TRANSACTIONS ON SCIENCE AND TECHNOLOGY

Identification of *Trichoderma* Species From Wet Paddy Field Soil Samples

Azriah ASIS & Shafiquzzaman SIDDIQUEE*
Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, Sabah, MALAYSIA.
*Corresponding author: shafiqpab@ums.edu.my; Tel: +6088-32000 (ext. 8467); Fax: +6088-320993

Received: *13 August 2015*
Revised: *27 August 2015*
Accepted: *27 August 2015*
In press: *31 September 2015*
Online: *1 April 2016*

Keywords:
Biocontrol agents; ITS; TEF1; *Trichoderma*.

Abstract

Trichoderma species has gained immense economic importances because of their production of industrial enzymes and antifungal antibiotics, used as biocontrol agents, used in textile industry and as plant growth promoter. Thus, the correct identification of the species is necessary for its commercial demand. Preliminary identification of the species is usually based on its morphological properties, but the result is inadequate for species level identification. Molecular approaches using a single gene to multiple genes have applied for valid species identification. The main aim of this study is to characterize the genetic variability among twenty isolates of *Trichoderma*, obtained from wet paddy field soil. Data analysis of the internal transcribed spacer (ITS) regions of the rDNA and a partial sequence of the translation elongation factor 1-alpha (TEF1) were constructed in a phylogenetic analysis and were positively identified as *Trichoderma asperellum* (85%), *T. harzianum* (10%) and *T. reesei* (5%). The result confirmed the potential of molecular data in differentiating the species-specific level among all *Trichoderma* isolates.

Introduction

Trichoderma is cosmopolitan fungal genus, mainly found in soils, decaying wood and plant debris (Migheli *et al*., 2009). Their potential in the biological control of plant diseases are well known. It always plays an important role in bio-control mechanisms including antibiotics and ergosterol production that can suppress different plant pathogens, especially those soil borne pathogenic fungi, stimulate growth of plants and induce resistance to the disease (Woo *et al*., 2007). Taxonomic study of *Trichoderma* aim to discover several new potential strains that might exhibit the biocontrol potential.

Conventional method for identification of *Trichoderma* strains was solely based on the morphological species concept (Gams & Bissette, 2008). The morphological-based characterization can easily lead to erroneous results because of the difficulties and it requires special expertise (Hatvani *et al*., 2014). Introduction of molecular approach enabled researchers to identify and delimit species of *Trichoderma* more reliably. The ITS regions of ribosomal DNA (rDNA) were the first gene studied (Kindermann *et al*., 1998). Later, multigene approaches have been applied (Kullnig-Gradinger *et al*,. 2002), which used the combination of ITS with translational elongation factor 1-alpha (TEF1), endochitinase (ech42), large-subunit of ribosomal polymerase 11 (RPB1), actin (ACT), calmodulin (CAL) and tub2 region of β-tubulin gene (Yu *et al*., 2007; Alvindia *et al*., 2011; Shentu *et al*., 2014).

An important approach for the identification of *Trichoderma* species is the development of an oligonucleotide barcode *Trich*OKEY (Druzhinina *et al*., 2005) and *Tricho*BLAST (Kopchinskiy *et al*.,

2005) which available at www.isth.info. *Trich*OKEY allows the identification of a majority of species within *Hypocrea*/*Trichoderma*, based on the combination of certain markers (anchors, hallmarks) of vouchered ITS1 and ITS2 sequences, obtained by thorough analysis of these sequences compiled in a local database. *Tricho*BLAST is developed based on ITS, TEF1 and RPB2 sequences of vouchered specimens and provides more reliable identification of *Hypocrea*/*Trichoderma* than GenBank.

