
Morphological and molecular based identification of *Trichoderma* CB-Pin-01 biological control agent of plant pathogenic fungi in Thailand

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Abstract *Trichoderma* CB-Pin-01 is an efficient biocontrol agent of fungal plant diseases, commercially produced and legally registered in 1996 as a biological control agent against several plant diseases in Thailand. This isolate was primarily identified as *T. harzianum* based on morphological characteristics. To confirm the species identification of *Trichoderma* CB-Pin-01, conidial surface observation and DNA sequence analysis were conducted. *Trichoderma* CB-Pin-01 showed irregular pyramidal warts on conidia surface which is a typical characteristics of *T. asperellum*. Moreover, ITS 1 and 4 sequences of rDNA combined with *tefl* gene sequence indicated the phylogenetic placement of *Trichoderma* CB-Pin-01 as *T. asperellum*. We concluded that the formerly *T. harzianum* CB-Pin-01 has to be re-classified as *Trichoderma asperellum*.

Keywords: *Trichoderma*, *Trichoderma asperellum*, Biological control, Plant disease

Introduction

Trichoderma species are worldwide used as biocontrol agent (BCA). It is important to use the correct identification of *Trichoderma* species in agroecosystem, since, many of species of this genus produce mycotoxins. For example, *T. brevicompactum* produces trichothecins and some species such as *T. longibrachiatum* and *T. citrinoviride* were reported to be human pathogens (Mukherjee *et al.*, 2013). Unfortunately, the species identification of *Trichoderma* based on only morphological characters is sometimes perplexing and misleading.

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The scanning electron microscopy of the conidial surface reported by Meyer and Plaskowitz (1989) advocated that *T. viride* consisted of at least two subgroups. Later, Watanabe *et al.* (2005) revealed that the conidial surface of Group I was broadly round, whereas the Group II was irregular pyramidal wart. *Trichoderma asperellum* was in group II and separated from the complex of *T. viride* on the basis of its inconspicuous conidial warts, then this species was proposed as a new species by Samuels *et al.* (1999). This conclusion was confirmed by Lieckfeldt *et al.* (1999), who showed the differences in ITS rDNA (ITS1 5.8S and ITS2) nucleotidesequences between *T. viride* type I (group I) and type II (group II) isolates and found that *T. asperellum* was closed to *T. hamatum*. Furthermore, based on multilocus genealogies, along with morphological and proteome data supported that a new species *T. asperelloides* is distinctly different from *T. asperellum* (Samules *et al.*, 2010). Therefore, the original *T. viride* isolates were now comprised of three distinct species including *T. viride*, *T. asperellum* and *T. asperelloides*.

Based on the conidial surface structure, *T. asperellum* was once classified as *T. viride* Group II. However, it was re-classified as *T. asperellum* by Samuels *et al.* (1999) with the supporting data of the difference in nucleotide sequences of ITS rDNA (ITS1 5.8S and ITS2) among Group I and Group II (Lieckfeldt *et al.*, 1999). These data also revealed the more similarities of *T. asperellum* and *T. hamatum*. Moreover, the combination of morphological data, multilocus genealogies and proteomes could distinctly identify two cryptic species, *T. asperelloides* and *T. asperellum* (Samules *et al.*, 2010). Therefore, based on the combining information, the originally *T. viride* was then classified into three species, *T. viride*, *T. asperellum* and *T. asperelloides*. Corresponding to these more informative data, *Trichoderma* T203, a BCA that was once classified as *T. harzianum* (Yedidia *et al.*, 2003) and later as *T. asperellum* T203 (Harman *et al.*, 2004; Viterbo and Chet, 2006) is now *T. asperelloides* (Mukherjee *et al.*, 2013).

