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Variation in morphology and ribosomal DNA among isolates of *Metarhizium anisopliae* from Thailand

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Metarhizium anisopliae is an important entomopathogenic fungus that mainly used for the biological control. The objectives of this work were to isolate and identify varieties of *Metarhizium anisopliae* mainly based on the morphological and molecular characteristics. We investigated 24 *Metarhizium* isolates which collected from different insect hosts and soil from different sites in Thailand and one isolate was collected from the Philippines. These isolates have been partially characterized using morphological traits such as features of colony morphology, size and shape of conidia that were grown on Potato-dextrose-agar (PDA) at room temperature. Colony features could be separated into five groups. The length/width ratio of the conidia were calculated and generated into three groups: ≤ 2.18 is low ratio group, between 2.33 and 2.87 is medium ratio group and ≥ 2.91 is high ratio group. Internal transcribed spacer (ITS) and 5.8S DNA sequences analysis was employed to identify genotypes. The ITS regions were amplified using the ITS1 and ITS4 primers that was a unique fragment of approximately 550 bp. The sequences were aligned using the ClustalX program and compared with 19 isolates of *Metarhizium* available in the GenBank/NCBI database and 2 isolates *Beauveria bassiana* sequences as outgroup. Phylogenetic trees constructed using the Neighbor-joining algorithm in the Phylip package. Our study showed that *M. anisopliae*, *M. album*, *M. anisopliae* var. *acridum* and *M. flavoviride* can be clearly differentiated and generated the 25 isolates into four main groups (Clade B, C, E and F). However, it is not clearly difference between *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus* and most of them are related to *M. anisopliae* var. *anisopliae*. This study indicated that *M. anisopliae* are highly divergent. Nevertheless, most of *M. anisopliae* isolates from Thailand distantly related to *M. anisopliae* from other countries. It would be interesting to compare them using other molecular technique in the future.

Key words: *Metarhizium anisopliae*, Internal Transcribed Spacers (ITS) Regions

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Introduction

An entomopathogenic fungus, *Metarhizium* is a genus of asexual stage (anamorph) which produces sexual stage (telomorph) namely *Cordycep* sp. belongs to phylum Ascomycota. *Metarhizium anisopliae* is a well known as biological control agents of insect pests. *M. anisopliae* infects a wide range of insect orders and a broad geographical distribution (St. Leger *et al*., 1992; Bidochka *et al.,* 1994; Bridge *et al*., 1997; Leal *et al*., 1997; Driver *et al*., 2000; Dong *et al*., 2007). Traditionally, *Metarhizium* spp. has been classified based on its phenotypic characteristics as *M. anisopliae* and *M. flavoviride* (Tulloch, 1976). Including, *M. anisopliae* has been subdivided into two varieties (*M. anisopliae* var. *anisopliae* and var. *major*) based on the length of conidia. *M. anisopliae* var. *anisopliae* has smaller conidia (5.0 to 8.0 µm in length) than var. *major* (10.0 to 14.0 µm) (Tulloch, 1976), now referred to as *majus* (Rombach *et al*., 1986).

