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Isolation and Identification of Trichocomaceae from Soil by Morphology and Three Regions DNA Sequencing

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Abstract The Trichocomaceae contains some of the most familiar fungi, such as *Penicillium* and *Aspergillus*. This family is cosmopolitan in distribution, ubiquitous in soil communities and extremely common associates of decaying plant materials and food stuffs. The objectives of this work were to isolate and identify varieties of Trichocomaceae mainly based on the morphological and molecular characteristics. The samples were isolated from soil collected in Chiang Mai's forest, Thailand. These fungi have been partially characterized using morphological traits such as features of colony morphology, size and shape of ascospores that were grown on potato dextrose agar (PDA) at 25℃. The three regions: ITS, *β*-tubulin and calmodulin were amplified using the primers that was a unique fragment of approximately 550, 450 and 650 bp, respectively. The nucleotide sequences demonstrated the level of genetic diversity of Trichocomaceae and related to the two genera: *Talaromyces flavus* (EU02, EU03, EU07, EU12 and EU14), *Talaromyces trachyspermus* (EU10 and EU23), *Neosartorya hiratsukae* (EU06) and *Neosartorya pseudofischeri* (EU13). However, a new classification system including both anamorph and teleomorph species will be investigating the relationship of some genera of Trichocomaceae.

Keywords: Trichocomaceae, *Talaromyces*, *Neosartorya*

Introduction

The Trichocomaceae is a large family of well-known fungi for their advantage and disadvantage. They are associated with food spoilage and mycotoxin production and can occur in the indoor environment. The most well-known species of this family belongs to the genera *Aspergillus*, *Penicillium* and *Paecilomyces*. Species belonging to Trichocomaceae are predominantly saprobic and represent some of the most catabolically and anabolically diverse microorganisms are known (Houbraken and Samson, 2011). It belongs to Ascomycota which is the largest phylum of fungi with over 64,000 species (Kirk *et al*., 2008). Ascomycota which do not have sexual stage to form asci

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and ascospores, previously placed to Deuteromycota with asexual stage or anamorph which are now identified based on morphology and phylogeny analyses of DNA sequences. Ascomycota have been grouped of absence of asci. Sexual and asexual isolates of the same species commonly carry different binomial species names, for example: *Aspergillus nidulans* for asexual and *Emericella nidulans* for sexual isolates of the same species (Alexopoulos *et al*., 1996). Asexual reproduction is the dominant form of Ascomycota which occurs through vegetative reproductive spores namely conidia. Trichocomaceae is a widespread and abundant which are known as asexually reproducing fungi. This asexual stage is interested because it implies to maintain competitive edge without the benefits of genetic recombination. Some mycologists have argued that these fungi do have sexual stages but do not know how to find them. Others believe that there are no sexual stages and so many Trichocomaceae are large amount of genetic diversity through mutations themselves. The relatives in genera of Trichocomaceae are still unclear according to the single-name nomenclature (Houbraken and Samson, 2011).

Trichocomaceae is an excellent presentation with a key, illustrations, discussions and descriptions of the species commonly found on foods and indoor environments (http://website.nbm-mnb.ca/mycologywebpages/NaturalHistoryOfFungi/Eurotiales.html)**.** Trichocomaceace is identified as higher classification known as Eurotiales. Teleomorphs of *Aspergillus* species belong to different genera in Trichocomaceae, Eurotiales, Eurotiomycetes. Many species of *Aspergillus* are known to reproduce sexually, producing asci and ascospores. The asci are nearly spherical and borne in nearly spherical cleistothecia. The cleistothecia may themselves be borne within stromatic tissues that range from simple masses of hülle cells to hard sclerotium-like structures. These are discussed in more detail in the section dealing with Eurotiales, Phylum Ascomycota (Webster and Webster, 2007; Yazdani *et al*., 2011). This research finding was to identify soil fungi based on morphological and three regions DNA sequencing.

Materials and methods

Samples collection

The soil samples were collected from Doi Suthep and Doi Inthanon mountains (Chiang Mai, Thailand) in May 2012. All samples were kept in sealed plastic bags and brought to laboratory at Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok,

Thailand. The samples were originally isolated by soil plate technique according to the method described by Soytong (1992).

Fungus isolation

The Trichocomaceae fungi were isolated from soil sample by soil plate technique using glucose ammonium nitrate agar (GANA: glucose 20 g, NH_4NO_3 1 g, difco bacto yeast extract 1 g, K_2HPO_4 0.5g, rose bengal 0.06 g, $MgSO_4$ $7H_2O$ 0.05g, agar 20 g in 1L distilled water). Soil samples were ground into a powder and put onto GANA media, then incubated for 15 days at room temperature (27-30 ℃). Fungi growing out as a colony was transferred to potato dextrose agar (PDA) to get pure culture and maintained on PDA for the duration of the experiment.

