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Morphological and molecular phylogeny studies on Eurotiales isolated from soil

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Seven isolates belonging to Eurotiales were isolated from forest soils in the North of Thailand. These isolates were identified and confirmed down to species level by morphological and molecular phylogeny. Six isolates namely: EU02, EU03, EU04, EU07, EU12 and EU14 were identified as *Penicillium verruculosum* and isolate EU06 was identified as *Neosartorya hiratsukae*.

Keywords: *Penicillium verruculosum*, *Neosartorya hiratsukae*.

Introduction

Eurotiales is an order of sac fungi, also known as the green and blue molds. The order contains 3 families, 49 genera, and 928 species. It was circumscribed in 1980. 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 and ascospores, previously placed to Deuteromycota with asexul 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. Eurotiales are widespread and abundant fungi which are known as asexually reproducing fungi. This asexual stage are interested because it implies that 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

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no sexual stages and so many Eurotiales are large amount of genetic diversity through mutations themselves.

Trichocomaceae (Eurotiales) (Klich, 2002; Samson, 1979; Samson and Varga, 2007; Samson *et al.*, 2011; Samson *et al.* (2004) have a excellent presentation with a key, illustrations, discussions and descriptions of the species commonly found on foods and indoor environments and this reveals for most species of *Aspergillus* found in environments in the tropics (http://website.nbm-mnb.ca/mycologywebpages/NaturalHistoryOfFungi/Eurotiales.html)**.**

Eurotiales are identified as higher classification known as Eurotiomycetes and lower classification as Elaphomycetaceae and Trichocomaceae. Teleomorphs of *Aspergillus* species are considered to belong to different genera of family Trichocomaceae of the order Eurotiales, class Eurotiomycetes. Many species of *Aspergillus* are known to reproduce sexually, producing asci and ascospores. The asci are nearly spherical and are 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). The research was done to identify soil fungi based on morphological and molecular phylogeny in these species.

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).

Isolation of Eurotiales

The Eurotiales fungi were isolated from soil sample by soil plate technique using glucose ammonium nitrate agar (GANA: glucose 20 g, NH4NO3 1 g, difco bacto yeast extract 1 g, K2HPO4 0.5g, rose Bengal 0.06 g, MgSO4 7H2O 0.05g, Streptomycin 0.03 g, Agar 20 g in 1L distilled water). Soil samples were ground into a powder and put onto GANA then incubated for 15 days at room temperature (27-30 °C). 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 Eurotiales fungi were grown on PDA for 10-15 days at room temperature (27-30ºC). Colony characters and microscope feature were observed. Each species was identified based on the methods of Domsch *et al*. (1993) and Soytong (1992).

Nucleic acid preparation, PCR amplification and sequencing

All Eurotailes fungi colonies were cultured on potato dextrose broth (PDB) for 10-15 days at 25ºC. 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 were amplified by PCR using oligonucleotide primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) X (White *et al*., 1990). PCR reaction mixture (a total volume is 25 µl) contained 50 ng of DNA template, 20 pmol of each primer, 1.25 mM dNTPs, 1x buffer, 1 unit of *Taq* DNA polymerase. PCR condition were programed as follow: initial denaturation at 95 ºC for 1 min, followed by 35 cycles of denaturation at 95 ºC for 1 min, annealing at 50,52 ºC for 1 min, and extension at 72 ºC for 2 min, and final extension at 72 ºC for 5 min. The PCR products were purified with PCR purified kit. Sequencing was performed at first base laboratories Sdn Bhd, 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 discussions

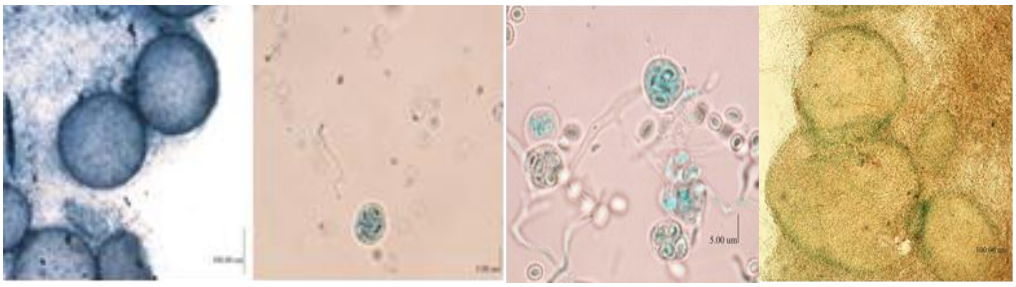
Isolation and morphological identification

In this study, seven isolates showed slow growing property with pigment on PDA at 25 °C. The morphological study was observed as microscopic

