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Efficacy of soil-borne entomopathogenic fungi against subterranean termite, *Coptotermes curvignathus* Holmgren (Isoptera: Rhinotermitidae)

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Abstract

Background: *Coptotermes curvignathus* Holmgren (Isoptera: Rhinotermitidae) is a subterranean termite that poses serious damage to oil palm and rubber trees. Chemical pesticides could cause negative effect to human and the environment in long-term usage. The use of entomopathogenic fungi (EPF) to suppress the population of subterranean termites is in favour when compared to chemical pesticides because they do not harm to the environment and non-target organisms. The study aimed to isolate and identify the EPF from the soil using yellow mealworm larvae of *Tenebrio molitor* Linnaeus in the baiting method and assessed their efficacy against subterranean termite, *C. curvignathus*.

Result: Eleven EPF isolates were successfully isolated from the oil palm plantation in Universiti Putra Malaysia, namely: *Aspergillus auricomus* (UPM-A1C-1), *A. caelatus* (UPM-A1C-2), *Metarhizium anisopliae* var *anisopliae* (UPM-A2C-1, UPM-A3C-1, UPM-A3C-2, UPM-A5C-1 and UPM-A10C-1), *Purpureocillium lilacinum* (UPM-A2C-3 and UPM-A7C-1), *Cordyceps javanica* (UPM-A2C-5), and *M. pinghaense* (UPM-A13C-2). The identity of these EPF were confirmed by morphological and molecular characteristics. All EPF yielded 100% mortality in *C. curvignathus* in 10 days after inoculation (DAI), except UPM-A1C-1 and UPM-A1C-2 after exposure to 1×10^7 conidia ml^{-1}. UPM-A2C-5 *Cordyceps javanica* yielded the highest mycelia formation (69%) after 6 DAI. The LT_{50} values varied from 3.90 to 7.75 days. UPM A2C-1 *M. anisopliae* var *anisopliae* showed the lowest LT_{50} (3.90 days), while UPM-A1C-1 *Aspergillus auricomus* showed the highest LT_{50} (7.75 days). The lowest LC_{50} value (1.49×10^5 conidia ml^{-1}) was recorded in UPM A2C-1 *M. anisopliae* var *anisopliae*.

Conclusions: The present study confirmed the soilborne EPF with potential insecticidal activity against *C. curvignathus*. UPM-A2C-1 *M. anisopliae* var *anisopliae* was a potential biological control agent against Subterranean termite, *C. curvignathus* due to its virulence score and high percentage of mycelia formation after 6 DAI. The data reported in the present study, particularly using *P. lilacinum, M. pinghaense, Aspergillus auricomus, A. caelatus* and *C. javanica* with potential insecticidal activity against *C. curvignathus*, are new records.

Keywords: Entomopathogenic fungi, *Coptotermes curvignathus*, Biological control, Concentration dependent bioassay, Mycoses

Background

The subterranean termite, *Coptotermes curvignathus* Holmgren (Isoptera: Rhinotermitidae) was recorded in 1927 in Malaya attacking oil palm, *Elaeis guinensis* Jacq. (Deasy 2008) and it proved to be the main pest of oil

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palm, particularly in the immature palm trees. They are the most aggressive and largest subterranean termites among the genus *Coptotermes* spp. (Wong et al. 2015). They feed vigorously on the fresh tissue of oil palm tree as the main diet rather than fed on wood-based materials. This pest attacks palm trees by creating earthy coloured mound and permanent working trails from the centre part of the trees, after which the affected trees gradually dry up and die (Kon et al. 2012).

The common insecticides used in controlling insect pest could cause negative effect to human and the environment in long term usage (Aktar et al. 2009). Development of resistance to chemical pesticides has become the major concern in the oil palm industry. One of the alternatives to overcome it is the application of EPF. EPF such as *Metarhizium*, *Beauveria*, *Aschersonia*, *Lecanicillium*, *Aspergillus*, *Tolypocladium*, and *Hirsutella* are members of phylum Ascomycota and have been commercially manufactured as biopesticides (Bischoff et al. 2006). The mode of infection is the same for all EPF. Fungal conidia will bind to the cuticle of the hosts by hydrophobic binding under desirable condition and geminate into germ tubes (Inglis et al. 2000). The germ tubes penetrate the cuticle by producing metalloid proteases and amino peptidases. EPF form hyphal during parasitic stage, and live as a saprophyte by obtaining nutrients from the carcass while maintaining hypha development upon death of their hosts. They will form fungal mycelia when the insect dies (Inglis et al. 2000). Most of the EPF produced secondary metabolites known as destruxins that consist of an a-hydroxy acid and 5 amino residues which could weaken the insect immune response (de Bekker et al. 2013). Some EPF have been successfully developed for commercial purposes. *Beauveria bassiana* and *Metarhizium anisopliae* were among the EPF commercialized as biopesticide to target for a wide range of insect hosts. Till date, majority of the EPF based biopesticides (approximately 50 products in the global market) contain *Metarhizium* spp. as the active agent. In Malaysia, the trend in using biopesticide against insect pest of oil palm plantation has become popular.