Morphological identification

The Trichocomaceae fungi were grown on PDA for 10-15 days at 25 ℃. Colony characters and microscope feature were observed. Each species was identified based on the methods of Domsch et al. (1993) and Soytong (1992).

DNA extraction, amplification and sequencing

All Trichocomaceae fungal colonies were cultured on potato dextrose broth (PDB) for 10-15 days at 25 ℃. Fungal genomic DNA was obtained from the mycelia of the PDB cultures using the GF-1 plant DNA extraction kit (vivantis, USA).

The ITS ribosomal DNA regions was amplified by PCR using the universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) (White *et al*., 1990). The *β*-tubulin gene was amplified using Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC) (Hubka and Kolarik, 2012). The calmodulin gene was amplified with primers CF1L (5'-GCCGACTCTTTGACYGARGAR) and CF4 (5'-TTTYTGCATCATRAGYTGGAC) (Peterson, 2008). Twenty-five microliter PCR reaction mixture contained 50 ng of DNA template, 0.8 pM of each primer, 200 µM dNTPs, 1x buffer, 1 unit of *Taq* DNA polymerase. PCR condition for the ITS regions were programed as follows: initial denaturation at 95 ℃ for 1 min, followed by 35 cycles of denaturation at 95 ℃ for 1 min, annealing at 50,52 ℃ for 1 min, and extension at 72 ℃ for 2 min, and final extension at 72 ℃ for 5 min. PCR amplification for *β*-tubulin gene and calmodulin gene were

performed using the conditions which comprised 32 cycles under the following temperature regime: 1 cycle of 95 ℃/3 min, 55 ℃/30 sec and 72 ℃/1 min, follow by 32 cycles of 95 ℃/30 sec, 55 ℃/30 sec and 72 ℃/1 min and final cycle of 95 ℃/30 sec, 55 ℃/30 sec and 72 ℃/10 min (Hubka and Kolarik, 2012). The PCR products were purified with PCR purified kit. Sequencing was performed at First Base Laboratories, Malaysia.

Molecular phylogenetic analysis

The nucleotide sequences were conducted by comparing the DNA sequences against those available in the NCBI Genbank database using a BLASTn search. The DNA sequences were aligned using BioEdit program and performed maximum parsimony in MEGA5. Bootstrap value was determined using heuristic searches with 1000 replications.

Results and discussion

Isolation and morphological identification

In this study, nine isolates showed slow growing property with pigment on PDA at 25 ℃. The morphological study was observed as microscopic characters which these isolates were shown two different groups of characters in colony, cleistothecia, asci and ascospores (Fig. 1-2) as reported by Domsch and Gams (1993). Nine isolates coded were morphologically compared as shown in Table 1. Seven isolates (EU02, EU03, EU07, EU10, EU12, EU14 and EU23) were showed yellow colony, ascomatal walls composed of loose hyphae, ascospores are broadly ellipsoidal to ellipsoidal; mostly 1.5~3.5 x 2.5~5.3 μm. Two isolates (EU06 and EU13) were showed white colony, ascospores are subglobose to broadly ellipsoidal with furrow and ridges. Therefore, EU02, EU03, EU07, EU10, EU12, EU14 and EU23 are identified as *Talaromyces* sp. EU06 and EU13 are identified as *Neosartorya* sp.