In Thailand, the study on *Trichoderma* spp. as plant disease control started in 1985. *Trichoderma* CB-Pin-01 was originally isolated from pineapple field soil in Chonburi Province. This isolate was primarily identified as *T. harzianum* based on morphological characteristics such as colony characters, growth rate of mycelia, conidiophores and conidial characters under the compound microscope. *Trichoderma* isolate CB-Pin-01 provided the most promising efficacy for the control of fungal plant diseases and became commercially produced and legally registered as biological control agent, *Trichoderma* isolate CB-Pin-01 (UNIGREEN UN-1®) for several plant diseases in Thailand (Chamswarnng and Tanangsnakool, 1996). There were reports during 1986-2016 which proved that *Trichoderma* CB-Pin-01 was the most effective antagonistic BCA for controlling various diseases caused by fungi and promoting plant growth of

many kinds of crops, including cereal, fruit, vegetable, ornamental and hydroponic lettuces (Chamswarng and Intanoo, 2002; Charoenrak and Chamswarng, 2015; Lamool *et al.*, 2005). This antagonistic isolate as well as other misidentified *T. harzianum* isolates also provided the benefit through the colonization of plant roots which has been proven to induce resistance in plants against fungal diseases (Charoenrak *et al.*, 2012). Moreover, promotion of plant growth attributed to phosphate solubilization and the production of auxins as well as other plant growth promoting-like substances such as pentylpyrone, harzianic acid and harzianic acid isomer had been reported (Promwee *et al.*, 2014). Beside *Trichoderma* isolate CB-Pin-01, there were many effective isolates needed to be appropriately re-identified. The accurately species identification for commercial purposes and patent registration is strongly required. Moreover, the genetic markers are very useful for species identification and strain specific detection. There are many DNA markers used for detection the fungal strain and their diversity such as RAPD, RFLP, AFLP and SCAR markers (Hermosa *et al.*, 2001; Dodd *et al.*, 2004). Rubio *et al.* (2005) generated the SCAR marker for *T. harzianum* 2413 using BR1 and BR2 primers which were designed to amplify of 1.5 kb fragment from *T. harzianum* 2413. The strain specificity was determined by inoculation of mixed strain of *Trichoderma* including *T. harzianum* 2413 in the sterile soil and non-sterile soil. The results indicated that BR1 and BR2 primers could specifically amplify the DNA fragment from only *T. harzianum* 2413 (Cordier *et al.*, 2006).

The purpose of this study was to determine the phylogenetic placement of *Trichoderma* CB-Pin-01 using the nucleotide sequences of ITS regions of ribosomal DNA and *tefl* gene coupled with the conidial surface structure under scanning electron microscope (SEM).

Materials and methods

Trichoderma species and isolates

Twenty-six *Trichoderma* species or isolates were appointed for nucleotide sequence analysis. All isolates have been reported as biocontrol agents (BCA) against various plant pathogenic fungi causing diseases on economic crops in Thailand. Isolate numbers, *Trichoderma* biocontrol agents (BCA), sources (province, mutant, fusant) and phytopathogenic fungi to be controlled by BCA were described in Table 1.

Morphological observation and conidial surface observation

Trichoderma CB-Pin-01 and *Trichoderma* spp. were cultured on PDA for a week at 27 °C. Then, all samples were observed for colony characteristics, conidial shape and size under the light microscope. The conidial surface observation was conducted with *Trichoderma* CB-Pin-01

sample. The agar disc with fungal hypha and conidia were pre-fixed in 2.5% Glutaraldehyde in Sodium Phosphate Buffer (0.2 M and pH 7.2) for 12 hrs or overnight at 4°C. The materials were then post-fixed in 1% Osmium tetroxide in distilled water for 1 hr and washed with distilled water for 10-15 min., three times repeating. Later, the materials were dehydrated in a series of acetone 15-30 min per step. The specimens were dried for 1 hr and coated with gold and examined using scanning electron microscope (SU8020, Hitachi) at the Scientific Equipment and Research Division, KURDI, Kasetsart University, Thailand.

Fungal preparation and DNA extraction

Mycelial samples were collected, lyophilized for 6-8 hrs and stored at -20°C condition. For DNA extractions steps, the samples were ground in liquid nitrogen and 50 mg ground mycelia was extracted in 0.5 ml extraction buffer (50 mM Tris-HCl pH 8.0, 850 mM NaCl, 100 mM EDTA, and 1% SDS) and incubated at 65 °C for 30 min. Then, extraction solutions were added 0.5V phenol and 0.5V chloroform: IAA (24:1) and gently mixed. The extraction samples were centrifuged at 13,000 rpm for 10 min. The supernatant was taken and it was deproteinized with 1V chloroform: IAA (24:1); then, centrifuged at 13,000 rpm for 10 min. The DNA pellet was twice washed with 70% ethanol and dissolved in 10 µl TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The DNA was quantified using 1% agarose gel electrophoresis utilizing Lambda DNA standard marker (*EcoRI* and *HindIII* digestion) as the quantification standard (Zimand *et al.*, 1994).