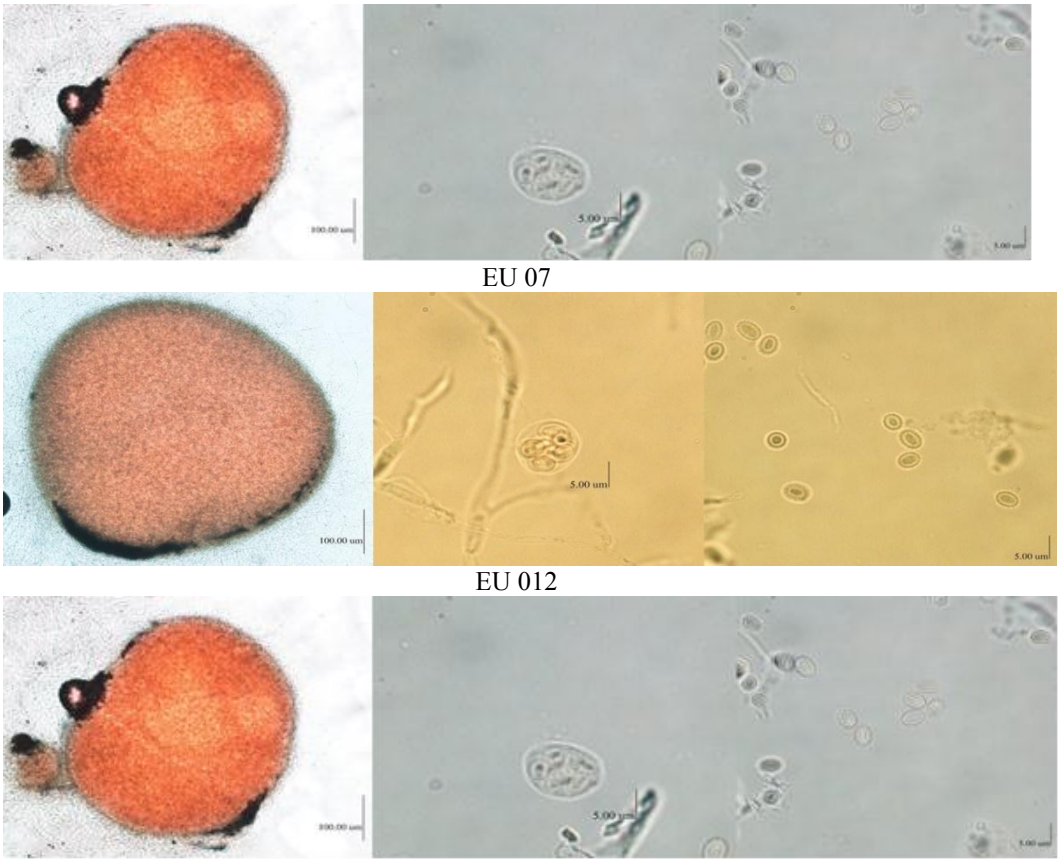
characters which some isolates were shown silmilar characters in colony, cleistothecia, asci and ascospores (Table 1 and Fig.1) as reported by Domsch *et al.* (1993). Therefore, the isolates coded as EU02, EU03, EU04, EU06, EU07, EU12 and EU14 were morphologically compared as shown in Table 1.

Table 1. Morphological characters of isolated Eurotiales

Code	**Cleistothecia**	**Asci**	**Ascospores**
EU02	subglobose 260.81~406.83 x 200.66~332.05	NA	ellipsoidal 1.92~3.35 x 1.13~2.91
EU03	NA	subglobose 8.12~9.16 x 7.16~8.89	ellipsoidal 2.90~4.56 x 2.14~3.44
EU04	globose 147.60~254.12 x 145.24~233.51	subglobose 7.99~9.96 x 6.92~8.97	ellipsoidal 3.35~4.85 x 2.90~3.82
EU06	NA	NA	NA
EU07	subglobose 244.40~482.06 x 220.78~420.59	globose 8.69~11.69 x 8.69~9.95	ellipsoidal 4.27~5.46 x 2.91~4.42
EU12	globose 400.68~867.84 x 256.28~769.80	globose 11.20~11.60 x 9.90~11.55	ellipsoidal 3.87~5.30 x 2.79~4.74
EU14	globose 93.66~213.11 x 91.18~189.55	NA	ellipsoidal 1.92~3.35 x 1.13~2.91



EU 02 EU 03 EU 04



EU 07

EU 012

EU 014

Fig. 1. Morphological characters of Eurotiales isolates

DNA sequencing identification

The sequences were compared with BLASTN in NCBI Genbank showed that EU02, EU03, EU04, EU07, EU12 and EU14 identified as *Penicillium verruculosum* and EU06 identified as *Neosartorya hiratsukae*. As seen the literatures that *Penicillium verruculosum* as anamorph stage (ATCC62396) (http://www.atcc.org/products/all/62396.aspx) and *Neosartorya hiratsukae* reported as teleomorph (ATCC MYA-1318) (http://www.atcc.org/Products/All/MYA-1318.aspx) as seen in Fig.2.