Methodology

DNA Extraction, PCR Amplifications and Sequencing

Genomic DNA of *Trichoderma* was extracted from fresh mycelium using the modified CTAB method Primers ITS1 (5'-TCTGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') described by White *et al.* (1990) and amplified a fragment of the rDNA including ITS1 and ITS2 and the 5.8S rDNA gene. Fragments of translational elongation factor 1 alpha (TEF1) were amplified as described previously by Druzhinina *et al*. (2008). The PCR products were resolved using a 1.5% agarose gel. PCR fragments of these genes were purified (PCR purification kit, Qiagen) and subjected to the sequencing at the First BASE Laboratories.

Sequence Analysis

DNA sequences were aligned using computer software package Clustal W in the BioEdit Sequence Alignment Editor. Single gaps were treated as fifth nucleotide.

Phylogenetic Analysis

A phylogenetic analysis was carried out using the MEGA 6 package. Kimura-2 distance model was applied for phylogenetic tree reconstruction using unweighted pair-group method using arithmetic average (UPGMA). Confidence value was assessed using 1000 bootstraps to examine the reliability of the interior branches and the validity of the trees obtained.

Results

DNA Extraction, PCR Amplifications and Sequencing

A total of 20 *Trichoderma* isolates were identified preliminarily via morphological observation. After that, their genomic DNA was amplified using two unlinked loci, ITS1 and 4 of the rDNA region and TEF1 gene. A specific fragment of the expected size about 600 bp and 400 bp were successfully amplified from DNA of all investigated *Trichoderma* isolates, both for ITS and TEF1 regions, respectively. The isolates were identified via Blast search of GenBank available in NCBI. NCBI GenBank accession number and isolation details are given in Table 1. The majority of the isolates were found to be *T. asperellum* (13 isolates) followed by *T. harzianum* (6 isolates) and *T. reesei* (1 isolate).

Table 1: Lists of *Trichoderma* isolates including NCBI accession numbers of corresponding sequences.

Isolate code	Species identified	GenBank accession number	
		ITS	TEF1
2P	*T. asperellum*	KM458788	KJ677263
3P	*T. asperellum*	LN846676	KJ677263
4P	*T. asperellum*	LN846676	AB935986
5P	*T. asperellum*	LN846676	AB935986
7P	*T. asperellum*	LN846676	KJ677268
8P	*T. asperellum*	KM604669	LN846754
9P	*T. asperellum*	LN846676	KJ677263
10P	*T. asperellum*	LN846676	AB935986
12P	*T. asperellum*	LN846676	LN846754
13P	*T. harzianum*	KP281701	KJ677263
27P	*T. harzianum*	KM386415	FJ463324
39P	*T. harzianum*	JX518920	AF469194
40P	*T. harzianum*	LN846719	AF469194
43P	*T. reesei*	KP263685	KJ713193
44P	*T. harzianum*	LN846721	AB568382
45P	*T. asperellum*	LN846676	JF964995
46P	*T. harzianum*	KC874893	KJ871183
49P	*T. asperellum*	LN846676	KJ677263
50P	*T. asperellum*	KM527926	JF964995
52P	*T. asperellum*	LN846676	JF964995

Phylogenetic Analysis

The phylogenetic tree obtained by sequence analysis of ITS and TEF1 regions among 20 isolates are represented in Fig. 1. An UPGMA analysis of the alienable ITS and TEF1 sequences of isolates were demonstrated into three (3) distinct clades and all the clades were phylogenetically distinct from each other.