DNA sequence analysis of ITS and tef1 gene

The ITS region including ITS1, ITS2 and the inverting 5.8S coding rDNA were amplified using the primers ITS1(5' -TCC GTA GGT GAA CCT GCG G -3') and ITS4 (5' -TCCTCC GCT TAT TGA TAT GC - 3') (White *et al.*,1990). Each PCR reaction mixture contained 5-10 ng of genomic DNA, 1 µM each of the primers ITS1 and ITS4, reaction buffer (50 mM KCl, 50 mM Tris-HCl; [pH 8.3] 0.1 mg/ml bovine serum albumin), 3 mM MgCl₂, 200 µM each of dNTP and 1 U of *Taq* DNA polymerase (Promega, Mannheim, Germany) in a total volume of 50 µl. The PCR program consisted of an initial denaturation of 2 min at 95 °C, followed by 30 cycles of 94 °C for 1 min, 54 °C for 30 sec and 72 °C for 1 min and final extension at 72 °C for 15 min. The TEF-1 (forward primer: 5'-ATGGGTAAGGARGACAAGAC-3') and TEF-2 (reverse primer: 5'-GGARGTACCAGTSATCATGTT-3') (O'Donnell *et al.*, 1998) primers were used in the PCR amplification assays of *tef1* gene. Each PCR reaction mixture contained 5-10 ng of genomic DNA, 1 µM each of the primers TEF-1 and TEF-1, reaction buffer (50 mM KCl, 50 mM Tris-HCl; [pH 8.3] 0.1 mg/ml bovine serum albumin), 3 mM MgCl₂, 200 µM each of dNTP and

2.5 U of *Taq* DNA polymerase (Promega, Mannheim, Germany) in a total volume of 50 μ l had an initial DNA denaturation step of 3 min at 94 °C, followed by 34 cycles of 30 s at 94 °C, primer annealing for 45 sec at 61 °C, primer extension for 1 min at 72 °C. The last step for the final primer extension reaction consisted of an incubation at 72 °C for 15 min.

The PCR products were evaluated using 1% (W/V) agarose gel electrophoresis in a TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 8.0). Then, the PCR products were purified using Illustra™ MicroSpin S-400 HR columns (GE Health care UK Limited). The purified PCR products were sequenced at 1st BASE DNA Sequencing Services, Malaysia. All generated sequences of *Trichoderma* species were submitted to the GenBank.

Sequence data analysis

A multiple sequence alignment was carried out using Clustal W (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the MEGA version 7.0 (Tamura *et al.*, 2007). The ITS and *tef1* datasets were analyzed using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap value was estimated at 1,000 replications of bootstrap resampling of the original nucleotide sequence alignments (Felsenstein, 1985). Moreover, *Trichoderma* CB-Pin-01 sequence (ITS region) was identified using DNA barcode program in the international subcommission for *Hypocrea/Trichoderma* taxonomy (ISTH) database.

Results

Morphological and conidial surface observations

Colony characteristics were observed on 26 isolates of *Trichoderma* spp. The growth rate was 19.00-30.00 mm. per day. The colony characters were different among species of *Trichoderma*. The conidia production of *Trichoderma* CB-Pin-01 (*T. asperellum*) on PDA was denser at the center with green conidia while dense white mycelia grew toward the margins. There were no concentric rings of mycelial growth formed by *Trichoderma* CB-Pin-01 (Figure 1A). While, isolate T14 (*T. viride*) provided dark green conidia distributed thoroughly without concentric rings (Figure 1B). Isolates T18 and T19 (*T. virens*) produced green conidia at the center and light green-yellowish conidia were formed around the colony margins (Figure 1C and 1D). Conidia of isolate T22 (*T. longibrachiatum*) were compact in shades of green in the center, then mycelia grew toward the margin with white or light green conidia. The yellow pigment was released into the PDA. Conidial sizes were not different among species and isolates (1.67-3.44 \times 1.61-2.84 μ).

