CHAPTER 3

Collection, Isolation and Identification of Entomopathogenic Fungi

3.1 Introduction

Nowadays the development of environmentally sustainable systems of controlling pests in crop production is the use of BCAs that are less reliant on chemical pesticides as alternate approach. Promoting natural fungal epizootics by adopting appropriate cultural and crop protection practices is as alternative way of harnessing EPFs for pest control (Charnley and Collins, 2007). Generally, entomogenous fungi can be divided into two groups; necrotrophic fungi which act against insects (killing host cells and utilizing the nutrient source) and biotrophic fungi which form mutualistic associations with insects (living host cells required) parasites (Benjamin *et al*., 2004; Blackwell and Vega, 2005). The necrotrophic parasites are pathogenic to insects and those EPFs are particularly important for controlling insect pests. These fungi have been documented to occur naturally in over 750 species of host and development of microbial insecticide in advanced countries (Hejek and St. Leger, 1994; Inglis *et al*., 2001; Shah and Pell, 2003). Naturally occurrence pathogens in invertebrate populations contribute to the regulation of injurious pests of humans, crop production, households and domestic animals. Humid, tropical forests have a rich, varied entomopathogenic mycoflora (Evans, 1982), and it has been suggested that these fungi have a significant role in the regulation of insect population because of the stable microclimates in such habitats (Charnley and Collins, 2007). On the other hand, non-specialized pathogens such as *Beauveria* and

Metarhizium are poorly represented in forest habitats, but commonly encountered by agricultural entomologists and have potential as BCAs (Madelin, 1966; Samson *et al*., 1988).

Asexually produced fungal spores or conidia are generally responsible for infection and are dispersed throughout the environment in which the insect hosts are present. Studies on EPFs from Thailand have been carried out since 1990 and published (see Hywel-Jones, 2001; Jones and Hyde, 2004; Luangsa-Ard *et al*., 2007) and more than 400 morphotaxa have been recorded (Luangsa-Ard *et al*., 2007). Aung *et al*. (2008) found 34 entomogenous taxa belonging to 15 genera (18 telomorphs and 16 anamorphs) from Chiang Mai province.

The concern for the development of hyphomycete fungi as suitable BCAs of insect pests leads to the isolation of various insect pathogenic fungi (Dhar and Kaur, 2010). According to these reasons, we focused on the efficacy of naturally infested EPFs in crop production. Therefore, this study was initiated with the following objective.

3.2 Objective

To isolate and identify the entomopathogenic fungi from natural habitat.

3.3 Methodology

3.3.1 Sample site

Four locations in Chiang Mai province were studied for the collection of entomopathogenic fungi.

(A) Mokh Fa waterfall, located near 18km marker on Highway 1095.

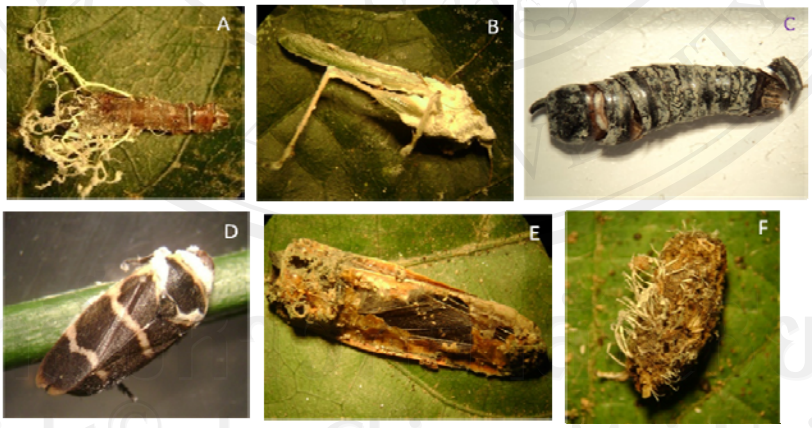
(B) Medicinal plant garden, located on Doi Suthep 930m above sea level.

(C) Monthatarn waterfall, located on Doi Suthep.

(D) Chomthong district.