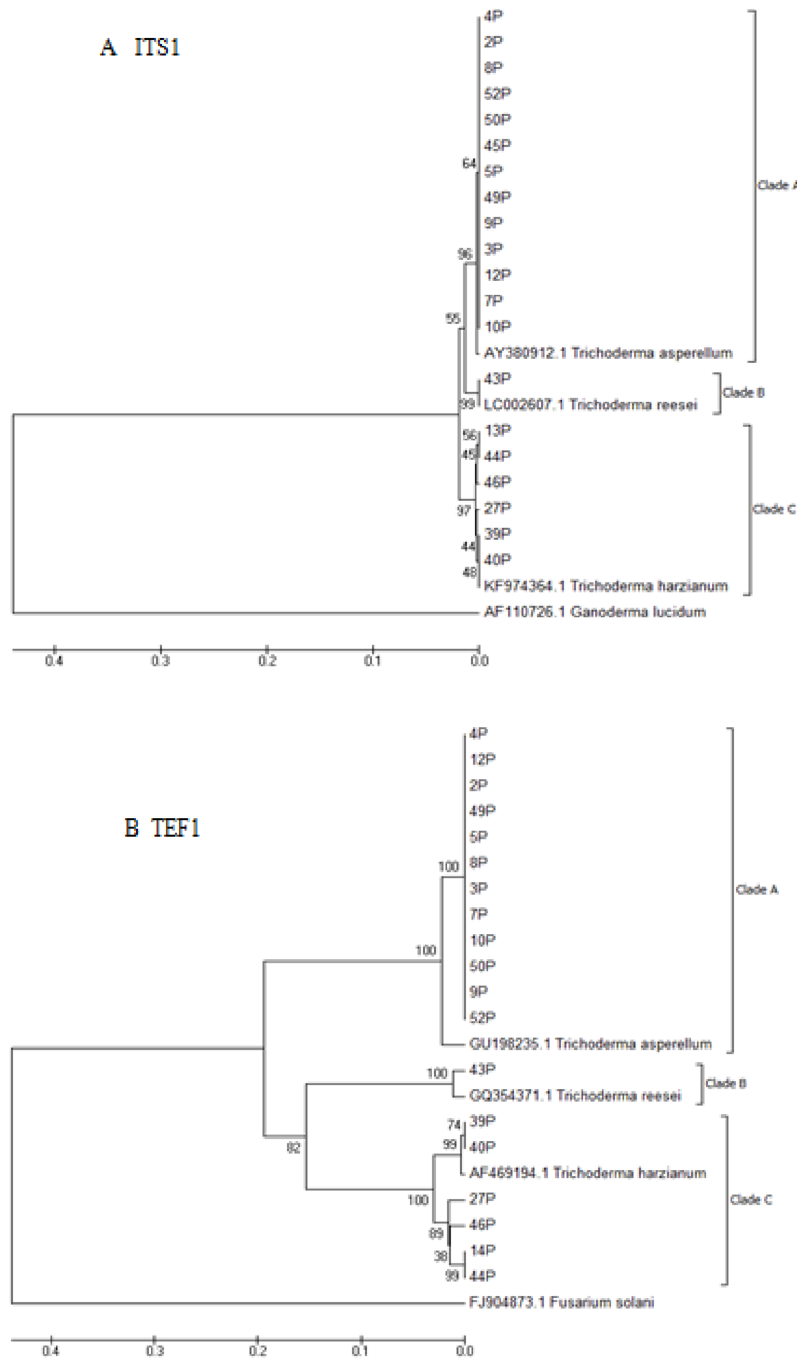

Figure 1: A UPGMA tree based on (A) ITS1 and (B) TEF1 sequences of *Trichoderma* isolates and its closest sequences obtained from GenBank. Values on the nodes indicate bootstrap percent confidence.

The individual gene trees for ITS1 and TEF1 are shown in Fig. 1(A) and (B), respectively. Clade A comprised mainly of *T. asperellum*, which represents the biggest group of *Trichoderma* isolates which were supported by a bootstrap value of 96% and 100% for ITS1 and TEF1 phylogenetic inference, respectively. Clade B had a close match with *T. reesei* species supported by a bootstrap value of 99% for ITS1 and 100% for TEF1 phylogenetic inference respectively. Clade C represents five isolates of *T. harzianum* with bootstraps value of 97% and 100% for ITS1 and TEF1 phylogenetic inference, respectively.

The best resolution of phylogenetic tree was obtained by TEF1, which were superior to ITS1. Clade C (13P, 44P, 46P, 27P, 39P and 40P) consistently clustered at the basal position. Clade B was clustered differently from ITS1 and TEF1 trees. In ITS1, Clade B was branched with all the isolates of T. *asperellum*, meanwhile it were clustered with *T. harzianum* isolates in TEF1 tree.