Yip *et al*. (1992) used the ability to germinate at low temperatures (i.e. 5°C) of *M. anisopliae* var. *anisopliae* as *M. anisopliae* var. *frigidum*. Therefore, it is difficult to identify species or varieties based only on morphological characteristics that affected by environmental conditions. In recent years, molecular techniques have provided valuable insights into genetic relationships within and among species. So, morphological and molecular methods were used to identify and characterized *Metarhizium* species. For example, allozyme analysis (St. Leger *et al*., 1992; Rakotonirainy *et al*., 1994; Bridge *et al*., 1993), Restriction Fragment Length Polymorphisms: RFLP (Bridge *et al.,* 1997; Pipe *et al.,* 1995; Mavridou and Typas, 1998), the nuclear ribosomal DNA (rDNA) sequence data comparisons (Rakotonirainy *et al.,* 1994; Curran *et al.,* 1994), Random Amplified Polymorphic DNA: RAPD (Cobb and Clarkson, 1993; Bidochka *et al.,* 1994; Leal *et al.,* 1994) and restriction analysis of the protease *Pr1* gene (Leal *et al.,* 1997). In the taxonomic revision of the *Metarhizium* spp., Driver *et al.* (2000) recognized ten distinct clades or lineage of organisms from 123 isolates based on internal transcribed spacer (ITS) regions, 5.8S rDNA and the D3 region of 28S (LSU) of rDNA. This study was divided into three species; *M. album*, *M. anisopliae* and *M. flavoviride* that represent nine varieties; *M. flavoviride* type E, *M. flavoviride* var. *flavoviride*, *M. flavoviride* var.*minus*, *M. flavoviride* var. *novazealandicum, M. flavoviride* var. *pemphigum*, *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *majus*, *M. anisopliae* var. *lepidiotum* and *M. anisopliae* var. *acridum*. However, it is not yet clearly understood because they did not comment on the positions and validity of *M. pingshaense, M. cylindrosporae, M. guizhouense* and *M. taii* taxa from china. Furthermore, they were studied only one isolate from Thailand.

In the most recent with the advent of genetic profile, Bischoff *et al.* (2009) recognized nine distinct species, *M. anisopliae* that described as *M. anisopliae* var. *anisopliae*, *M. guizhouense* (syn. *M. taii*), *M. pingshaense, M. acridum* stat. nov. (*M. anisopliae* var. *acridum*), *M. lepidiotae* stat. nov. (*M. anisopliae* var. *lepidiotae*), *M. majus* stat. nov. (*M. anisopliae* var. *major*), *M. globosum* sp. nov., *M. robertsii* sp. nov. and *M. brunneum*. However, there were non isolate from Thailand had been studied. In this study, we investigated both morphological and molecular analyses that based on ITS regions and 5.8S rDNA for *Metarhizium* that have been isolated and collected in Thailand.

Materials and methods

Fungal isolation and morphological characteristics

The 25 isolates of *Metarhizium* spp. were studied, 13 isolates from insects and 11 isolates from soils in different regions in Thailand, except TISTR 3158 isolate was obtained from soil in the Philippines. All fungal isolates studied are listed in Table 1. The fungi from insects were isolated from samples by incubating or streaking a sub-sample on surface of selective media (potato dextrose agar: PDA, Criterion, USA with 0.1% chloramphenicol and 0.05% cyclohexamind). Soil samples were processed by diluting 10 g in 100 ml of sterile water that was added 0.01% Tween-80. One milliliter of the soil dilution was spread over those selective media. Then, single colony of *Metarhizium* spp. was transferred to PDA for purification. All were propagated and maintained with a 5 mm diameter mycelial plug taken from the growing edge of a 7 days old culture grown on PDA plates for 28 days at room temperature. The identification was done by observation of conidia, colony and mycelia morphology.

DNA extraction and amplifications

Mycelia and conidia from each isolate were plated on potato dextrose agar (PDA) and single spore colony was grown on potato dextrose broth (PDB), incubated on shaker (150 rpm) at room temperature for 5-7 days. Mycelium was recovered by centrifugation and filtration through Whatman No. 1 filter paper, washed twice with sterilized water, adding liquid nitrogen and ground until a powder mycelium was obtained. The powder was extracted by DNeasy Plant Mini Kit® (QIAGEN) following the manufacture's instructions and stored genomic DNA at 4 ºC. The ITS1-5.8s-ITS2 region of rDNA was amplified using the universal primers, ITS 1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990).

Amplification reactions were performed at a total volume 25 µl, consisted of template DNA, 200 µMol dNTPs, 0.8 *p*Mol each primer, 10X PCR buffer and 1U *Taq* DNA polymerase (Biolabs, England). The condition of temperature in thermal cycling was one cycle of initial denaturation at 95 ºC for 5 minutes, followed by 35 cycles with denaturation at 94 ˚C for 1 minute 30 seconds, annealing at 55 ºC for 2 minutes, and extension at 72 ºC for 3 minutes and a final extension at 72 ºC for 5 minutes. PCR products were separated by electrophoresis in 1% agarose gels by comparison with 100 bp DNA Ladder. The PCR products were purified and then used directly for rDNA sequencing.