3.3.2 Collection and isolation of collected entomopathogenic fungi

The dead cadavers with external signs of mycosis were collected (Figs. 3.1) and transported promptly to the laboratory. Single spore isolation was carried out using a modified procedure of Thakur and Sandhu (2010). The fungi were isolated directly from dead cadavers by taking conidia onto 1.5% water agar with 0.05 mg/l chloramphenicol and incubated at 25±2˚C. All cultures were examined daily under the stereomicroscope. For pure culture isolation, the mycelial tip of a small fungal colony was cut and transferred to PDA plates. Cultures were incubated at 25±2˚C. The collected EPFs used in this study are presented in Table 3.1.



Figures 3.1 Collected entomopathogenic fungi from different hosts in Chiang Mai province, Thailand. (A) *Isaria tenuipes* CMUCDMF02, (B) *Beauveria bassiana* CMUCDMF03, (C) *Metarhizium flavoviride* CMUCDCT01, (D) *Beauveria bassiana* CMUCDMG03, (E) *Metarhizium anisopliae* CMUCDMF04, (F) *Paecilomyces lilacinus* CMUCDMT02.

3.3.3 Identification of entomopathogenic fungi

3.3.3.1 Morphological examination

Morphological identification of collected EPFs was conducted 14 days after incubation at 25±2˚C by using a slide culture technique according to Riddell (1950). The morphotaxonomic characteristics of conidia-forming mycelia and conidia structure were examined based on Humber (1998) and Luangsa-Ard *et al.* (2007).

3.3.3.2 DNA extraction, PCR and sequencing

To obtain target DNA, PCR amplification was performed using the primer pairs ITS5 (5′- GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′- TCCTCCGC TTATTGATATGC-3′) described by White *et al*. (1990). Genomic DNA from collected fungal isolates was extracted by a modified CTAB method (Chutima *et al.,* 2010). Mycelia were scraped off from culture plates and transferred into 1.5 ml eppendorf tubes. To the mycelia sterile white quartz sand and 500 µl sterilized extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0) were added and the sample was grounded and incubated at 65 ˚C for 60 min. Five hundred micro liters of chloroform- isoamyl alcohol (24:1) was added to each tube and mixed. The mixture was centrifuged at 13,000 rpm for 30 min, and the aqueous extraction layer was transferred into new eppendorf tubes. The addition of chloroform- isoamyl alcohol (24:1) and centrifugation was repeated two more times until no interface was visible. Eight hundred micro liters of cold absolute ethanol was added to each tube and left at -20˚C for 5-10 min, and centrifuged at 13,000 rpm for 15 min at 4˚C. The upper layer was discarded and the DNA pellet was dried overnight. The dried pellet was re-suspended in 30 µl of TE buffer (10 mM Tris-HCl,

1 mM EDTA, pH 8.0) containing 20 μg/ml RNase and incubated at 37˚C for an hour. The purity of DNA was checked by electrophoresis in 0.8 % agarose gel stained with ethidium bromide under UV light. The internal transcribed spacer (ITS) regions of nuclear rDNA were amplified by polymerase chain reaction (PCR) with primers ITS4 and ITS5 under the following thermal conditions: 95˚C for 2min, 30 cycles of 95˚C for 30s, 50˚C for 30s, 72˚C for 1min, and a cycle of 72˚C for 10min. Amplicons were checked on 1% agarose gels stained with ethidium bromide under UV light. The PCR products were purified using PCR clean up Gel extraction NucleoSpin® Extract II purification Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. Sequencing reactions were preformed and the sequences were automatically determined in a genetic analyzer (1st Base, Malaysia) using the PCR primers mentioned above. Sequences were queried in GenBank via BLAST (http://blast.ddbj.nig.ac.jp/top-e.html).

3.3.4 Phylogenetic analysis

A phylogentic tree of DNA sequences was constructed using the neighbor-joining method according to Nimnoi *et al.* (2010). Multiple alignments of sequences determined in this study and reference sequences obtained from databases were taken together in the calculations of levels of sequence similarity using CLUSTALW 1.74 (Higgins *et al.*, 1992) with arithmetic averages tree-making algorithms taken from the MEGA package version 4.0 (Tamura *et al.*, 2007). The topologies of the neighbor-joining phylogenetic trees were evaluated based on bootstrap analyses of 1,000 replicates (Saitou and Nei, 1987).