Discussion

The present study on the occurrence and diversity of *Trichoderma* species from Tuaran were carried out for the first time in this region. The results from this study stress the importance of the use of molecular identification tools to describe the *Trichoderma* diversity. The result obtained from phylogenetic analysis based on the ITS and TEF1 sequences of 20 *Trichoderma* isolates showed three distinct clades. *T. asperellum* represents the dominant group of *Trichoderma* spp. followed by *T. harzianum* and *T. reesei*.

Generally, the results presented here differ from all diversity studied of *Trichoderma* from soil which listed *T. harzianum* as a predominant species complex (Mpika *et al*., 2009; Naeimi *et al*., Sun *et al*., 2012). Difference in agricultural fields influenced the species composition and abundance of the individual *Trichoderma* species. Gherbawy *et al*. (2004) found very low *Trichoderma* biodiversity in agricultural soils of the Nile valley in Egypt, which contained only *T. harzianum* and the anamorph of *H. orientalis.* The alkalinity occurance of the soils (pH=7.3-7.4) may influence the low degree of diversity. The rDNA-ITS1 analysis of *Trichoderma* isolates obtained from rice field soils in Philippines comprised only *T. viride* and *T. harzianum* (Cumagun *et al*., 2000). *T. atroviride*, *T. harzianum* and *T. virens* are only species isolated from rice paddy field habitats in Northern Iran (Kredics *et al*., 2011).

The distribution of *Trichoderma* species are influenced by genetic structure, biological niches, soil type as well as the geographical regions. Xia *et al*. (2011) found that the genetic diversity of *T. asperellum* and *T. virens* within the epiphyte is lower than that within the epiphytes of banana roots. The biodiversity study of *Trichoderma* in Southest Asia including Burma, Cambodia, Malaysia, Singapore, Taiwan, Thailand and Western Indonesia revealved the high occurance of high metabolic diversity species, *T. harzianum* (Kubicek *et al*., 2003). Tsurumi *et al*. (2011) reported the mostly occurred of the genus *Trichoderma* within four countries of Asia (Indonesia, Japan, Mongolia and Vietnam) are *T. crassum*¸ *T.hamatum*, *T. harzianum* and T. *virens,* whereas *T. polysporum* and *T. viridescens* are frequently in cooler region. Wuczkowski *et al*. (2003) isolated *Trichoderma* strains soil collected from original European river-floodplain in Austria. The ITS and TEF1 gene analysis reported the presence of *T. harzianum*, *T. rossicum*, *T. cerinum*, *T. hamatum*, *T. artroviride*, *T. koningiopsis* and *Trichoderma* spp. MA3642 from section of *Longibrachiatum.*

Trichoderma strains have reported as potential biocontrol agents against plant pathogens of *Gibberella fujikuroi* in rice (Watanabe *et al*., 2005), *Sclerotinia sclerotium*, *Fusarium oxysporum* f. sp. *cubense* in banana (Xia *et al*., 2011), *Collectotrichum falcatum* causing red rot of sugarcane (Joshi

TRANSACTIONS ON SCIENCE AND TECHNOLOGY

& Misra, 2013) and *Rhictozonia solani* in wheat (Yang *et al*., 2005). They also have been commercially used on cultivated plants for inducing systemic resistance, increasing nutrient availability and uptake, promoting plant growth, improving crop yields and degrading xenobiotic pesticides (Harman, 2006). *Trichoderma* strains identified in this study may be a suitable candidate for the biocontrol agent.

Conclusion

The results underline the importance of using molecular approach which may not only serve as a valuable tool in inferring phylogenetic relationships among species in *Trichoderma* but also provide valuable information on the species identification of *Trichoderma* strains.

Acknowledgements

The authors are grateful for the financial support provided by the Biotechnology Research Institute through Seed money under this research project. Thanks to the laboratory staffs for their valuable assistance.