Several EPF have been isolated, identified and tested pathogenic to *C. curvignathus* in Malaysia. The pathogenicity of different species of EPF may be influenced by factors such as percentage of mortality (Lo Verde et al. 2015), effective lethal time (LT_{50}) (Sileshi et al. 2013) and the ability of spore to germinate fast on target pest (Montesinos-Matías et al. 2011). The above factors can be used as the criteria for selection of good EPF candidate for target pest. The present study was carried out to evaluate the efficacy of 11 soil borne EPF against subterranean termite, *C. curvignathus* under laboratory conditions.

Methods

Research experiments were conducted at the Laboratory of Insect Pathology, Faculty of Agriculture, Universiti Putra Malaysia from 2019 to 2021.

Sampling of termites

Termites were collected from the infested oil palm trees at the oil palm plantation in Universiti Putra Malaysia, Serdang, Selangor. Termites were baited using termite trap with some modifications of the design developed by Evans and Gleeson (2006). The specimens of *C. curvignathus* were kept at room temperature at the Laboratory of Insect Pathology for 7 days for habituation before they were used in the pathogenicity study.

Sampling of soil

The soil samples were obtained from 30 different sampling sites at the oil palm plantation in Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The distance between each sampling site was more than 10 m. The sampling locations were divided into 2 groups depending on the pesticide exposure. Group 1 consisted of area with pesticide (malathion) application, while group 2 consisted of area without pesticide application. Both areas were free from biopesticide exposure. Approximately 300 g of soil was collected by scrapping 10–15 cm deep into the soil and deposited separately in clean plastic containers. Five soil samples were collected from each sampling location. Soil samples were brought back to the Laboratory of Insect Pathology at the Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. The containers containing the soil were covered tightly with lid containing small holes to allow gaseous exchange and air-dried at 27 ± 2 °C for 72 h.

Isolation of EPF

Yellow mealworms, *T. molitor* were used in baiting EPF from the soil samples. Mealworms were surface-sterilized with 0.1% (v/v) sodium hypochlorite and then heat-treated at 60 °C for 30 s prior to experiment. The mealworms may appear dead at first after the heat treatment. Three quarters of the plastic containers (4.0 cm × 7.5 cm) were filled with soil. The soil was rehydrated by spraying with distilled water. Ten larvae (alive) were placed on the surface of the soil. The plastic containers were kept at room temperature for 30 days in dark with the soil sample and the larvae were gently inverted every day during the first week of the baiting process. The dead larvae were harvested and placed on moist filter paper in a Petri dish and incubated for 5 days until EPF had fully grown on the larval body. The larvae infected with EPF were disinfected with 2% (v/v) sodium hypochlorite for 2 min, followed by rinsing 3 times with

distilled water before incubated at 27 ± 2 °C on malt extract agar (MEA). EPF grown on MEA were sub-cultured to potato dextrose agar (PDA) a few times until pure cultures were obtained.

Morphological identification of EPF

Fungal discs (3 × 3 mm) of 7 days old culture were placed in the middle of fresh PDA and incubated at 27 ± 2 °C for 14 days, following the method of Bischoff et al. (2006). The culture near to the margin was harvested by using an inoculation needle for morphological study as this area contained mycelia, hyphae, young and matured conidiophores, while deeper toward the centre colony area contained older conidiophores that were heavily sporulated (Bischoff et al. 2006). The mycelia were mounted on a microscopic slide with a drop of lactophenol cotton blue mounting medium. A cover lip was positioned over the mount by placing one edge of the cover lip to the slide and slowly covered the whole mount so that no air bubble was trapped within the mount. Morphological characteristics of fungal culture such as the texture, form, and colour on upper and lower surfaces of media were recorded. The size, length, width and length/width ratio of conidia were also calculated. The micro-morphological features of all EPF isolates were observed under compound microscope (Olympus BX 41, Olympus Corporation, Tokyo, Japan) at 100X magnification and the photomicrographs of the EPF cultures were taken using Dino-Capture 2.0 Software.

Molecular identification of EPF

One week old actively grown EPF were sub-cultured by placing 3 mm mycelia plugs into Erlenmeyer flasks containing 100 ml of potato dextrose broth added with 1% (w/v) yeast extract. The broth cultures were agitated in a refrigerated orbital thermostat shaker at 160 rpm at 27 ± 2 °C for 5–7 days until a thin layer of mycelia formed on the broth. The mycelia were harvested, dried on a Whatman 2.0 filter paper and then ground with liquid nitrogen in a 1.5 ml microcentrifuge tube to a fine powder by using a sterile polypropylene disposable pestle. Approximately 500 mg of mycelial powder was used in the DNA extraction. DNA was extracted with EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit (BIO BASIC INC) according to the manufacturer's protocol. The ITS region of rDNA was amplified using the primers ITS1 (5′-TCGGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Gardes and Bruns, 1993), the Beta-Tubulin (βTub2) region with primers Bt2a (5′-GGT-AAC-CAA-ATC-GGT-GCT-GCT-TTC-3′) and Bt2b (5′-ACC-CTC-AGT-GTA-GTG-ACC-CTT-GGC-3′) (Glass and Donaldson, 1995), and the regions of translation elongation factor 1 alpha (TEF1-α), with primers EF1-1251R (5′-CCT-CGA-ACT-CAC-CAG-TAG-CG-3′) and EF1-668F (5′-CGG-TCA-CTT-GAT-CTA-CAA-GTG-C-3′) (O'Donnell et al.1998). Amplification reaction was prepared in a final volume of 25 µl. The amplification program for ITS region was performed with initial denaturation at 95 °C for 5 min, followed by 35 cycles with denaturation at 95 °C for 1 min, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplification program for βTub2 region was performed with initial denaturation at 94 °C for 1 min, followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 5 min, and a final extension at 72 °C for 7 min. Lastly, the amplification program for TEF1-α region was performed with initial denaturation at 95 °C for 4 min, followed by 30 cycles with denaturation at 95 °C for 1 min, annealing at 57 °C for 75 s, and extension at 94 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated on a 1% (w/v) agarose gel with CSL-MDNA-1KbPLUS DNA Ladder RTU used as DNA marker. The PCR products were then sent out for sequencing services.