The scanning electron micrograph of *Trichoderma* CB-Pin-01 observed by SEM showed many irregular pyramidal warts on the conidial surface (Figure 2). This type of wart was different from the broadly rounded wart on conidial surface of *T. viride* and smooth surface wart of *T. harzianum*. Therefore, *Trichoderma* CB-Pin-01 was identified as *T. asperellum* based on the type and shape of warts produced on conidial surface.

Table 1. Isolate numbers, *Trichoderma* biocontrol agents (BCA), sources and phytopathogenic fungi controlled by BCA

Isolate No.	<i>Trichoderma</i> spp. (BCA)	Source	Phytopathogenic fungi controlled by BCA
T1	<i>T. harzianum</i> CB-Pin-01	Chonburi province	<i>Alternaria</i> spp., <i>Aspergillus</i> sp., <i>Bipolaris oryzae</i> , <i>Cercospora oryzae</i> , <i>Colletotrichum</i> spp., <i>Corynespora cassicola</i> , <i>Curvularia lunata</i> , <i>Fusarium</i> spp., <i>Greenaria uvicola</i> ., <i>Helminthosporium sativum</i> , <i>Lasiodiplodia theobromae</i> , <i>Phomopsis asparagi</i> , <i>Phytophthora</i> spp., <i>Pseudocercospora</i> spp., <i>Pseudoperonospora cubensis</i> , <i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Rigidoporus microporus</i> , <i>Sarocladium oryzae</i> , <i>Sclerotium rolfsii</i> , <i>Xanthomonas axonopodis</i> pv. <i>citri</i> , <i>Meloidogyne incognita</i>
T2	<i>T. harzianum</i> T-50	Songkla province	<i>Alternaria padwickii</i> , <i>Bipolaris oryzae</i> , <i>Colletotrichum</i> spp., <i>Curvularia lunata</i> , <i>Greenaria uvicola</i> ., <i>Phytophthora</i> spp., <i>Pseudocercospora</i> spp., <i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Sclerotium rolfsii</i> , <i>Xanthomonas axonopodis</i> pv. <i>citri</i> ,
T6	<i>T. harzianum</i> M 4	Mutant strain of CB-Pin-01	<i>Alternaria padwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Sclerotium rolfsii</i>
T7	<i>T. harzianum</i> M 23	Mutant strain of CB-Pin-01	<i>Alternariapadwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Sclerotium rolfsii</i>
T8	<i>T. harzianum</i> M 35	Mutant strain of CB-Pin-01	<i>Alternariapadwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Sclerotium rolfsii</i>
T9	<i>T. harzianum</i> M 49	Mutant strain of CB-Pin-01	<i>Alternariapadwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Sclerotium rolfsii</i>
T12	<i>T. harzianum</i> F014	Suphanburi province	<i>Alternaria padwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Pyricularia oryzae</i> , <i>Sarocladium oryzae</i>

Table 1(Continued)

