*	AJ224020	T-3, Hermosa *et al*., 2000	100
T-45	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-49	*T. virens*	AF222865,	CBS 249.59, Kullnig and Kubicek 2000	100
T-55	*T. harzianum*	AJ224020	T-3, Hermosa *et al*., 2000	100
T-58	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-67	*T. virens*	AF222865,	CBS 249.59, Kullnig and Kubicek 2000	100
T-86	*T. harzianum*	AJ224020	T-3, Hermosa *et al*., 2000	100
T-106	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100
T-126	*T. harzianum*	AJ224021	ThVA, Hermosa *et al*., 2000	100

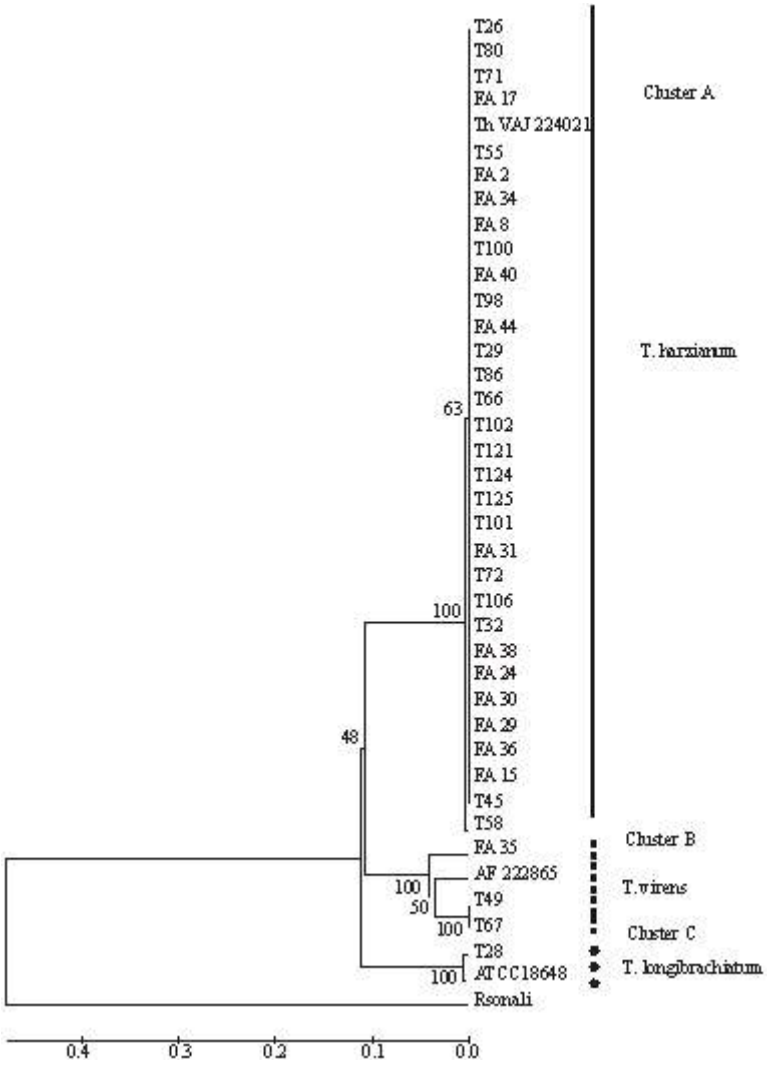

Fig. 2: Phylogenetic relationships of 36 isolates of *Trichoderma* strains inferred by UPGMA bootstrap tree analysis of ITS sequences.

genus of *Trichoderma*. In order to evaluate whether the grouping pattern obtained on the basis of the ITS 1-sequences of the ex-type strains would be useful frame to identify and align, thus were investigated a total of 36 Malaysian *Trichoderma* isolates, most of which had been deposited as *Trichoderma sp* belong to sect. *Pachybasium*.

A total of 36 strains of *Trichoderma* in these studies were isolated from infected and healthy oil palm. These strains were identified at the species level by morphological character using the existing taxonomic criteria analysis and by analysis of their ITS 1 region gene sequences. The strains of *Trichoderma virens* and *Trichoderma longibrachiatum* were used as an outgroup because it had shown to be more closely relative species of *T. harzianum*. The strains of *Rhizoctonia solani* used as an outgroup for totally difference gene sequence between ingroups the genus of *Trichoderma*. On the bases of a UPGMA bootstrap tree analysis with 1,000 bootstrap replications demonstrated three major cluster according to A, B and C (Figure 2).