Table 1. Morphological characters of isolated Trichocomaceae

Code	Colony Color	Cleistothecia (µm)	Asci (µm)	Ascospore (µm)
EU02	yellow			
- shape		subglobose	subglobose	broadly ellipsoidal
- size		200.66~332.05 x 260.75~406.83	7.32~11.44 x 7.95~11.93	1.13~2.91 x 1.92~3.35
EU03	yellow			
- shape		globose	subglobose	ellipsoidal
- size		154.79~261.76 x 160.81~231.39	7.61~8.89 x 8.12~9.16	2.14~3.44 x 2.14~4.56
EU06	white			
- shape		globose	globose	broadly ellipsoidal
- size		90.7~263.05 x 91.98~276.76	9.86~13.63 x 8.63~13.97	3.86~6.81 x 3.70~6.48
EU07	yellow			
- shape		subglobose	globose	ellipsoidal
- size		220.78~420.59 x 244.4~482.06	8.51~9.95 x 8.69~11.69	2.91~4.42 x 4.27~5.46
EU10	yellow			
- shape		subglobose	subglobose	ellipsoidal
- size		266.63~429.15 x 221.18~404.67	7.1 1~9.01 x 5.36~8.00	2.05~4.11 x 2.04~3.92
EU12	yellow			
- shape		subglobose	subglobose	broadly ellipsoidal
- size		219.28~769.80 x 219.95~867.84	8.19~11.18 x 9.32~12.17	2.60~4.84 x 2.95~5.30
EU13	white			
- shape		globose	subglobose	subglobose
- size		151.70~458.70 x 148.06~526.58	10.33~14.19 x 10.63~15.87	4.22~6.07 x 4.49~7.41
EU14	yellow			
- shape		subglobose	globose	broadly ellipsoidal
- size		161.15~377.71 x 172.48~408.21	6.31~10.45 x 8.38~11.46	2.79~3.93 x 3.27~5.27
EU23	yellow			
- shape		globose	subglobose	ellipsoidal
- size		401.62~763.75 x 401.91~892.28	5.63~7.76 x 5.85~9.54	2.09~3.92 x 2.40~4.37

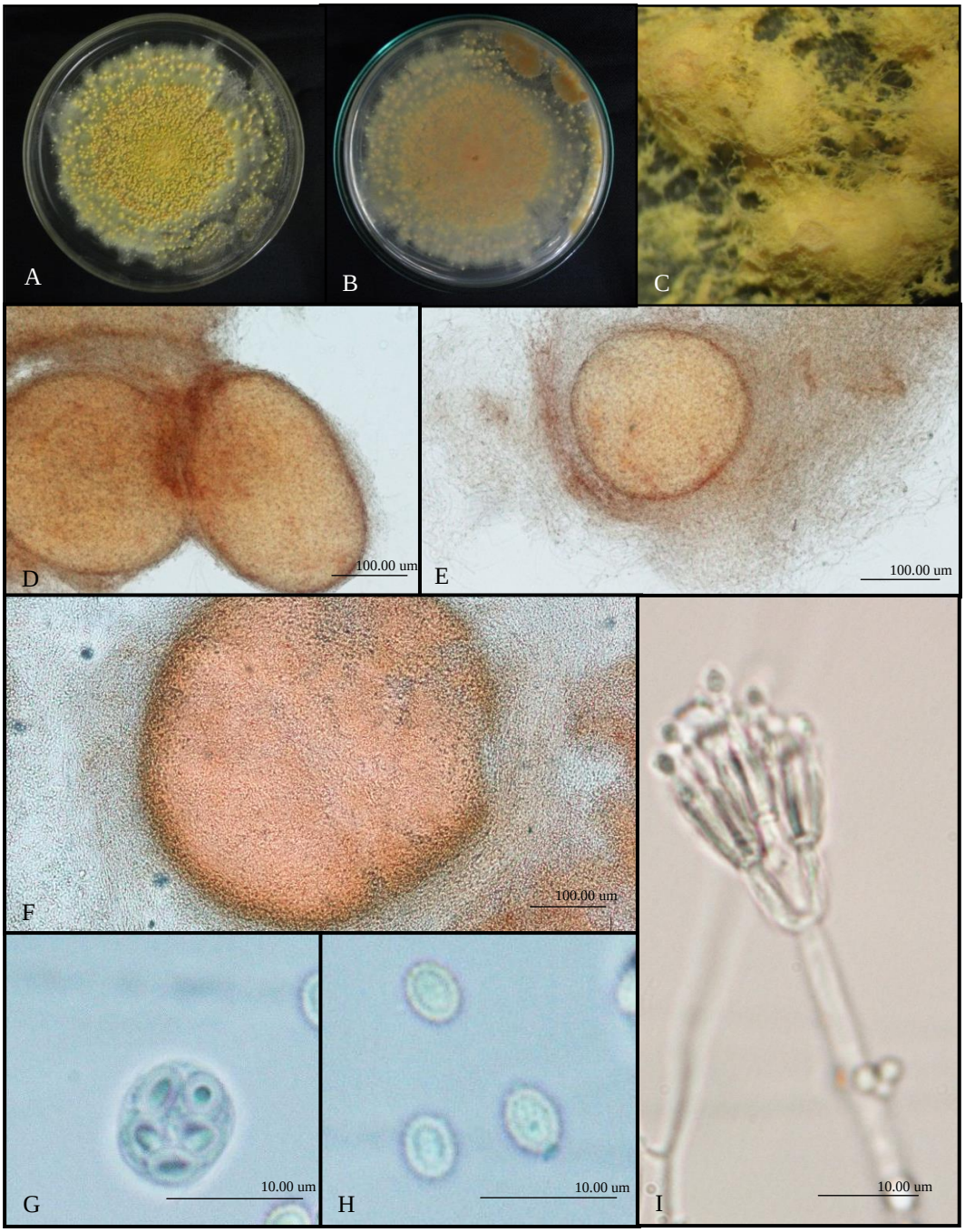

Figure 1. Morphological features of *Talaromyces* sp. (EU12) A-B: Colony grown for 15 day at 25 ℃, C-E: Cleistothecia, F: Asci exposed from cleistothecia, G: Ascus, H: Ascospores and I: Thallus, phialophores and phialides

