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*Research article*

# Occurrence of Entomopathogenic Fungi from Natural Ecosystems in Phetchabun, Thailand and Their Virulence Against Brown Planthopper

**Araya Bunsak[1,2], Wipa Homhaul[1,3], Weerathep Pongprasert[1,3], Krissana Ruangrit[4] and Suphannika Intanon[1,3]***

[1]*Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand*
[2]*Agricultural Science Program, Faculty of Food and Agricultural Technology, Pibulsongkram Rajabhat University, Phitsanulok, Thailand*
[3]*National Biological Control Research Center, Lower Northern Regional Center, Naresuan University, Phitsanulok, Thailand*
[4]*Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Phetchaburi Information Technology Campus, Phetchaburi, Thailand*

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## Abstract

**Keywords**

biological control; brown planthopper; *Metarhizium* spp.; phylogenetic analysis; rice

Brown planthopper (BPH), *Nilaparvata lugens* Stål, is a serious insect pest of rice. The invasion of BPH causes heavy losses in rice production, both quality and quantity. The objective of this research was to search for *Metarhizium* spp., a genus of entomopathogenic fungi in the Clavicipitaceae family, from forest soil in three districts of Phetchabun Province, Thailand: Khao Kho, Lom Kao and Nam Nao to control BPH. *Metarhizium* spp. fungi were isolated by soil dilution plate technique and determined their efficacy using conidia suspension at the concentration of $1X10^8$ conidia/mL on BPH at the 2-3$^{th}$ instar nymph fed on seedlings of susceptible rice cultivars, Taichung Native 1 (TN1). The most effective *Metarhizium* sp. isolate was identified using the ITS region of 18S rDNA sequencing, Basic Local Alignment Search Tool (BLAST) and evolutionary history. The result showed that 126 isolates of *Metarhizium* spp. were found and coded as PB-01 to PB-126. All isolates infected BPH nymph with 26.7 to 100% mortality within 6 days after contacting conidia suspension. The isolate of PB-75 showed the highest efficacy (100% BPH mortality) with the lethal times of 50% mortality ($LT_{50}$) within 2.9 days. The species identification showed that the DNA sequence of *Metarhizium* sp. isolate, PB-75, was 98.6% similar to *M. anisopliae* Genbank ID JQ889704.1. Evolutionary history based on phylogenetic analysis using neighbor-joining and maximum parsimony methods confirmed that PB-75 formed the same phylogenetic clade with *M. anisopliae* Genbank ID JQ889704.1 and *M. anisopliae* var. *anisopliae*.

*Corresponding author: E-mail: suphannikai@nu.ac.th

## 1. Introduction

Brown planthopper (BPH) is a major insect pest of rice in Thailand and other Asian countries [1-4]. Both nymphs and adults feed on the sap from the leaf sheath of rice just at the water level of the paddy field [5, 6]. This results in the complete drying in the vast area called "hopper burn" symptoms [7]. Moreover, BPH can transmit several viral diseases causing rice ragged stunt and grassy stunt [8-10]. BPH and viruses are direct and indirect invaders causing severe damage to the rice crop, resulting in a loss of rice yield of 50% or more [11-13]. The control of BPH is commonly achieved through the application of synthetic chemical insecticides because of their high efficiency (obvious and fast control) with less labor, cost and time [14]. However, after pesticide usage for the past 40 years, several adverse effects on humans, the ecology and the environment have been identified [15], including insect resistance [16-18], insect resurgence, chemical residue effects, harmful effects on humans, and animals and natural enemies. Thus, alternative control methods have been considered and developed continuously to solve these problems [19, 20].

A biological control using entomopathogenic fungi (EPF) has infected and killed particularly harmful insects [21]. Such EPF should have a high ability to control BPH without any adverse effects on humans or the environment, and is also suitable as a replacement for harmful insecticides [22-25]. EPF can control BPH because the high moisture condition of paddy fields is appropriate to the EPF activities including fungal conidia germination and penetration [23, 19, 26]. The accumulation of EPF by frequent application improves their continuing efficacy via the higher concentration of fungal conidia in soil and wider dispersion over the paddy field by insects, water, or wind [27]. In addition, BPH does not easily become resistant to EPF [24]. Currently, many species of EPF are commercially available as products which are ready to use for controlling many insect pests including BPH [28, 26].