Phylogenetic analysis

All sequences were compared to others in GenBank using BLASTN and the best match recorded and selected. Two sequences of *Beauveria bassiana* were used as an outgroup. The entire DNA sequences of 25 *Metarhizium* spp. isolates from this study, 19 public sequences of *Metarhizium* spp. and *B. bassiana* from the GenBank database were edited within Bioedit version 7.0.5.2. The sequences were aligned using the ClustalX 1.83 software. The consensus trees were constructed using Consense program in the Phylip package version 3.6 with Neighbor-joining method by 1000 bootstrap resembling. Phylogenetic inferences were performed and exposed using TreeView 1.6.6.

Results and discussion

Identification of *Metarhizium* was done by observation of colony morphology, size and shape of conidia. The conidia are cylindrical with round ends which vary color from light green to dark green and the conidiophores are simple and branch by apical phialides produce conidia. Twenty-five isolates in this study, most common isolates were identified as *M. anisopliae*. Conidia color may differ in colony size and condition (Latch, 1964). The growth of colony was observed on PDA at room temperature (25-30 ºC) for 28 days. Fungal colonies are initially white or creamy mycelium, becoming shades of yellow, shades of green/yellow to shades of dark green during sporulation. Most isolates are brightly dark green colony, except MA 017 is duller dark green.

Table 1. Morphological and phylogenetic clade of *Metarhizium* spp.

Isolates	Host	Geographic Origin	Conidia size (μm) Width		Length/Width Ratio	Conidia Ratio	Morphology Group	Clade
			Width	Length				
			Mean	Mean				
MA001	Coleoptera	Nakhonratchasima	3.34	7.29	2.18	Low	3	C
MA002	Coleoptera	Bangkok	2.96	8.62	2.91	High	5	F
MA003	Coleoptera	Samutprakan	2.60	9.10	3.49	High	5	F
MA007	Coleoptera	Nakhonsawan	3.73	10.26	2.75	Medium	1	B
MA011	Isoptera	Suphanburi	3.22	9.36	2.91	High	2	B
MA012	Coleoptera	Khonkaen	3.10	9.75	3.15	High	1	B
MA014	Coleoptera	Kanchanaburi	3.33	9.10	2.73	Medium	1	B
MA015	Coleoptera	Not available	3.56	9.50	2.67	Medium	2	B
MA017	Dermaptera	Nakhonratchasima	3.50	9.22	2.64	Medium	4	E
MA018	Coleoptera	Kanchanaburi	3.18	9.15	2.87	Medium	2	B
MA019	Coleoptera	Kanchanaburi	3.39	9.72	2.87	Medium	1	B
MA025	Coleoptera	Prachuapkhirikhan	2.92	9.42	3.23	High	3	F
MA026	Coleoptera	Prachuapkhirikhan	2.90	9.17	3.17	High	3	F
TISTR 3158	Soil	Philippins	2.28	6.21	2.73	Medium	3	E
TISTR 3607	Soil	Thailand	2.35	5.12	2.18	Low	3	C
SEC04	Soil	Chanthaburi	2.26	6.37	2.82	Medium	3	F
SED07	Soil	Sakaeo	2.29	5.32	2.33	Medium	3	C
SNA01	Soil	Maehongson	2.25	6.75	3.00	High	5	F
SNB02	Soil	Chiangmai	3.85	10.77	2.80	Medium	3	C
SNB03	Soil	Chiangmai	2.42	5.85	2.42	Medium	5	F
SNB07	Soil	Chiangmai	3.04	9.01	2.96	High	5	F
SNB08	Soil	Chiangmai	3.50	10.23	2.92	High	5	F
SNEB17	Soil	Nakhonratchasima	2.39	6.30	2.64	Medium	3	F
SNEB18	Soil	Nakhonratchasima	2.06	6.41	3.11	High	3	E
SWC03	Soil	Prachuapkhirikhan	3.00	8.80	2.94	High	3	E