3.4 Results

3.4.1 Isolation and identification of entomopathogenic fungi

Six isolates belonging to four genera of entomopathogenic fungi from Chiang Mai Province, Thailand were identified by both morphological and molecular technology. Colonies of fungal isolates on culture media are illustrated in (Fig. 3.2). Microscopic examination after mounting in lactophenol cotton blue showed these fungi to be *Beauveria*, *Metarhizium*, *Isaria* and *Paecilomyces* based on macro- and micro-morphological criteria such as the shape and size of colonies, conidiophores, and conidia. The measurements of morphological criteria of each fungus are shown in Table 3.1.

Figures 3.2 Colony characteristic of collected entomopathogenic fungi on half-strength PDA. (A) *Isaria tenuipes* MF02, (B) *Beauveria bassiana* MF03, (C) *Metarhizium flavoviride* CT01, (D) *B. bassiana* MG03, (E) *M. anisopliae* MF04, (F) *Paecilomyces lilacinus* MT02. Bars=50mm.

3.4.2 Molecular identification of fungal isolates

The purified DNA of ITS regions of collected EPFs run on gel electrophoresis and PCR products are shown in Figure 3.3. The partial internal transcribed 1, 5.8S ribosomal RNA gene and the internal transcribed spacer 2 sequences of six entomopathogenic fungi isolates were obtained and compared with the GeneBank database. Sequence data obtained from the PCR products are deposited in GeneBank (Table 3.1). In the neighbor-joining tree, isolates MF03 and MG03 were placed in *B. bassiana* (Fig. 3.4). Isolates MF02 and MT02 were closely related with high bootstrap support (100%) to *I. tenuipes* and *P. lilacinus* respectively, and the fungus isolate MF04 was closely related to *M. anisopliae*. The remaining entomopathogenic fungus isolate, CT01, was closely related to *M. flavoviride*.

Figure 3.3 Purified PCR products of ITS regions of six collected insect pathogenic fungi. JT1 (CT01), *M. flavoviride*; MT2 (MT02), *P. lilacinus*; MF4 (MF04), *M. anisopliae*; MF3 (MF03), *B. bassiana*; MG2 (MF02), *I. tenuipes*; MG3 (MG03), *B.bassiana*

3.5 Discussion

In this study, a total of six insect pathogens were isolated from dead arthropods in natural habitats Chiang Mai province. Based on their colony characteristic and sequence analyses, these isolates were identified as *B. bassiana*, *M. anisopliae*, *M. flavoviride*, *P. lilacinus* and *I. tenuipes* according to analyzed sequences. From this study we observed two *B. bassiana* isolates and two species of *Metarhizium* from different hosts at different locations. Differences in colony morphology of these *B. bassiana* isolates were recorded in culture medium. Because these isolates were recovered from different geographical locations and their different morphological, it is likely that genomic variation exists among them (McCoy *et al.*, 1985). Similar reports that *B. bassiana* has been isolated from numerous insects, with diversification to specific habitats and hosts (Hajek, 1997; Glare and Inwood, 1998; Rehner and Buckley, 2005; Rehner *et al*., 2006a, b; Meyling and Eilenberg, 2007; Quesada-Moraga *et al*., 2007; Zimmermann, 2007). In agreement, this study reported 99% similarity of two *B. bassiana* isolates originating from different sites and hosts. *Paecilomyces lilacinus,* a common soil parasite, well known as biological control agent of nematode egg parasite and as a controller of greenhouse insects and pests (Kerry, 1990: Schenck, 2004: Fiedler and Sosnowska, 2007). In our collection, we obtained one isolate of *P. lilacinus* from unknown insect.

From this study, we continued further experiments for the future studies to find out their potential efficacies such as pathogenicty, metabolic enzyme activity etc. as biocontrol agents to control agricultural pests for human requirements in the near future.