*Metarhizium* spp. is a member of the genus of entomopathogenic fungi in the Clavicipitaceae Family, which is a large family of EPF that has been known to infect more than 200 insect species covering many Orders such as Coleoptera, Homoptera, Hemiptera, Lepidoptera, Orthoptera and Isoptera [28]. Research on *Metarhizium* spp. has been developed continuously to identify the promising isolates that are suitable to control many insect pests [29]. Although *Metarhizium* spp. is highly effective to control BPH and is commonly used in the paddy field, its efficiency and efficacy can be suppressed and reduced after applications by various environmental stress conditions, and in particular, temperature [30], humidity [31], UV radiation [32], and soil chemicals [33]. Since large genetic variations of *Metarhizium* spp. are found among varieties and strains or types or isolates, the screening of *Metarhizium* spp. based on pathogenicity and virulence against the insect hosts, and the tolerance potential to the environmental influences, have frequently been performed [34]. The origin or source of *Metarhizium* isolates also plays an important role in the effectiveness and specificity to infect and kill insect hosts [35], which means that the search for the new significant *Metarhizium* spp. isolate can be carried out in various conditions of both disturbed and undisturbed areas such as crop fields, living areas, ocean shores, riverbanks, deserts, and forests. The processes of collecting, screening, identifying, and formulating *Metarhizium* spp. samples based on microbiology, chemistry, and toxicity methods can be applied to select effective *Metarhizium* spp. strains for the control of BPH.

The objectives of this research were to screen and identify *Metarhizium* spp. isolates which are specific to the control of BPH hosts from undisturbed rainforest areas in Phetchabun, Thailand, and to select promising *Metarhizium* spp. isolates that are conducive to their effective implementation in a BPH management program.

## 2. Materials and Methods

### 2.1 Description of soil sample collection sites

Soil samples were collected from 15 locations, mainly in the forest areas around Phetchabun Province in Thailand, covering areas: 1) Khao Kho District (16°35'42"N 100°56'04"E), 2) Lom Kao District (16°54'20"N 101°05'19"E), and 3) Nam Nao District (16°43'48"N 101°33'39"E) (Figure 1). These areas have an annual average temperature of 22°C and precipitation at 1.425 mm/year. At each sampling site, 5 picking points were randomly assigned at each of the 15 locations, 2 m apart, and a soil sample of approximately 100 g was collected from the top 5 cm of the soil. The soil samples from each of the 5 picking sites at a location were mixed thoroughly in plastic bags, containing 500 g of sample soil representative of the whole sampling site, and the sampling date and location were recorded. The 500 g sample bags were kept in styrofoam boxes at approximately 5°C during transport to the laboratory [36]. At the laboratory, the soil samples were mechanically crushed and passed through a 149 µm sieve and stored at 4°C for further processing.

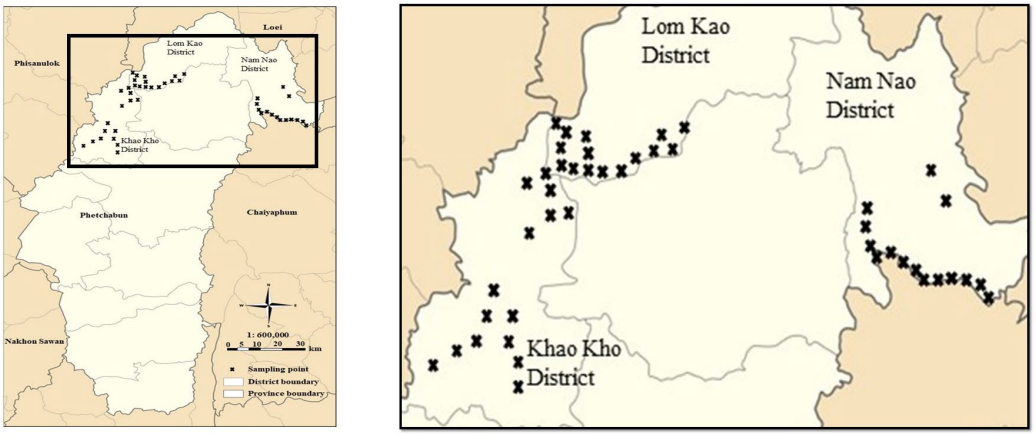


**Figure 1.** Sampling locations of the soil collected from forest areas in Phetchabun in 2020

### 2.2 Isolation of *Metarhizium* spp.

A 10 g soil sample was added to a Schott tube (250 mL) containing 90 mL of sterile distilled water, mixed for 30 min using a vortex to obtain homogenous suspension and left for 1 h at 28°C to precipitate the soil particles. The supernatant of each soil suspension was then diluted serially from $10^{-1}$ to $10^{-5}$. Each dilution of 0.1 mL was spread on a plate (3 replications) of a CTC selective medium [37] containing 0.5 g/L chloramphenicol (Sigma Aldrich, St. Louis, MO, USA), 0.001 g/L thiabendazole (Sigma Aldrich, St. Louis, MO, USA), 0.25 g/L cycloheximide (Sigma Aldrich, St. Louis, MO, USA) and incubated at a temperature of 28±1ºC for 1 week [38]. The colonies of *Metarhizium* spp. were preliminarily identified based on the morphology of the colony and then confirmed, under light microscopy (40X), by the shape and structure of the conidial chains, hyphae, and conidia [39, 40]. All growing single colonies were picked up, coded and transferred to PDA, incubated at 28°C for 2 weeks, and subcultured as necessary to purify and increase the number of conidia for further processing.