References

[1] Alvindia, D. G. & Hirooka, Y. (2011). Identification of *Clonostachy* and *Trichoderma* spp. from banana fruit surfaces by cultural, morphological and molecular methods. *Mycol*., **2**(2): 109-115.

[2] Cubero, O., Crespo, A., Fatehi, J. & Bridge, P. D. (1999). DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized and other fungi. *Plant Systematics and Evolution*, **216**(34), 243-249.

[3] Cumagun, C. J. R., Hockenhull, J. & Lübeck, M. (2000). Characterization of *Trichoderma* isolates from Philippine rice fields by UP-PCR and rDNA-ITS1 analysis: Identification of UP-PCR markers. *J. Phytopatho.,* **148**(2), 109-115.

[4] Druzhinina, I. S, Kopchinskiy, A.G., Komoń, M., Bissett, J., Szakacs, G. & Kubicek, C. P. (2005). An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet Biol*., **42**(10), 813-828.

[5] Druzhinina, I. S., Komoń- Zelazowska, M., Kredics, L., Hatvani, L., Antal, Z., Belayneh, T. & Kubicek, C. P. (2008). Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. *Microbiology*, **154**(Pt 11), 3447-3459.

[6] Gams, W. & Bissette, J. (1998). Morphology and identification of *Trichoderma*. In: Kubicek, C. P. & Harman G. E. (Eds.). *Trichoderma and Gliocladium*, *Basic Biology* (Vol. 1). London: Taylor & Francis.

[7] Gherbawy, Y., Druzhinina, I. S., Shaban, G. M., Wuczkowsky, M., Yaser, M., El-Naghy, M. A., Prillinger, H. & Kubicek, C. P. (2004). *Trichoderma* populations from alkaline agricultural soil in the Nile valley, Egypt, consists of only two species. *Mycol. Prog*., **3,** 211-218.

[8] Harman, G. E. (2006). Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology*, **96**(2), 190-194.

[9] Hatvani, L., Vágvölgyi, C., Kredics, L. & Druzhinina, I. S. (2014). DNA barcode for species identification in *Trichoderma*. *In*: Gupta, V. K., Schmoll, M., Herrera-Estrella A., Upadhyay, R. S., Druzhinina, I. S. & Tuohy, M.G. *Biotechnology and biology of Trichoderma*. Elsevier.

[10] Joshi, D., & Misra, S. C. (2013). Characterization of *Trichoderma* isolates from sugarcane agro-ecosystem and their efficacy against a *Colletotrichum falcatum* causing red rot of sugarcane. *Sugar Tech*., **15**(2), 192-196.

[11] Kindermann, J., El-Ayouti, Y., Samuels, G. J. & Kubicek, C. P. (1998). Phylogeny of the genus *Trichoderma* based on sequence analysis of the internal transcribed spacer region 1 of the rDNA cluster. *Fungal Genetics and Biology*, **24**(3), 298-309.

[12] Kopchinskiy, M., Komoń- Zelazowska, M., Kubicek, C. P. & Druzhinina, I. S. (2005). TrichoBLAST: a Multiloci database for *Trichoderma* and *Hypocrea* identification. *Mycological Research,* **109**(6), 657-660.

[13] Kredics, L., Láday, M., Körmöczi, P., Maczinger, L., Rákhley, G., Vágvölgyi, Cs and Szekeres, A. (2011). Trichoderma communities of the winter wheat rhizosphere. *Agr. Vidėkfejl. Szeml*., **6**(Suppl), 413-418.

[14] Kubicek, C., Bissette, J., Druzhinina, I. S., Kullnig-Gradinger, C. & Szakacs, G. (2003). Genetic and metabolic diversity of *Trichoderma*: a case study on South East Asian isolates. *Fungal Genet. & Biol*., **38**(3), 310-319.

[15] Kullnig-Gradinger, C. M., Szakacs, G. & Kubicek, C. P. (2002). Phylogeny and evolution of the genus *Trichoderma*; a multigene approach. *Mycol. Res*., **106**(7), 757-767.