Morphological features of colonies were studied and could be separated into five groups on the basic of colony color and surface. The surface layer of the group 1 (MA 007, MA 012, MA 014, MA 019 and SWC 03) colony is flat and mycelium is smooth. Colonies have green/yellow pigmented within 4-5

days and came to brighter dark green and conidia showing zonation (Fig. 1A). Group 2 (MA 011, MA 015 and MA 018) colony is similar to group 1 except the color of media is shades of light yellow (Fig. 1B). The most common are group 3 (MA 001, MA 026, SEC 04, SED 07, SNEB 17, SNEB 18, SNB 02, TISTR 3158 and TISTR 3607) that colony is flat, conidia are medium size and showing both zonation and non-zonation (Fig. 1C). Group 4 are MA 017 and MA 025 similarity to group 3 but conidia mass like a mat (Fig. 1D). The last group (MA 002, MA 003, SNA 01, SNB 03, SNB 07 and SNB 08) showed differ from the others, because the colonies are not smooth, but mycelia upright as aerial mycelia, and the media color is yellow to orange (Fig. 1E).

Morphology of conidia in PDA under bright field light microscope showed cylindrical shapes and usually light green in color and string together (Fig. 2A-C). Those demonstrate overlap in ranges of conidia sizes; length and width of 40 conidia per isolate were measured and presented in table 1. Conidia sizes were varied from the width of 1.40 µm (TISTR 3158) to 4.75 µm (MA 007) and length 4.10 µm (TISTR 3607) to 12.59 µm (MA 019). The width of conidia ranged from 2.06 to 3.73 µm and the length of conidia ranged from 5.12 to 10.77 µm. This is similar to Riba *et al*. (1986) and Yip *et al*. (1992) who reported conidia dimension of *M. anisopliae*. Length/width ratio of the conidia was calculated and divided into three groups: isolates with length/width ratio ≤ 2.18 is low ratio group (Example; MA 001: Fig. 2A), isolates with length/width ratio between 2.33 and 2.87 is medium ratio group (Ex.; MA 018: Fig. 2B), and isolates with length/width ratio ≥ 2.91 is high ratio group (Ex.; MA 026: Fig. 2 C, Table 1).

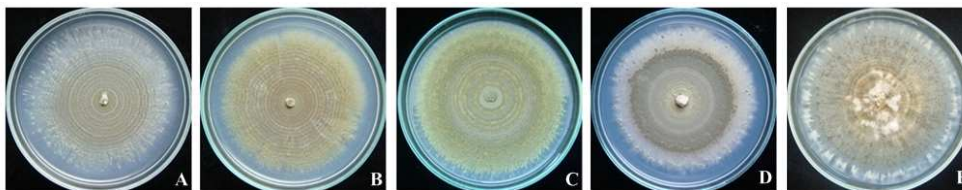

Fig. 1. *Metarhizium* colonies were grown on PDA at room temperature. Colony from group 1; MA 012 (A), group 2; MA 011 (B), group 3; SNEB 17 (C), group 4; MA 017 (D) and group 5; SNA 01 (E).

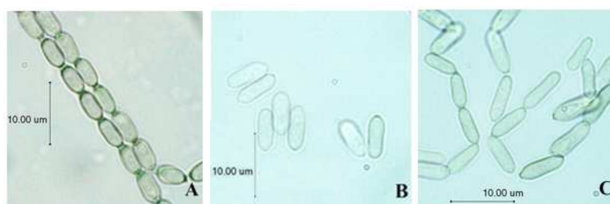

Fig. 2. The morphology of conidia under bright field light microscope (40X). Example image in low ratio group: MA001 (A), medium ratio group: MA018 (B) and high ratio group: MA026 (C).