## 2.3 Brown planthopper preparation

Brown planthopper populations were obtained from the Phitsanulok Rice Research Center, and continuously reared and maintained on five pots of 60-day-old rice plants, Tai Chung Native 1 (TN1). The rice plants were then placed in a 0.6 x 0.6 x 1.0 m insect cases (width x length x height) in the insectary greenhouse at the National Biological Research Center at Naresuan University, Phitsanulok, located in the lower central north of Thailand, under 30±2°C with 70% relative humidity and a 12-h photoperiod. The BPH were reared continuously in a system to maintain the amount for use in further experiments.

## 2.4 Pathogenicity screening

Pathogenicity screening was a completely randomized design (CRD) with three replications. A total of 20 BPH nymphs at the $2^{nd}$-$3^{rd}$ instar were fed on the TN1 rice planted in pots. The insect rearing cases (0.3 x 0.3 x 1.0 m size) were used as the experimental units. Each isolate of *Metarhizium* spp., at 3 mL of a conidial suspension with the concentrations of $1x10^8$ conidia/mL [41], was applied with an applicator on rice leaves, onto which the BPH nymphs were released. Distilled water was used as a control. The mortality and morphological changes of the BPH nymphs were observed and recorded daily for 7 days. The mortality of BPH as a result of fungal infection was considered based on the visible mycelium or conidia of *Metarhizium* spp. growing on the BPHs' body surfaces. The mortality data were corrected according to Abbott's formula [42], analyzed by analysis of variance (ANOVA) followed by Duncan's new multiple range test (DMRT). Then, the median lethal time ($LT_{50}$) was calculated by probit analysis [43]. The effective fungal isolates were screened and selected based on the highest percentage of mortality and shortest $LT_{50}$ values. Polymerase Chain reaction (PCR) and the sequence of ITS region of 18S rDNA were assigned to confirm the identity of the selected species.

## 2.5 Molecular identification of promising *Metarhizium* spp. isolates

The most virulent *Metarhizium* sp. isolate with the lowest $LT_{50}$ was selected for this study. The *Metarhizium* isolate was grown in PDA at 25°C for one week and sent to Microgen Asia company (Incheon, Korea) for sequencing on ITS of 18S rDNA using the primers ITS1 (forward: TCCGTAGGTGAACCTTGCGG) and ITS4 (reverse: TCCTCCGCTTATTGATATGC) [44]. The sequence quality was determined using BIOEDIT v. 7.2.5 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) [45]. The ITS of 18S rDNA sequences of selected *Metarhizium* spp. were compared with the sequences placed in the Gen Bank Database (National Center for Biotechnology Information, NCBI) using the BLAST (Basic Local Alignment Search Tool, NCBI), and aligned by CLASTALX [46]. The close relatives with high similarity values were retrieved from the Gen Bank Database and aligned, trimmed, and edited with the obtained sequence using BioEdit v.7.0.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) [45]. The phylogenetic analysis of the edited ITS of 18S rDNA sequences was performed based on the neighbor-joining and maximum parsimony approaches. A majority consensus tree that relied on 1,000 bootstrap replicates was reconstructed using MEGA 11 (http://www.megasoftware.net/) [47, 48].

## 3. Results and Discussion

### 3.1 Survey and collection from soils

Generally, the *Metarhizium* spp. behaves as two phases of living: parasitic and saprophytic stages [49] and can be found abundantly in soil from various geographical locations [50, 51]. However, differences in their prevalence and distribution are found among cultivated farmland soil, grazing land, and forest soil [52-54]. In this study, *Metarhizium* spp. were isolated from soil samples collected from 45 collecting sites in undisturbed rainforest areas in Phetchabun Province, Thailand. Based on the determination of the morphological characteristics of colony and conidia, a total of 126 isolates (PB01-126) were identified (Figure 2). All the obtained isolates had a round shape and greenish color, with a white color at the edge of the colony, and produced ellipsoid conidia with 5-7 µm in size that confirmed the classification as *Metarhizium*. Although the variation of color and size of *Metarhizium* conidia among isolates or strains was reported [55], they were not recognized in this study.