Figure 2. Morphological features of *Neosartorya* sp. (EU06) A-B: Colony grown for 15 day at 25 ℃, C-E: Cleistothecia, F: Asci exposed from cleistothecium, G: Ascus and H: Ascospores

DNA sequencing identification

The nucleotide sequences were compared with BLASTn in NCBI Genbank demonstrated the level of genetic diversity of Trichocomaceae (Table 2) that related to the two genera. The *Talaromyces* sp. group is identified as *T. flavus* (EU02, EU03, EU07, EU12 and EU14) and *T. trachyspermus* (EU10 and EU23). The *Neosartorya* sp. group is identified as *N. hiratsukae* (EU06) and *N. pseudofischeri* (EU13).

Table 2. The three regions of nucleotide sequences compared with BLASTn in NCBI Genbank

Code	ITS regions	β-tubulin gene	Calmodulin gene
EU02	*P. verruculosum*	*T. marneffei*	*T. flavus*
EU03	*P. verruculosum*	*T. marneffei*	*T. flavus*
EU06	*N. hiratsukae*	*N. hiratsukae*	*N. hiratsukae*
EU07	*P. verruculosum*	*T. marneffei*	*T. flavus*
EU10	*T. assiutensis*	*T. trachyspermus*	*T. trachyspermus*
EU12	*P. verruculosum*	*T. marneffei*	*T. flavus*
EU13	*N. pseudofischeri*	*N. pseudofischeri*	*N. pseudofischeri*
EU14	*P. verruculosum*	*T. marneffei*	*T. flavus*
EU23	*T. trachyspermus*	*T. trachyspermus*	*T. trachyspermus*

The nucleotide sequences from the ITS regions confirmed all isolates identified as the Trichocomaceae. The three region: ITS, *β*-tubulin and calmodulin were amplified at 550, 450 and 650 bp fragments in each isolate, respectively. Maximum parsimony tree of three regions of Trichcocomaceae were represented the same relatives with the sample from NCBI-based to confirm the species. The phylogenic tree of calmodulin gene and the three regions were demonstated representative of this group (Fig. 3-4).

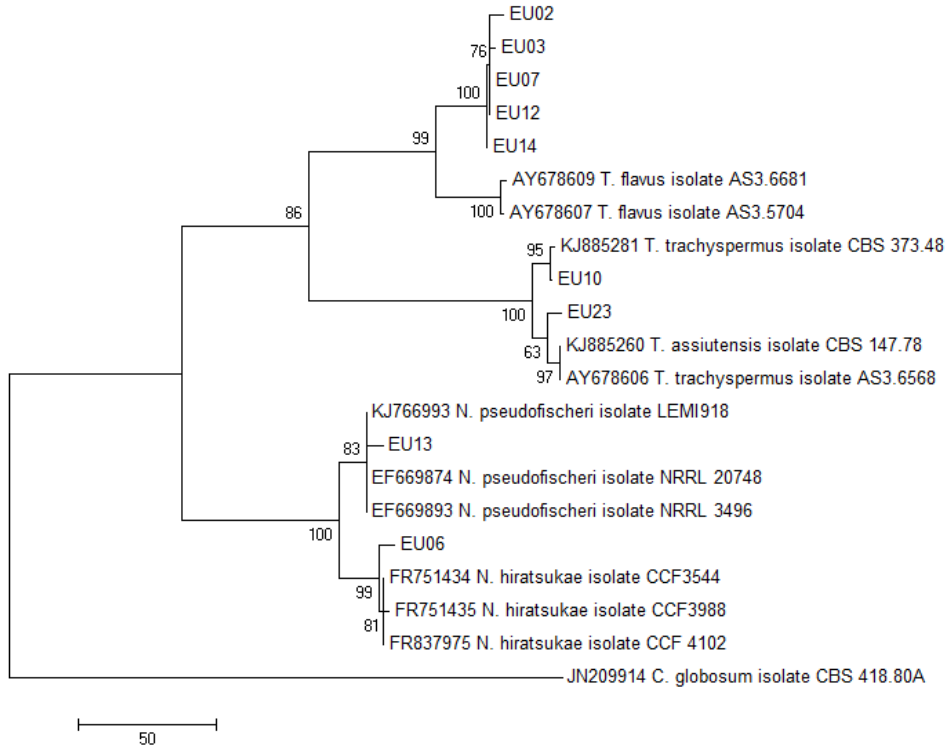

Figure 3. Maximum parsimony tree of some Trichocomaceae based on calmodulin gene using *Chaetomium globosum* as the out group species with bootstrap test (1000 replicates)