This study confirmed that isolates: EU02, EU03, EU04, EU07, EU12 and EU14 were *Penicillium verruculosum* according to morphology and molecular taxonomy. The activity of these isolates is still doubtful but Gyorgy (1981) stated that *P. verruculosum* WA 30 expressed cellulase productivity and successfully enhanced nearly threefold in medium experiments. The pH and

temperature optima for WA 30 cellulase complex were pH 4.2 and 60°C. It is interesting to find out on enzyme activity among these isolates.

As a result, *Neosartorya 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 *Neosartorya hiratsukae*, an Ascomycetes which the asexual conidia resembles *Aspergillus fumigatus* causes a brain infection in a Brazilian woman. However, Hawksworth (2009) reported that *Neosartorya fumigata* is given to the sexual stage which is also asexually produced as *A. fumigates* and the analogy of *Aspergillus nidulans* where its teleomorph *Emericella nidulans.* The two names for the same species which are same genomic sequence is certainly a confusing aspect of mycology. The morphological description of species and that before DNA or molecular approaches to identification it was difficult to decide its anamorph or teleomorph. Hawksworth makes an important point especially when discussing something where the anamorphs and teleomorphs are unified. A number of genera have been proposed to teleomorphs, including *Emericella*, *Eurotium*, and *Neosartorya* which genetic studies have shown these genera to be rather closely related and possibly not distinct from *Aspergillus*.

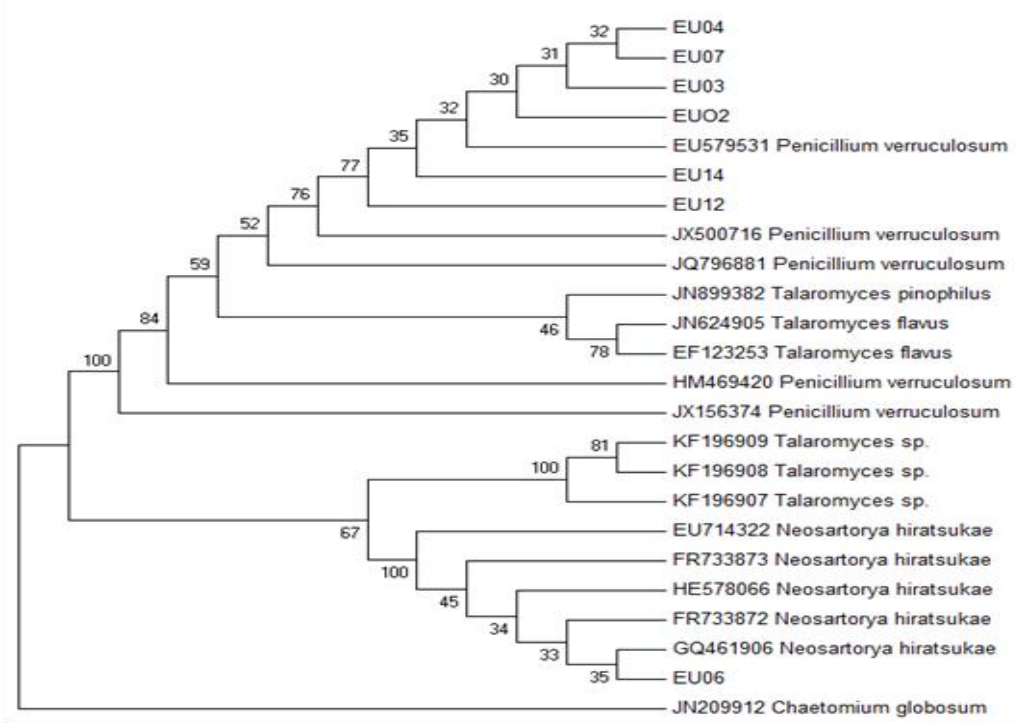


Fig. 2. Maximum parsimony tree of Eurotiales isolation based on ITS1, 5.8S rDNA, ITS2 sequence using *Chaetomium globosum* as the out group speciesc with bootstrap test (1000 replicates).

Conclusion

Seven isolates belongs to Eurotiales were isolated from forest soils. These isolates were identified and confirmed species by morphological and molecular phylogeny. Six isolates of EU02, EU03, EU04, EU07, EU12 and EU14 identified as *Penicillium verruculosum* and one isolate EU06 identified as *Neosartorya hiratsukae*. Further study will be tested biological activity of these fungal metabolites against some human and plant pathogens and possible testing for some enzyme activity.

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