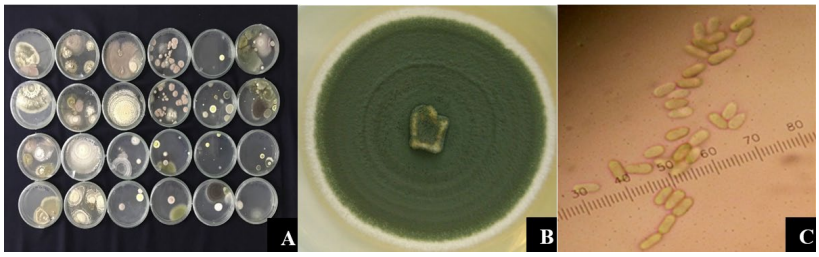


**Figure 2.** Colonies of *Metarhizium* spp. and other fungi growing on the selective CTC media plates (A), morphology of *Metarhizium* colony (B), and *Metarhizium* conidia (C)

### 3.2 Pathogenicity screening

The *Metarhizium* spp. in this study were isolated directly from the soil samples using CTC selective media [37] because this method is convenient, easy, cheap, and fast. The isolation on CTC media involves a simple process and equipment, and tends to recover many more fungal colonies. However, the differences in pathogenicity and virulence that can occur among the colonies of the various *Metarhizium* spp. that were obtained, make it essential to evaluate and screen for the best one [20, 56]. Generally, the entire infection process of *Metarhizium* spp. is relatively long and takes approximately 7-14 days after infection. The process involves many stages in the following order: dissemination, adhesion, germination, appressorium formation, penetration, invasion, colonization (in the hemocoel, tissues, and organs), extrusion, and sporulation. However, first symptoms of infection usually occur around 7 days or earlier depending on the species or isolates of *Metarhizium* spp., insect host and environmental conditions [57]. In case of a small insect as the brown planthopper, the entire process takes shortly around 5-6 days and the symptoms of infection probably appear at the first day after inoculation [58, 59]. Therefore, the promising *Metarhizium* spp. species or isolates can be determined and screened using the maximum mortality rate at the shortest time of death along with the median lethal time value ($LT_{50}$).

The results of the pathogenicity screening showed that 30 out of 126 *Metarhizium* spp. isolates were able to infect BPH at a mortality higher than 50% and only 16 isolates, or about 0.13% of the isolates, were highly virulent with a mortality rate of 100% at 6 days after contacting the fungal conidia (Table 1). The period of infection and insecticidal activity of those *Metarhizium* isolates was in the same range as Tang *et al*. [58] and Bunsak *et al*. [59].

**Table 1.** Location of soil collection, codes, and number of isolates of *Metarhizium* spp. retrieved from soil samples in Phetchabun Province and the effect of the *Metarhizium* spp. isolates on the BPH larvae at 6 days after inoculation

| Locations (Districts) | *Metarhizium* spp. Isolates* | No. effective isolates (isolates) | Mortality ranges (%) | No. isolates with mortality rate >50% (isolates) | Isolate code with mortality rate >50% (%) |
|---|---|---|---|---|---|
| Khao Kor | PB-01-08 | 8 | (86.7-100.0%) | 8 | PB-01 (100.0%)<br>PB-02 (100.0%)<br>PB-03 (96.7%)<br>PB-04 (90.0%)<br>PB-05 (96.7%)<br>PB-06 (90.0%)<br>PB-07 (86.7%)<br>PB-08 (93.3%) |
| | PB-09-10 | 2 | (83.3-100.0%) | 2 | PB-09 (83.3%)<br>PB-10 (100.0%) |
| | PB-11-23 | 13 | (33.3-100.0%) | 2 | PB-16 (96.7%)<br>PB-19 (100.0%) |
| | PB-24-25 | 2 | (46.6-50.0%) | - | - |
| | PB-26-30 | 5 | (50.0-100.0%) | 1 | PB-28 (100.0%) |
| | PB-31-32 | 2 | (33.3-43.3%) | - | - |
| | PB-33-37 | 5 | (40.0-96.7%) | 2 | PB-33 (93.3%)<br>PB-34 (96.7%) |
| | PB-38-41 | 4 | (43.3-50.0%) | - | - |
| | PB-42-43 | 2 | (46.7%) | - | - |
| | PB-44 | 1 | (26.7%) | - | - |
| | PB-45-48 | 4 | (43.3-100.0%) | 1 | PB-47 (100.0%) |
| | PB-49-50 | 2 | (33.3-43.3%) | - | - |
| | PB-51 | 1 | (100.0%) | 1 | PB-51 (100.0%) |
| | PB-52 | 1 | (46.7%) | - | - |
| Lom Kao | PB-53-55 | 3 | (43.3-50.0%) | - | - |
| | PB-56 | 1 | (43.3%) | - | - |
| | PB-57-59 | 3 | (33.3-46.7%) | - | - |
| | PB-60-67 | 8 | (50.0%) | - | - |
| | PB-68-71 | 4 | (43.3-100.0%) | 1 | PB-71 (100.0%) |
| | PB-72 | 1 | (50.0%) | - | - |
| | PB-73-81 | 9 | (36.7-100.0%) | 2 | PB-75 (100.0%)<br>PB-76 (100.0%) |
| | PB-82-85 | 4 | (40.0-46.7%) | - | - |
| | PB-86-88 | 3 | (50.0%) | - | - |
| | PB-89-91 | 3 | (40.0-96.7%) | 1 | PB-89 (96.7%) |