Cluster A, the majority of *Trichoderma* strains (30 isolates) exhibited ITS 1 sequences which were closely resembled those described of *T. harzianum* as the gene sequence very similar. For 30 isolates of *Trichoderma*, the ex-type strain (THVA) of *T. harzianum* was indicated that all isolates were fitted well into cluster A as established by UPGMA analysis. All these strains could be referred to *T. harzianum*. In order to propose that *T. harzianum* isolates could be grouped into several haplotypes (term "genotype") on the basis of ITS 1 hallmark region. In these cluster was supported at a bootstrap

stability of 100% on the basal position (Figure 2).

Three *Trichoderma* isolates were closely similar ITS 1 sequence clustered at a statistically of 100% supported on the position of the cluster referring *T. virens*. *Trichoderma* isolates (FA35, T49 and T67) and ex-type strain (CBS 249.59) collected from *TrichoBLAST* showed that all isolates were well-appropriately located of cluster B. Thus strains were referred to *T. virens* (Figure 2). One isolates (T28) morphologically resembling *T. longibrachiatum*. After that these isolates exhibited a sequence and ex-type strain (CBS 816.68) sequences were showed very similar. The UPGMA analysis was high supported of 100% at the position of Cluster C.

Discussion: The genus *Trichoderma* poses a major challenge for systematists because the phylogenetic relationships of many of its members still now are unclear. So the concepts of "species aggregate" and "section" introduced by Bissett,[5] and Rifai,[4] have helped clarify placement of conflicting species such as *T. harzianum*, *T. viride* and *T. atroviride* within the genus. However, the influences of environmental conditions on morphological and physiological characteristics have made accurate identification very difficult[35]. (Lieckfeldt *et al.*, 1998).

In these works and in previous works[36] the sequence of the ITS 1 region of the most of the accepted species of *Trichoderma* has been examined. The information provided by these studies giving more useful for assessing the *Trichoderma* taxonomy. A total of 36 *Trichoderma* isolates, in which, initially morphological identified as *T. harzianum, T. aureoviride, T. virens and T. longibrachiatum*. After that, molecular phylogenetic analysis shows no species identity of *T. aureoviride*. The thirty-two isolates (Table 1) of *T. harzianum* as the same sequences have showed to the referred stain (ThVA) sequence of *T. harzianum*. The genetic distance among these strains virtually zero, thus suggesting that these strains morphological character very similar– are phylogenetically very closely related *T. inhamatum/ T. lixii/* was synonymized with *T. harzianum*. In view of the present data, the ITS 1 region sequence would suggest alone with the anamorph of *Hyrocrea lixii* are fully held among the genetically of *T. harzianum*.

Phylogenetic analysis placed ex-type strain (THVA) as most closely related to cluster-A, all identified strains of *T. harzianum*. The cluster-A is well supported by the bootstrap value of 100% in these analyses. After that these results indicate that the ITS 1 region gene sequence could be used as a reference sequence for future study involving the identification of *T. harzianum* taxonomy. Three *Trichoderma* isolates belong to *T. virens*, which are approximately similar to Cluster-A and clearly members of sect. *Trichoderma*. Then, it is owing to the small sequence differences of ITS 1 region in these clusters and, which have not appeared a closely interaction. In these *Trichoderma* isolates identified as '*T. virens*' and are markedly similar for the reference strain (CBS 249.59) of *T. virens*, which is collected from *TrichoBLAST* genbank. The isolate of T28 is morphologically consistent with the types of *T. longibrachiatum*, while the isolate has (T28, IMI 375055) conformed by International Morphology Institute, UK described for *T. longibrachiatum*. Phylogenetic analysis has showed at the same cluster-C defining as the ex-type strains of *T. longibrachiatum*. The cluster-C is supported by the bootstrap value of 100%.

In conclusion, above thus results strongly indicated of "*Trichoderma* aggregate species" with similar "DNA-based sequence", to accommodate with similar forms as referred the species identifying concept. Whether 32 *Trichoderma* isolates comprise the largest group and simialr gene sequence with respect to phylogenetic analyses and assigned to *T. harzianum*.

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