Isolate No.	<i>Trichoderma</i> spp.	Source	Biocontrol agent (BCA)
T12	<i>T. harzianum</i> F014	Suphanburi province	<i>Alternaria padwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Pyricularia oryzae</i> , <i>Sarocladium oryzae</i>
T14	<i>T. harzianum</i> F009	Suphanburi province	<i>Alternaria padwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Pyricularia oryzae</i> , <i>Sarocladium oryzae</i>
T16	<i>T. harzianum</i> A18+A50 No.3 (Fusant)	Protoplastfusion	<i>Alternaria padwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Phytophthora parasitica</i>
T17	<i>T. harzianum</i> A40+A50 No.7 (Fusant)	Protoplastfusion	<i>Alternaria padwickii</i> , <i>Bipolaris oryzae</i> , <i>Curvularia lunata</i> , <i>Phytophthora parasitica</i>
T18	<i>T. virens</i> Tv. 16	ChiangMai province	<i>Phytophthora parasitica</i> , <i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Sclerotium rolfsii</i>
T19	<i>T. virens</i> Tv. 24	Chumporn province	<i>Phytophthora parasitica</i> , <i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Sclerotium rolfsii</i>
T20	<i>T. virens</i> Tv. 100	Sukhothai province	<i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Sclerotium rolfsii</i>
T21	<i>T. longibrachiatum</i> TL-01	Bangkok province	<i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Sclerotium rolfsii</i>
T22	<i>T. pseudokoningii</i> DU-07	Chantaburi province	<i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>Sclerotium rolfsii</i>
T23	<i>T. harzianum</i> A47 (SY)	Mutant of T-152-CO3	<i>Phytophthora parasitica</i>
T24	<i>T. harzianum</i> A9	Mutant of T-58	<i>Phytophthora parasitica</i>
T25	<i>T. harzianum</i> A33	Mutant of T-58	<i>Phytophthora parasitica</i>
T26	<i>T. harzianum</i> A44	Mutantof CB-Pin-01	<i>Phytophthora parasitica</i>
T27	<i>T. harzianum</i> A46	Mutant of T-152-CO3	<i>Phytophthora parasitica</i>
T28	<i>T. harzianum</i> A54	Mutantof T-152-CO4	<i>Phytophthora parasitica</i>

Table 1(Continued)

Isolate No.	<i>Trichoderma</i> spp.	Source	Biocontrol agent (BCA)
T34	<i>T. harzianum</i> PRM49	Mutant of T-58	<i>Phytophthora parasitica</i>
T39	<i>T. harzianum</i> PRM58+ PRM23 (6) (Fusant)	Protoplastfusion	<i>Phytophthora parasitica</i>
T40	<i>T. harzianum</i> PRM58 + PRM23 (7) (Fusant)	Protoplastfusion	<i>Phytophthora parasitica</i>
T44	<i>T. harzianum</i> PRM58 + PRM23 (12) (Fusant)	Protoplastfusion	<i>Phytophthora parasitica</i>
T45	<i>T. harzianum</i> RB-NST-002	Nakhon Srithammaraj province	<i>Phytophthora palmivora</i> , <i>Rigidoporus microporus</i>

References: Chamswarnng and Tanangsnakool (1996), Chamswarnng and Intanoo (2002), Chamswarnng *et al.* (2010), Charoenrak and Chamswarnng (2015), Charoenrak *et al.* (2012), Gesnara (1994), Lamool *et al.* (2005), Promwee *et al.* (2014), Seemadua *et al.* (1997)

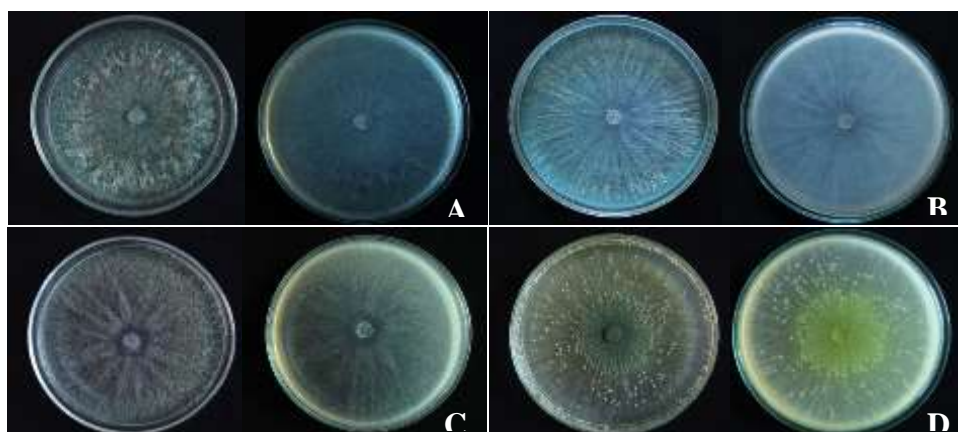


Figure 1. Colonies of four different species of *Trichoderma* grown on PDA for 5 days. *Trichoderma asperellum*CB-Pin-01 (A), *T. viride* T14 (B), *T. virens* T19 (C) and *T. longibrachiatum* T22 (D)