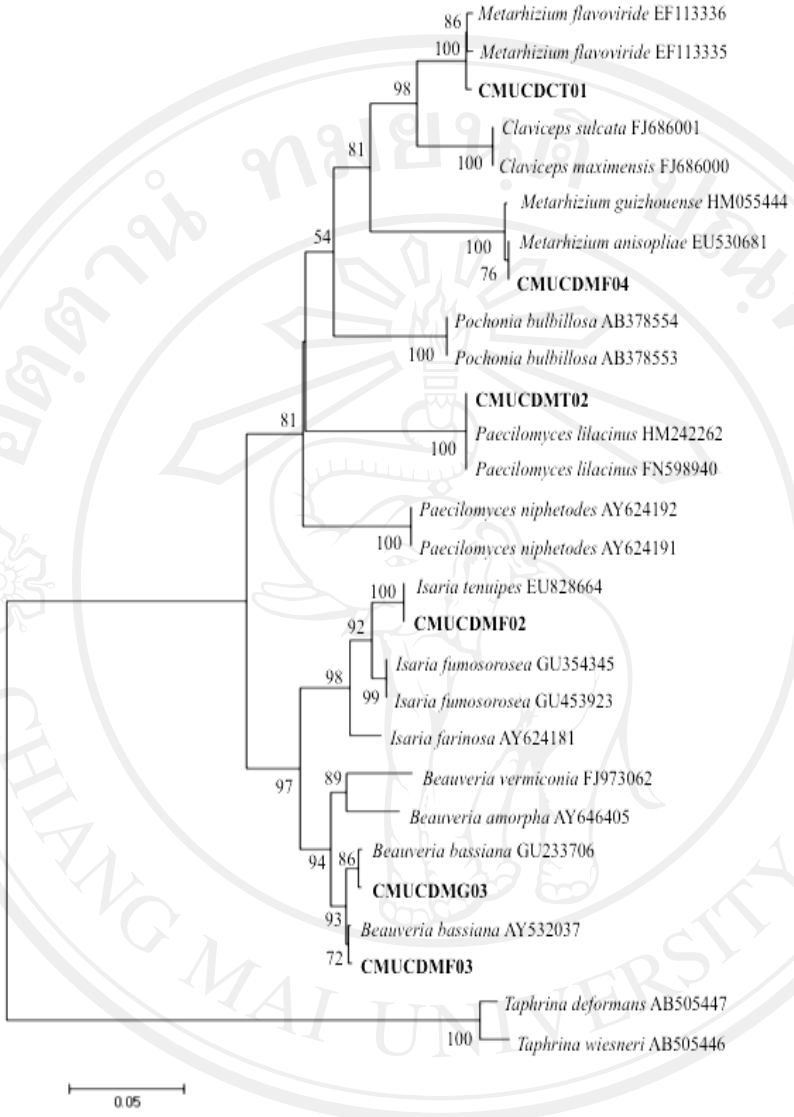


Figure 3.4 Neighbor-joining tree based on an alignment of internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2. The tree was rooted with *Taphrina deformans* and *T. wiesneri*. Bootstrap values (calculated from 1,000 resamplings) higher or equal to 50% are shown at each branch. The bar represents the number of mutations per sequence position.

Table 3.1 Origin of collected entomopathogenic fungi

Strains/ Isolates	Diameter of culture (cm)	Length of Conidia (µm)	Shape of Conidia	Color of Conidia	Conidiop-hore	Date of collection	Origin	Identification	Accession number	Host
CMUCD CT01	2.475	2.7-4.02 × 0.56-1.99	Cylindrical /elongate	Light green	Loosely aggregated	02/02/2010	Chomthong district	*Metarhizium flavoviride*	JN038191	Glomerides -mida
CMUCD MT02	4.825	2.1-3.9 × 1.5-2.5	Globose-subglobose	Pink	Slightly smooth and colored	06/01/2010	Monthatarn waterfall	*Paecilomyces lilacinus*	JN038196	unknown insect
CMUCD MF02	2.225	3.5-5.5 × 2.1-3.5	Cylindrical	Hyaline	Tightly	06/01/2010	Mokh Fa waterfall	*Isaria tenuipes*	JN038192	Pupa (Lepidoptera)
CMUCD MF03	3.225	1.5-2.57 × 1.1-1.7	Globose-subglobose	Hyaline	Smooth and colorless	06/01/2010	Mokh Fa waterfall	*Beauveria bassiana*	JN038193	Orthoptera
CMUCD MF04	4.025	3.15-4.35 × 1.3-2.1	Cylindrical/e longate	Light green to dark green	Branched, densely intertwined	06/01/2010	Mokh Fa waterfall	*Metarhizium anisopliae*	JN038194	Orthoptera
CMUCD MG03	2.65	1.5-2.13 × 1.1-1.5	Globose	Hyaline	Smooth and colorless	15/12/2009	Medicinal plant garden	*Beauveria bassiana*	JN038195	Coleoptera