**Table 1.** Location of soil collection, codes and number of isolates of *Metarhizium* spp. retrieved from soil samples in Phetchabun Province and the effect of the *Metarhizium* spp. isolates on the BPH larvae at 6 days after inoculation (continued)

| Locations (Districts) | *Metarhizium* spp. Isolates* | No. effective isolates (isolates) | Mortality ranges (%) | No. isolates with mortality rate >50% (isolates) | Isolate code with mortality rate >50% (%) |
|---|---|---|---|---|---|
| | PB-92-93 | 2 | (36.7-50.0%) | - | - |
| | PB-94-95 | 2 | (83.3-100.0%) | 1 | PB-95 (100.0%) |
| | PB-96 | 1 | (43.3%) | - | - |
| | PB-97 | 1 | (46.7%) | - | - |
| | PB-98-100 | 3 | (43.3%) | - | - |
| Nam Nao | PB-101 | 1 | (100.0%) | 1 | PB-101 (100.0%) |
| | PB-102-105 | 4 | (33.3-93.3%) | 1 | PB-103 (93.3%) |
| | PB-106-109 | 4 | (40.0-46.7%) | - | - |
| | PB-110 | 1 | (43.3%) | - | - |
| | PB-111 | 1 | (33.3%) | - | - |
| | PB-112 | 1 | (43.3%) | - | - |
| | PB-113 | 1 | (50.0%) | - | - |
| | PB-114-116 | 3 | (46.7-100.0%) | 1 | PB-114 (100.0%) |
| | PB-117 | 1 | (100.0%) | 1 | PB-117 (100.0%) |
| | PB-118-120 | 3 | (40.0-96.7%) | 1 | PB-118 (96.7%) |
| | PB-121 | 1 | (90.0%) | 1 | PB-121 (90.0%) |
| | PB-122-124 | 3 | (46.7-100.0%) | 1 | PB-123 (100.0%) |
| | PB-125-126 | 2 | (46.7-100.0%) | 1 | PB-125 (100.0%) |

*Range of isolate number based on the presence of *Metarhizium* spp. in each sampling location

The percentage of mortality of the 16 isolates with the mortality higher than 50% from 1-6 days are shown in Table 2. On day 1 of contact with the fungal conidia, the mortality rates of BPH ranged from 0.0-16.7% and this increased continuously as a sigmoid curve pattern to reach 100% at day 6 after contacting the fungal conidia. The degree of virulence ($LT_{50}$) of those ranged from 2.9 to 4.2 days after contacting the fungal conidia. This $LT_{50}$ value is lower than that reported in many other reports of the effect of *Metarhizium* spp. on BPH. Tang *et al*. [58] reported an $LT_{50}$ of *M. anisopliae* CQMa421 at the rate of $1x10^8$ of approximately 4.5 days. Similarly, Mohan *et al*. [60] showed an $LT_{50}$ of the *Metarhizium* (M1) strain as 4.4 days with the spore concentration of $1 X 10^8$ and a mortality percentage of 76.67% against *N. lugens* under *in vitro* conditions. Samuels *et al*. [61] found that the $LT_{50}$ value of 24 wild-type isolates of *M. anisopliae* on *N. lugens* ranged from 5 to more than 14 days, where 5 days were considered to be highly pathogenic and 14 or more days were nonpathogenic. However, the lowest $LT_{50}$ value (2.9 days) of *Metarhizium* spp. in our study was PB-75 which was isolated from the forest soil sample at Lom Koa District, and this isolate was selected for species confirmation.