The ITS regions and 5.8S rDNA of *Metarhizium* were amplified using the ITS1 and ITS4 primers that was a unique fragment of approximately 550 bp for all isolates. Destéfano *et al*. (2004) analyzed at the same region with 540 bp fragment for *M. anisopliae* var. *anisopliae* strain E9, B/Vi and C isolated in Brazil and 600 bp for *M. anisopliae* strain 14 isolates in Australia. The PCR products were sequenced and compared to others in GenBank using BlastN. Sequencing data conformed that all sample isolates are *M. anisopliae.* This study indicates that it is very similar that *M. anisopliae* is the predominant variety in this part of the world as stated by Roberts *et al.* (2004). Genetic distances between isolates are presented by branch length. Phylogenetic analysis showed other *Metarhizium* species or varieties and outgroup (*B. bassiana*) from GenBank can be separated clearly differentiated in clade A. The data support the monophyly of the *M*. *anisopliae* group except *M*. *anisopliae* var. *acridum*, and recognize five clades (clades B-F) within it. The isolates within clade A were subdivided into four groups with *B. bassiana*, *M. album*, *M. flavoviride* and *M. anisopliae* var. *acridum*. *M. album* and *M. flavoviride* are clearly distinguished from *M. anisopliae*. In this study, *M. anisopliae* var. *acridum* sequences were different from other *M. anisopliae* that was significantly supported by Diver *et al*. (2000) and Bischoff *et al*. (2009). Based on the results of morphological and molecular data, this study was confirmed by Bischoff *et al*. (2009) that the 5' region of EF-1ά is to date the most informative region to use for routine species identification with this genus. *M. anisopliae* var. *anisopliae* is pathogenic of innumerable insect variety. Whereas, *M. anisopliae* var. *acridum* is much more host specific only Orthoptera insects (Bridge *et al*., 1997; Goettel and Jaronski, 1997; Milner *et al*., 2002; Alston *et al*., 2005). So, Bischoff and coworker (2009) were introduced *M. anisopliae* var. *acridum* to *M. acridum* stat. nov. species. This study confirms that rDNA sequence data can be used to resolve evolutionary relationships within *Metarhizium* that *M. anisopliae* separate evolutionary lines.

The dendrogram can be generated the 25 isolates into four main clades (clade B, C, E and F) (Fig. 3). These works confirm the high variability of ITSs

and 5.8S rDNA within *M. anisopliae*. These results agree with Fegan *et al.* (1993) who found that *M. anisopliae* to be extremely diverse using RAPD. This study indicated that *M. anisopliae* are highly divergent, whereas MA 017, SNEB 18, SWC 03 and TISTR 3158 are closely related to isolates from other world regions than isolates from Thailand. Clade D and E contained isolates from elsewhere in the world, while the clade B, C and F contained only isolates from Thailand. However, Bootstrap resampling that used to estimate reliability of the tree shows less than 50% frequency. Despite their origin, the isolates collected from soil do not correlate together.

The most important taxonomic reviews of *Metarhizium* are the revisions by Tulloch (1976) and Rombach *et al.* (1986) that contains three species, *M. anisopliae*, *M. flavoviride* and *M. album*. *M. anisopliae* that was separated into two varieties; *M. anisopliae* var. *anisopliae* the short spored and *M. anisopliae* var. *major* the long spored (Tulloch, 1976). Great diversity in *M. anisopliae* supports those of other workers using both biochemical and molecular markers (Fegan *et al.*, 1993; Mavridou and Typas, 1998; Diver *et al.*, 2000). Using biochemical profiles, Rombach *et al.* (1987) could distinguish groups of var. *anisopliae* more divergent than var. *majus*. Using allozyme profiles, St. Leger and coworker (1992) propose that *M. anisopliae* may be composed of at least five varieties. Howevery, *M. anisopliae* was separated into 4 clades by RAPD-PCR method and sequence data from the ITS, 5.8S rDNA and 28S rDNA D3 regions: *M. anisopliae* var. *anisopliae*, var. *major*, var. *acridum* and var. *lepidiotum* (Diver *et al.*, 2000).