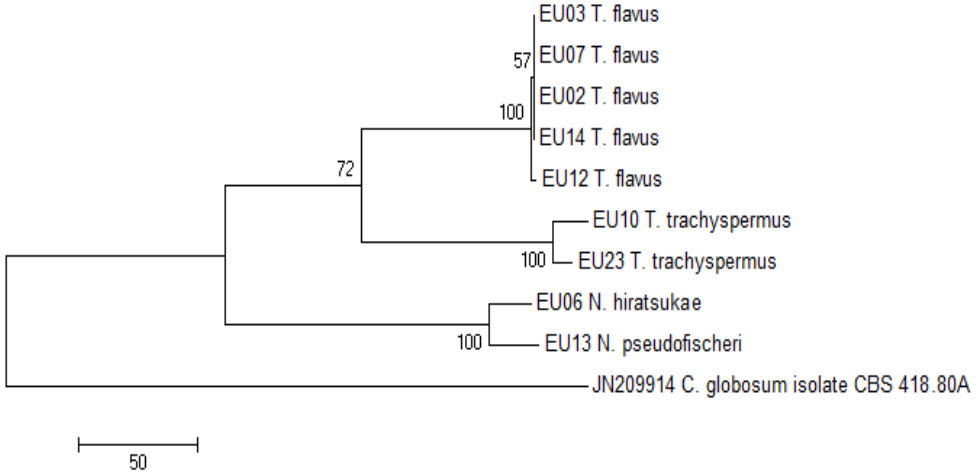

Figure 4. Maximum parsimony tree of nine isolates in this study based on the three regions using *Chaetomium globosum* as the out group species with bootstrap test (1000 replicates)

This study confirmed that five isolates (EU02, EU03, EU07, EU12 and EU14) are identified as *T. flavus* according to morphology and molecular taxonomy. There is yellow colony, ascomatal walls composed of loose hyphae, colony spreading broardly, ascoma intials consisting of vermiform (Domsch and Gams, 1993). The anamorph synonym of this species is *P. vermiculatum* which is the holotype of *P. dangeardii*. Both of these names were *T. flavus* which is the anamorph name (Samson *et al*., 2011). EU10 and EU23 are identified as *T. trachyspermus*. There is yellow colony, ascomatal walls composed of loose hyphae, ascomata white to creamish, ascospores 2~2.5 x 3~3.5 µm (Domsch and Gams, 1993). *P. spicucillium* is the anamorph of *T. trachyspermus* (Samson *et al*., 2011). One isolate (EU13) is identified as *N. pseudofischeri*. It shows white colony, ascospores are subglobose to broadly ellipsoidal with furrow and ridges. *N. pseudofischeri* anamorph synonym is *Aspergillus thermomutatus* which cause invasive fungal infections of human disease (Balajee *et al*., 2005). As a result, *N. hiratsukae* (EU06) is confirmed by molecular phylogeny but the activity of this isolate is not known. It must carefully work on this species because Guarro *et al.* (2002) reported for the first time that *N. hiratsukae*, an Ascomycetes which the asexual conidia resembles *A. fumigatus* causes a brain infection in a Brazilian woman. However, Hawksworth (2009) reported that *N. fumigata* is given to the sexual stage which is also asexually produced as *A. fumigatus* and the analogy of *A. nidulans* where its teleomorph *Emericella nidulans*.

Nine isolates belongs to Trichocomaceae were isolated from forest soils. These isolates were identified and confirmed species by morphology and three regions of ITS, β-tubulin and calmodulin. The morphology were shown two different groups of characters which identified as *Talaromyces* and *Neosartorya*. The nucleotide sequences were demonstrated and related to the four species: *Talaromyces flavus*, *T. trachyspermus*, *Neosartorya hiratsukae* and *N. pseudofischeri*. Some species of Trichocomaceae occur commonly and are important to both industry and medicine. Therefore, pathogenic fungi and their ability to secondary metabolite were also studied. However, a new classification system including both anamorph and teleomorph species will be investigating the relationship of some genera of Trichocomaceae.

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