**Table 2.** Percentage of mortality with the number of observed days and median lethal time ($LT_{50}$) of BPH infected by *Metarhizium* spp. isolates

| Isolates | % Mortality (days)* | | | | | | $LT_{50}$ (Days) |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| PB-01 | $6.7^{ab}$ | $16.7^{bc}$ | $23.3^{efg}$ | $40.0^{ef}$ | $86.7^{abcd}$ | $100.0^{ns}$ | 3.7 |
| PB-02 | $16.7^{a}$ | $30.0^{a}$ | $43.3^{abc}$ | $60.0^{ab}$ | $90.0^{abc}$ | 100.0 | 3.1 |
| PB-10 | $6.7^{ab}$ | $16.7^{bc}$ | $30.0^{def}$ | $43.3^{def}$ | $76.7^{abcde}$ | 100.0 | 3.8 |
| PB-19 | $6.7^{ab}$ | $16.7^{bc}$ | $33.3^{cde}$ | $46.7^{cde}$ | $73.3^{bcde}$ | 100.0 | 3.7 |
| PB-28 | $16.7^{a}$ | $23.3^{abc}$ | $36.7^{bcd}$ | $53.3^{bcd}$ | $93.3^{ab}$ | 100.0 | 3.2 |
| PB-47 | $6.7^{ab}$ | $20.0^{abc}$ | $30.0^{def}$ | $43.3^{def}$ | $80.0^{abcd}$ | 100.0 | 3.7 |
| PB-51 | $10.0^{ab}$ | $16.7^{bc}$ | $20.0^{fg}$ | $36.7^{ef}$ | $66.7^{de}$ | 100.0 | 4.0 |
| PB-71 | $16.7^{a}$ | $23.3^{abc}$ | $46.7^{ab}$ | $60.0^{ab}$ | $86.7^{abcd}$ | 100.0 | 3.2 |
| PB-75 | $15.0^{a}$ | $29.0^{ab}$ | $51.0^{a}$ | $68.0^{a}$ | $96.0^{a}$ | 100.0 | 2.9 |
| PB-76 | $16.7^{a}$ | $26.7^{ab}$ | $30.0^{def}$ | $46.7^{cde}$ | $80.0^{abcd}$ | 100.0 | 3.5 |
| PB-95 | $0.0^{b}$ | $3.3^{d}$ | $23.3^{efg}$ | $53.3^{bcd}$ | $80.0^{abcd}$ | 100.0 | 3.9 |
| PB-101 | $13.3^{a}$ | $20.0^{abc}$ | $26.7^{defg}$ | $43.3^{def}$ | $80.0^{abcd}$ | 100.0 | 3.6 |
| PB-114 | $6.7^{ab}$ | $16.7^{bc}$ | $26.7^{defg}$ | $36.7^{ef}$ | $70.0^{cde}$ | 100.0 | 3.9 |
| PB-117 | $16.7^{a}$ | $16.7^{bc}$ | $30.0^{def}$ | $56.7^{abc}$ | $93.3^{ab}$ | 100.0 | 3.3 |
| PB-123 | $16.7^{a}$ | $26.7^{ab}$ | $36.7^{bcd}$ | $43.3^{def}$ | $83.3^{abcd}$ | 100.0 | 3.4 |
| PB-125 | $6.7^{ab}$ | $13.3^{cd}$ | $16.7^{g}$ | $33.3^{f}$ | $56.7^{e}$ | 100.0 | 4.2 |

*Mean values followed by different lowercase superscript letters in the same column are significantly different at the 5% level by Duncan's multiple range test, ns = not significant.

### 3.3 Similarity of ITS region of 18S rDNA of *Metarhizium* PB-75 compared to data of those in BLAST and phylogenetic analysis

The sequence of ITS region of 18S rDNA of *Metarhizium* PB-75 was 1,218 base pairs in length, with a molecular weight at 367,667.00 for single-stranded and 739,779.00 Daltons for double-stranded, with slightly high AT content (53.28%). Ribosomal DNA sequences are commonly used to identify and determine the phylogenetic relationships of organisms to taxa species [62]. The organisms which have a similarity value of 100% can be stated as the same strain and a similarity value of 99% is stated as the same species, while the similarity value of 89-99% belongs to the same genus [63]. In this study, the ITS-1 and ITS-4 regions along with the 5.8S rRNA gene [44] of promising *Metarhizium* sp. isolate, PB-75, was sequenced and submitted to BLAST search for identification of closely related species. The result revealed that the sequence of *Metarhizium* PB-75 showed 99.63% similarity to the *M. anisopliae* Genbank ID JQ889704.1, FJ589649.1, FJ609311.1, MT114694.1, EU307893.1 AB099510.1, AY646382.1, and KM215662.1. It was therefore confirmed that this isolate was classified as *M. anisopliae*.