Among these groups in this study, most of the isolates in clade B perfectly homogeneous group were highly differed from other *M. anisopliae*. Those differ from the others by their biological origin and colony pattern. The most isolates in clade B included of both of group 1 and 2 according to morphological characterization and showed medium conidia ratio which isolated from insect hosts in order Coleoptera except MA 011 that host is order Isoptera (Table 1). Our result indicates that this group may correlate with insect host.

The four isolates in clade C was characterized only in group 3 according to morphological characterization. TISTR 3607 isolate was more closely related to MA 001 than the other, and clustered to SNB 02 and SED 07 that showed low conidia ratio.

The phylogenetic tree, clade D and E do not show a clear relationship between *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus* (*major*). *M. anisopliae* var. *majus* fell within range of genetic diversity of *M. anisopliae* var. *anisopliae* indicated that genetic relationship does not differences. *M. anisopliae* was divided into two varieties based on conidia size, short conidia (*M. anisopliae* var. *anisopliae*) and long conidia (*M. anisopliae* var.

majus). In general the conidia of *M. anisopliae* var. *majus* are rarely less than 10 µm long and are usually 12-13 µm (Rombach *et al*., 1986; Boucias and Pendland, 1998; Bischoff *et al*., 2009). Nevertheless, DNA-based studies were placed *M. majus* separate group from *M. anisopliae* (Yoon *et al*., 1999; Driver *et al*., 2000). The delimitation of *M. majus* only conidia size without molecular data is difficult. However, ITS regions data can be divided the MA 017 isolate into clade E that more closely related to *M. anisopliae* var. *majus* (AY781690) in clade E than *M. anisopliae* var. *anisopliae*. So, that problem warrants further investigation. Additionally, clade E were obtained the samples from various regions. TISTR 3158 isolate originating in Philippines was distinguished from isolates from Thailand but closely related to isolate from the other worldwide. There was not clear by associaed between the genetic and geographical distance separating the isolates.

Clade F, most isolates from soil were subdivided into two well-supported groups. The isolates in this clade were high conidia ratio as morphology in group 5. This clade was more closely related to *M. anisopliae* var. *anisopliae* in clade E than other clade which were remaining samples of *M. anisopliae* var. *anisopliae*. Pantou *et al*., 2003 indicated that IGS region using primers Ma-IGSspF and Ma-IGSspR could be identified. These primers amplified only a partial sequence of the IGS region in *M. anisopliae* var. *anisopliae*, but which did not amplify in other species or varieties of *Metarhizium* spp. (Hughes *et al*., 2004). From those data indicating that *M. anisopliae* may compose of at least five varieties (clade B-F). Clade B, C and F from Thailand were phylogenetically distantly related when compared to *M. anisopliae* from another country. Nevertheless, based on ITS regions, data did not provide sufficient resolution to clarify the relationships within isolate or variety.

Many researchers were interested in this fungus and many taxonomic studies were made because of their importance and potential in the biological control of pests. As the use of *M. anisopliae* as a biological control increases, a more adequate and accurate identification and separation of variety of *M. anisopliae* or genus *Metarhizium* become important to confirm species and to specify this fungus to colonize a specific insect host. Our data were revealed fundamental similarities between morphological and molecular groupings by morphological feature groups that correlated strongly with ITS and 5.8SrDNA sequence identity groups. The occurrence of *M. anisopliae* in Thailand is indicated that this fungus is an ubiquitous organism with a worldwide distribution and most of them more distantly related to *M. anisopliae* from other countries. For any line of *M. anisopliae* may include a variety of potential host insect species, sporulation, secondary metabolite as well as origin or source of collection. However**,** genetic materials may express distinguishing between the

isolates even similar in morphology. In contrast, morphological characteristic are generally complex and many involve genome expression. It is suggested that morphology and molecular phylogeny would be studied together to confirm identification of this fungus and their biological properties.