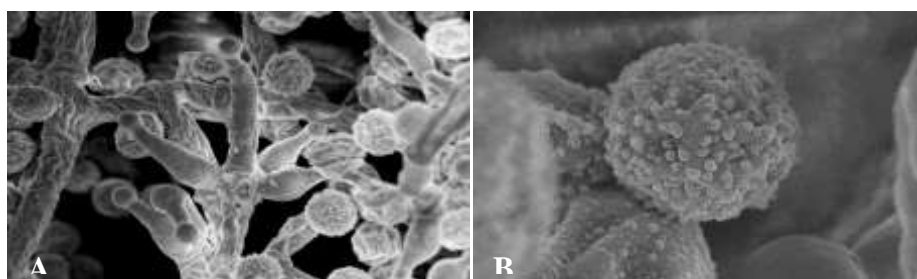


Figure 2. Morphological characteristics and conidial surface of *Trichoderma* (CB-Pin-01) observed in SEM. A, conidia and conidiophores; B, conidial surface with pyramidal warts Bars: A, 10 μm ; B 3 μm

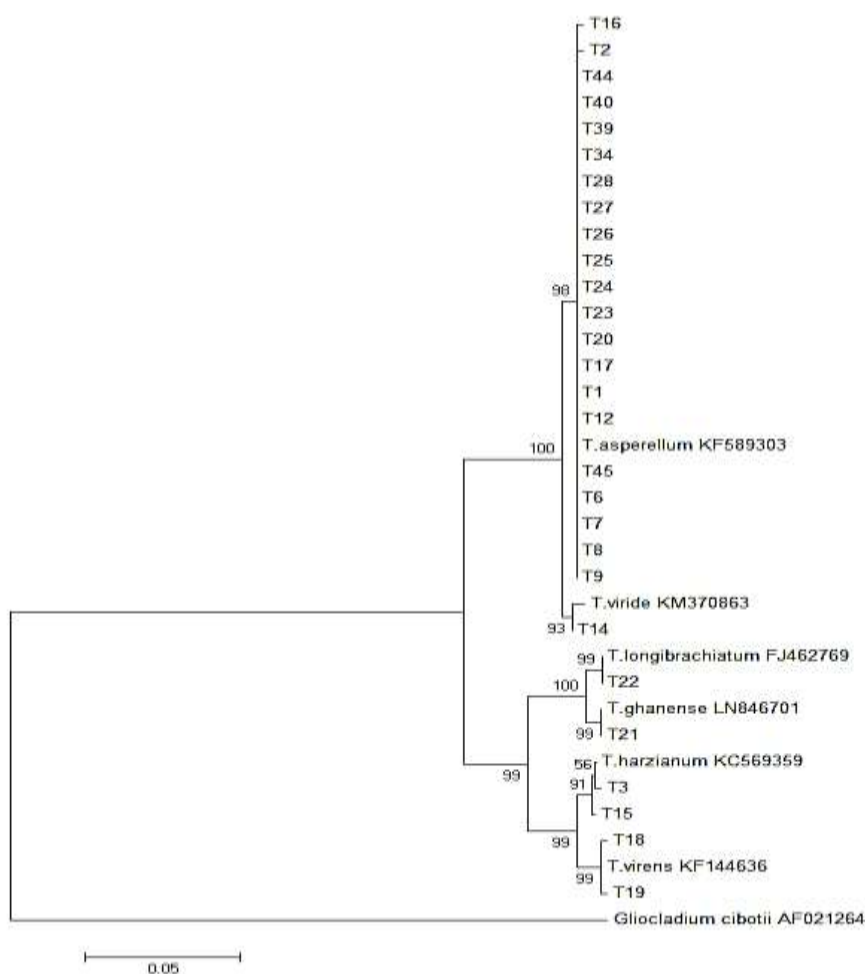


Figure 3. The Nj trees inferred from a heuristic search of the ITS region of rDNA of 28 sequences of *Trichoderma* species and *Trichoderma* sequences were used as the out group. Number above branches identify the bootstrap (1,000)