The total of the ITS region of 18S rDNA from 8 closely related *M. anosopliae* were selected including *M. minus* (HM055453.1) and *M. frigidum* (HM055448.1). Assigned out-groups were *Nomuraea rileyi* (AB100361.1) and *Dothidea sambuci* (AY930108.1). Those 13 ITS of 18S rDNA sequences were aligned, edited, and trimmed based on CLUSTAL W and the final data set was composed of 577 nucleotide base positions. Phylogenetic analysis was performed in MEGA11 using the Neighbor-Joining method [64]. The evolutionary distances were computed using the maximum

composite likelihood method [65] and the percentage of replicate trees which associated to clustered taxa using bootstrap method with 1000 replicates [66].

The obtained dendrogram clustered all sequences into 2 main clades: *Metarhizium* spp. clade and outgroup clade. The outgroup of compared fungal species (*N. rileyi* and *D. sambuci*) was arranged separately and connected to the whole *Metarhizium* group with 78% replicates supporting the bootstrap values. In *Metarhizium* spp. clade, all *M. anisopliae* were grouped together and other species of *Metarhizium* spp. (*M. minus* and *M. frigidum*) were arranged into another clade (Figure 3). The members of *M. anisopliae* clade were closely related with very few differences among aligned nucleotide bases. However, this group was separated into 2 subclades, with the subclade I being composed of 3 compared *M. anisopliae* sequences (AB099510.1, AY646382.1, and KM215662.1). The subclade II was composed of 5 *M. anisopliae* sequences (EU307893.1, FJ589649.1, MT114694.1, FJ609311.1, and JQ889704.1) and PB-75. The PB-75 sequence was grouped tightly to *M. anisopliae* JQ889704.1 as a separate small clade in the *M. anisopliae* subclade II.

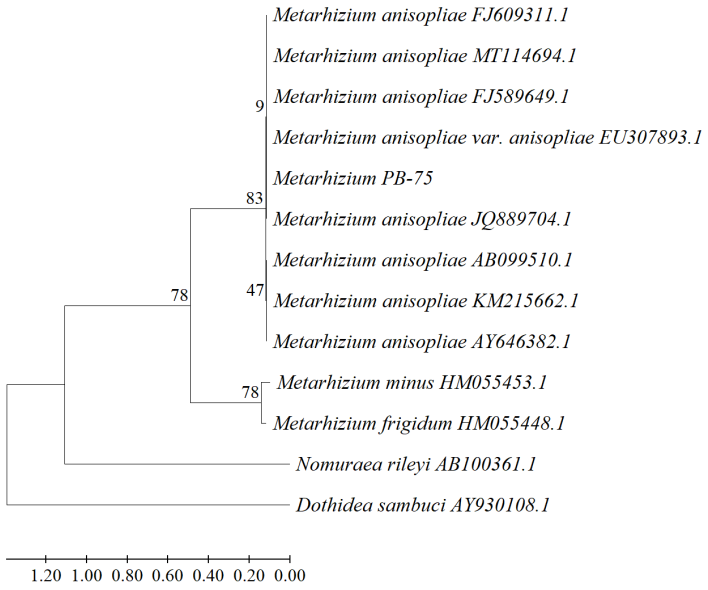


**Figure 3.** Phylogenetic tree (evolutionary relationships of taxa) obtained by the neighbor joining statistical method with evolutionary distance calculated by maximum composite likelihood based on the alignment of ITS region of 18S rDNA of *Metarhizium* spp. isolate PB-75 with 8 closely relatives including *M. frigidum* (HM055448.1) and *M. minus* (HM055453.1). Assigned out-groups were *Nomuraea rileyi* (AB100361.1) and *Dothidea sambuci* (AY930108.1). The confidence supporting number on tree branch calculated from bootstraps method with 1,000 replications. The scale bar corresponds to 0.01 substitutions per nucleotide position.

When the evolutionary history of those ITS regions of *Metarhizium* spp. was traced using the Maximum Parsimony method (MP), the trees were obtained using the subtree-pruning regrafting algorithm [67] and the consensus tree was inferred from the bootstrap method at 1,000 replications. The *Metarhizium* spp. members and out-group of the obtained consensus tree were slightly arranged in the same way as the neighbour-joining tree, discussed above. The out-group, *M. anisopliae* and its relatives were formed as an independent clade with 100% bootstrap support. The PB-75 sequence was grouped tightly to *M. anisopliae* JQ889704.1 and together with *M. anisopliae* var *anisopliae* as a separate small clade in the main *M. anisopliae* clade (Figure 4).