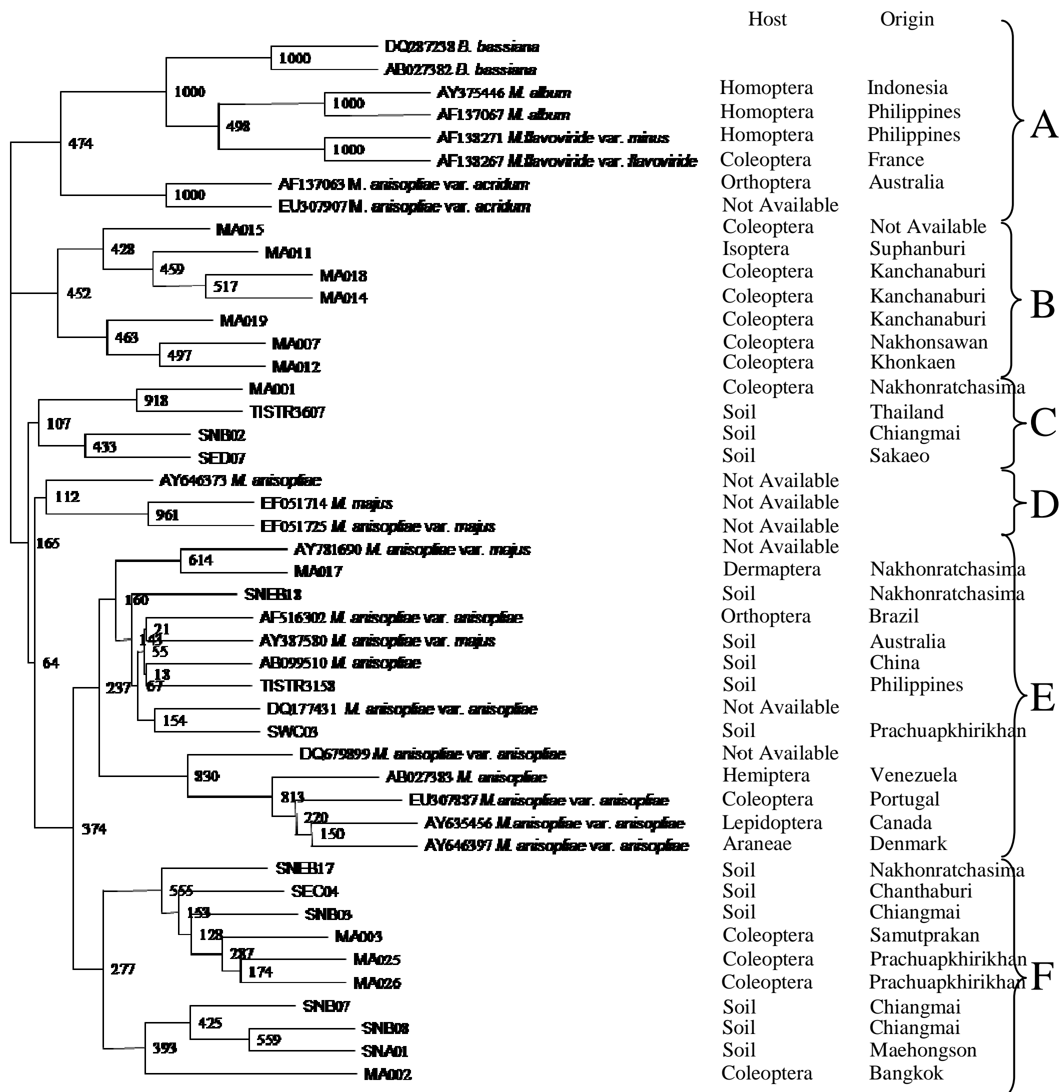

Fig. 3. Phylogenetic analysis on ITS regions and 5.8S of rDNA, 25 isolates of *Metarhizium* comparative with other isolates from GenBank using Neighbour-joining algorithm. Number on the branches represent 1,000 replicates of Bootstrap values.

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