[16] Migheli, Q., Balmas, V., Komoń-Zelazowska, M., Scherm, B., Fiori, S., Kopchinskiy, A. G., Kubicek, A. P. & Druzhinina, I. S. (2009). Soils of Mediterranean hot spot of biodiversity and endemism (Sardinina, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea*/*Trichoderma*. *Environmental Microbiology*, **11**(1), 35-46.

[17] Mpika, J., Kėbė, I. B., Issali, A. E., N'Guessan, F. K., Druzhinina, I. S., Komon-Zelazowska, M., Kubicek, C. P. & Akė, S. (2009). Antagonist potential of *Trichoderma* indigenous isolates for biological control of Phytophthora palmivora the causative agent of black pod disease on cocoa (*Theobroma cacao* L.) in Côte d'Ivoire. *Afr. J. Biotechnol*., **8**(20), 5280-5293.

[18] Naeimi, S., Khidaparast, S. A., Javan-Nikkhah, M., Vágvölgyi, C. & Kredics, L. (2011). Species pattern and phylogenetic relationships of *Trichoderma* strains in rice fields of Southern Caspian Sea, Iran. *Cereal Res. Commun*. **39**(4): 560-568.

[19] Shentu, X., Zhan, X., Ma, Z., Yu, X. & Zhang, C. (2014). Antifungal activity of metabolites of the endophytic fungus *Trichoderma brevicompactum* from garlic. *Brazilian Journal of Microbiol*., **45**(1), 248-254.

[20] Sun, R. -Y, Liu, Z. -C., Fu, K., Fan, L. & Chen, J. (2012). *Trichoderma* biodiversity in China. *J. Appl. Genet*., **53**(3), 343-354.

[21] Tsurumi, Y., Inaba, S., Kamijo, S., Widyastuti, Y., Hop, D., Balijinova, T., Sukarno., N., Nakagiri, A., Susuki, K. & Ando, K. (2011). Distribution of *Trichoderma* species in four countries of Asia. *9th International Mycological Congress*. 1 - 6 August, 2010. Edinburgh, Scotland.

[22] Watanabe, S., Kumakura, K., Kato, H., Iyozumi, H., Togawa, M. & Nagayama, K. (2005). Identification of *Trichoderma* SKT-1, a biological control agents against seedborne pathogens of rice. *J. Gen. Plant Pathol*., **71**(5), 351-356.

[23] White, T. J., Bruns, T., Lee, S. & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M.A., Gelfand, D.H., Sninsky, J. J. & White, T. J. (eds.). *PCR protocols: A guide to Methods and Applications*. San Diego: Academic Press.

[24] Woo, S. L. & Lorito, M. (2007). Exploiting the interactions between fungal antagonists, pathogens and the plant for biocontrol. *In:* Vurro, M. & Gressel, J. (eds.). *Novel Biotechnologies for Biocontrol Agent Enhancement and Management*. Netherlands: Springer.

[25] Wuczkowski, M., Druzhinina, I. S., Gherbawy, Y., Klug, B., Prillinger, H. & Kubicek, C. P. (2003). Species pattern and genetic diversity of *Trichoderma* in a mid-European, primeval floodplain-forest. *Microbiol. Res*., **158**(2), 125-133.

[26] Xia, X., Lie, T. K., Qian, X., Zheng, Z., Huang, Y. & Shen, Y. (2011). Species diversity, distribution and genetic structure of endophytic and epiphytic *Trichoderma* associated with banana roots. *Microb. Ecol*., **61**(3), 619-625.

[27] Yang, H. T., Ryder, M. H. & Tang, W. H. (2005). Characterisation and identification of *Trichoderma* isolates from a south Australian soil suppressive to *Rhizoctonia solani* on wheat. *Shandong Science*, **18**(3): 36-49.

[28] Yu, Z. F., Qiao, M., Zhang, Y. & Zhang, K. Q. (2007). Two new species of *Trichoderma* from Yunnan, China. *Antonie van Leeuwenhoek*, **92**(1), 101-108.