PCR and sequencing analysis

Twenty-eight ITS rDNA sequences of *Trichoderma* CB-Pin-01 and *Trichoderma* isolates were multiple aligned and analyzed together with the sequences obtained from NCBI database including *T. asperellum* (KF589303), *T. viride* (KM370863), *T. longibrachiatum* (FJ462769), *T. ghanense* (LN846701), *T. harzianum* (KC569359), *T. virens* (KF144636) and *Gliocladium cibotii* (AF021264). All 28 sequences of *Trichoderma* species have been deposited in the GenBank (Table 1). The sequence lengths were 503 bp when aligned together with other sequences from database. The Neighbor-Joining based analysis showed that *Trichoderma* CB-Pin-01 was placed in the same clade with *T. asperellum* (KF589303)

and other 21 isolates of *Trichoderma* with high bootstrap value supporting (98%). While, *Trichoderma* T14 was clustered with *T. viride* (KM370863) with 93% bootstrap value supporting. However, these two clades (*T. asperellum* and *T. viride*) were very close and separated from other groups or species. *Trichoderma* T21 and T22 were in the same group with *T. ghanense* (LN846701) and *T. longibrachiatum* (FJ462769) with 100% bootstrap value supporting, respectively. *Trichoderma* (T3 and T15) were placed with *T. harzianum* (KC569359) supported by 91% bootstrap value and isolates T18 and T19 were grouped with *T. virens* (KF144636) (99 % bootstrap value) which were closed with *T. harzianum* (99% bootstrap supporting). Two isolates, *Trichoderma* CB-Pin-01 and *Trichoderma* T18 were determined for the genetic relationship using *tef1* gene. The result revealed that *Trichoderma* CB-Pin-01 was in the same group with *T. asperelloides* (KP262478) and *T. asperellum* (AB568380). While, *Trichoderma* T18 was placed with *T. virens* (KP985651) with 1000 bootstrap. The results indicated that *Trichoderma* CB-Pin-01 should be *T. asperellum* confirmed by morphological characteristics, ITS rDNA sequence and *tef1* gene sequence data analysis. The ITS rDNA and *tef1* sequences of *Trichoderma* CB-Pin-01 have been deposited in GenBank database (LC123601 and LC155108 respectively). Moreover, *Trichoderma* CB-Pin-01 was detected the ITS rDNA sequence with molecular barcode program in *TrichOkeY*. The result showed that CB-Pin-01 sequence matched *T. asperellum* DNA barcode which was developed on the basis of 40 vouchered sequences with high identification reliability.

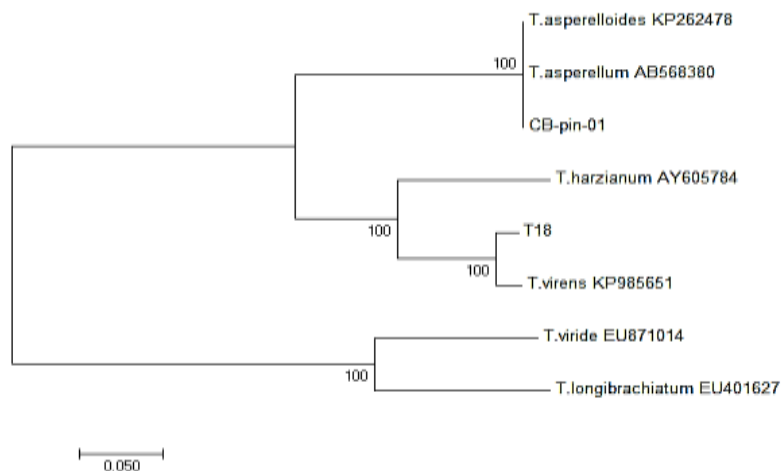


Figure 4. The Nj trees inferred from a heuristic search of the *tef1* gene sequence of *Trichoderma* CB-Pin-01 (T1), *T. virens* (T18) and *Trichoderma* sequences (AB568380, KP262478, AY605784, KP985651, EU871014, EU401627) obtained from Gen Bank database were used as the out group. Number above branches identify the bootstrap statistics percentages (1,000 replicates)