From the evolutionary history, derived by the two methods of analysis, *Metharhizium* spp. isolate PB-75 belonged to *M. anisopliae* and was tightly related to *M. anisopliae* JQ889704.1 along with *M. anisopliae* var. *anisopliae*. *Metharhizium anisopliae* (JQ889704.1) was recovered from the forest soil in Hainan, China; however, the details of its entomopathogenic property have not been available [68], whereas many studies of *M. anisopliae* var. *anisopliae* regarding the killing effect on insects have been reported. *Metharhizium anisopliae* var. *anisopliae* does not only infect many species of insect pests but also produces a family of cyclic peptide toxins, destruxins (DTX), both in the culture and in mycosed insects (*in vivo*). The contribution of these insecticidal toxins to the disease process has been investigated in the tobacco hornworm, *Manduca sexta* (Lepidoptera), the desert locust, *Schistocerca gregaria* (Orthoptera), and the vine weevil, *Otiorhynchus sulcatus* (Coleoptera) [69]. However, the high level of polymorphy of *M. anisopliae* var. *anisopliae* has been detected [70], indicating that the *M. anisopliae* var. *anisopliae* ITS sequences from strains E9, B/Vi and C were phylogenetically identical to each other and also to the *M. anisopliae* var. *anisopliae* sequences, AF516295 and AF134150, from GenBank. Meanwhile *M. anisopliae* var. *anisopliae* strain 14 from Australia formed a separate group.

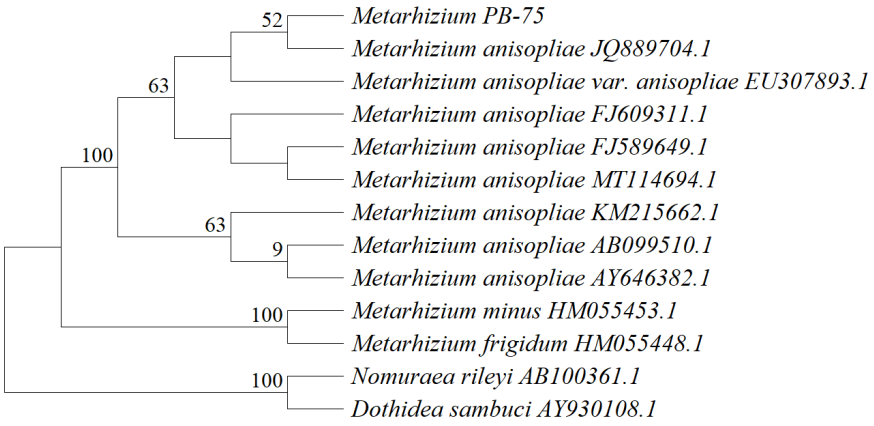


**Figure 4.** Phylogenetic tree (Evolutionary relationships of taxa) obtained by Maximum Parsimony method using the Subtree-Pruning-Regrafting (SPR) algorithm based on the alignment of ITS region of 18S rDNA of *Metharhizium* spp. isolate PB-75 with 8 closely relatives including *M. frigidum* (HM055448.1) and *M. minus* (HM055453.1). Assigned out-groups were *Nomuraea rileyi* (AB100361.1) and *Dothidea sambuci* (AY930108.1). The confidence supporting number on tree branch calculated from bootstraps method with 1,000 replications.

## 4. Conclusions

Entomopathogenic fungi occur in the natural ecosystems in Phetchabun Province, Khao Kor, Lom Kao and Num Now Districts. Their virulence towards BPH was tested to find promising new isolates of *Metarhizium* spp. For the control of BPH, forty-five soil samples were taken from 15 undisturbed rainforest sites in Phetchabun Province, Thailand. The 126 isolates of *Metarhizium* spp. (PB01-126) were identified based on the morphological characters of the colony and conidia. Under the pathogenicity test for the first round of the screening process, only 16 isolates (about 0.13%) of the obtained isolates were highly virulent with a mortality rate of 100% at 6 days after contact with the fungus conidia. The degree of virulence using the lethal times of 50% mortality ($LT_{50}$) value was

calculated on those 16 isolates. The isolate of PB-75 had the lowest $LT_{50}$ value at 2.9 days after contacting fungal conidia. The species identification of *Metarhizium* spp. isolate, PB-75 was performed using the ITS region of 18S rDNA sequences under 2 steps of identification: BLAST search and evolutionary history data based on phylogenetic analysis using neighbour-joining and maximum parsimony methods. The result attained by the identification methods was that the DNA sequence of *Metarhizium* spp. isolate PB-75 showed 98.6% similarity to *M. anisopliae* Genbank ID JQ889704.1, thereby confirming that it formed the same phylogenetic clade with *M. anisopliae* Genbank ID JQ889704.1 and *M. anisopliae* var. *anisopliae*.

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