Discussion

Although, *Trichoderma* CB-Pin-01 was not compared with the conidial surface with the type specimens of *T. viride* and *T. asperellum*, based on previously reported and reclassified *T. viride* complex by Samuels *et al.* (1999). The conidial surface of *Trichoderma* CB-Pin-01 and *T. asperellum* were irregular pyramidal warts, while the conidial surface of *T. atroviride* was smooth and *T. viride* had broadly rounded warts. According to proposal by Samuels *et al.* (1999) morphological observation with SEM, especially of the surface structure of conidia, could be used for differentiation among species of *Trichoderma*. In the present observation, the conidial surface of *Trichoderma* CB-Pin-01 was similar to *T. viride* type II described by Lieckfeldt *et al.* (1999). Lieckfeldt *et al.* (1999) confirmed that *T. viride* was divided into type I and II based on sequence of ITS regions and type I was *T. viride*, while type II represented as a new species, *T. asperellum*.

For the broad morphologically defined sense, *T. harzianum* is cosmopolitan, but *T. harzianum* in the strict sense is a species of the Northern Hemisphere, while several of the new segregate species are apparently exclusive tropical in distribution (Chaverri *et al.*, 2015). Based on ITS rDNA region and *tef1* gene sequences and SEM analyses, *T. harzianum* CB-Pin-01 was re-identified as *T. asperellum*. This finding was in agreement with a report of Samuels and Hebbar (2015) which concluded that *T. asperellum* is one from 12 *Trichoderma* commonly, or only, found in isolations from Tropical zones. Several *T. harzianum* strains such as T-22, ATCC 74058=PI and Ta040 (=IMI206039) were re-identified as *T. afroharzianum* (Chaverri *et al.*, 2015), *T. atroviride* (Kullnig *et al.*, 2001) and *T. atroviride* (Feng *et al.*, 2011), respectively. *Trichoderma harzianum* strain ICC012 (Bioten WP®, Tenet WP®) were re-identified as *T. asperellum* (European Food Safety Authority, 2013), while *T. harzianum* strain T203 was re-identified as *T. asperellum* and recently as *T. asperelloides* (Samuels *et al.*, 2010).

Since there were ultraviolet-irradiated mutant isolates (T6, T7, T8, T9, T26) and fusant isolates (T16, T17, T39, T40, T44) derived from *Trichoderma* CB-Pin-01 (Table 1), therefore, the species of those isolates were also re-identified as *T. asperellum* as well. The mutant isolate 01-52 provided comparable efficacy as *Trichoderma* CB-Pin-01 to control dirty-panicle, brown spot, narrow brown spot and sheath rot of rice (Charoenrak and Chamswarng, 2015). While mutant *Trichoderma* M23 effectively controlled seedling blight of barley and stem rot of tomato caused by *Sclerotium rolfsii* (Gesnara, 1994), reduced *Phytophthora palmivora*, a causal agent of root rot of durian (*Durio zibethinus*) and *Phytophthora parasitica*, a causal agent of root rot of tangerine (Seemadua, 1997). The fusant isolate, PM9 which was derived from protoplast fusion of mutant

isolates of *Trichoderma* CB-Pin-01 effectively suppressed anthracnose of chilli, mango and grapes (White Malaka), respectively (Chamswarnng *et al.*, 2010).

To avoid confusion regarding of Genbank database as some sequences deposited under the original species, the International Subcommittee for *Hypocrea/Trichoderma* Taxonomy (ISTH) of the International Commission on the Taxonomy of Fungi (Mycological Division of IUMS) had developed a computerized approach that allowed both authentic and revised sequences data to be included to classify *Trichoderma* in species level (www.isth.info). In the year 2005, Druzhinina *et al.* made the first move on fungal DNA barcoding software, TrichOKey version 1.0, that clearly identified species of *Hypocrea* and *Trichoderma* by combining the data of species, clade and genus-specific markers. This software is very useful for mycologist to verify the precision of their results. Though the sequences of the identified species can easily be retrieved from the database, the assigned level of identification reliability is yet to be strongly considered. Druzhinina *et al.* (2006) suggested that the DNA barcode that has been developed from 20 or more reference sequences collected worldwide exhibited highly reliability level. Therefore, in this study, based on morphological characterization and the ITS DNA barcode developed from 40 references, we confirmed that the *Trichoderma* CB-Pin-01 is *T. asperellum*.

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