Sequence analysis

Upon receiving the DNA sequencing data, the noises of the sequences were removed using Biological Sequence Alignment Editor (BioEdit) version 7.2. The sequences were checked for their alignment by using Mega X software and corrected manually. The nucleotide sequences were blasted with sequences of others species deposited in the data bank at the National Center for Biotechnology and Information (NCBI) (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). Phylogenetic tree was constructed using MEGA X software (Tamura et al. 2011) based on the Maximum Likelihood-Joining tree (Saitou and Nei 1987) by 1000 bootstrap value (Felsenstein 1985). Only branches with more than 30% bootstrap value are shown.

Preparation of conidia suspension

Conidia suspension of EPF isolates was prepared by adding 15 ml of sterile 0.05% (v/v) Tween 80 into the 4 weeks old EPF cultures and gently scraping the surface of the cultures with a sterile "L" shaped inoculation loop to dislodge the conidia from the surface of the PDA plates. The conidial suspensions were filtered through a 3 layer-sterile muslin cloth into 50 ml sterile plastic tubes to remove mycelium and residue of agar. The filtrate was vortexed for 5 min. The number of conidia was counted using a Neubauer haemocytometer with the help of a compound microscope (Olympus BX 41, Olympus Corporation, Tokyo, Japan) under 40X magnification. An initial stock suspension was prepared in 1×10^9 conidia ml^{-1} and

then centrifuged at 8000 × *g* for 1 min. After washing for 5–10 times, the final pellet was then resuspended in 1 ml of double-autoclaved distilled water.

Mortality test of EPF against subterranean termite, *C. curvignathus*

A screening test was carried out on *C. curvignathus* using the conidia concentration of 1×10^7 conidia ml^{-1} containing 0.05% (v/v) Tween 80. Treatment was carried out by inoculating each individual termite with 20 µl conidia suspension (topical application) onto the dorsal abdominal segment of the termite. *C. curvignathus* were surface-sterilized with 0.01% (v/v) sodium hypochlorite and washed 3 times with double-autoclaved distilled water. The treated *C. curvignathus* were dried for 2 min. 10 termites per treatment with ten replications were accomplished. *C. curvignathus* treated with 0.05% (v/v) Tween 80 were served as negative control. All treated and untreated *C. curvignathus* were then placed on a wet Whatman No. 1 filter paper located inside a 90 mm × 15 mm non-treated polystyrene petri dish at room temperature for 24 h in dark. The signs and symptoms of infection, and the mortality rate were recorded daily until 100% mortality was achieved. Mortality data were corrected using Abbott's formula (Abbott 1925). Graph of mean percentage of cumulative mortality against days was plotted using Prism GraphPad for Mac version 8.1.1. The effective lethal time (LT_{50}) was determined by using Probit analysis method in the SPPS software version 27.0 (SPSS Inc., Chicago, IL, USA).

Assessment of mycoses of *C. curvignathus*

C. curvignathus that showed mycelial growth on their carcass (mycoses) were placed on PDA and maintained at 27 ± 2 °C for fungal growth. The mean percentage of mycoses was recorded and the mean separation test using Tukey HSD at alpha 0.05 (SAS Institute Inc., USA, version 9.4).

Virulent score

The virulent score assessment consisted of 3 criteria, namely the mean mortality at 6 DAI, LT_{50} value and mean percentage of mycelia growth (Table 1). The virulent score consists of 5 levels as stated in Table 1.

Concentration dependent bioassay against *C. curvignathus*

EPF with virulent score of 3.1–5.0 were chosen for concentration dependent mortality test. The *C. curvignathus* were applied with five different conidia concentrations (1×10^9, 1×10^8, 1×10^7, 1×10^6 and 1×10^5 conidia ml^{-1}) prepared by serial dilution. *C. curvignathus* was surface sterilized with 0.01% (v/v) sodium hypochlorite and washed 3 times with double-autoclaved distilled water. After air-dried, each individual termite was inoculated with 20 µl conidia suspension containing 0.05% (v/v) Tween 80 on the dorsal abdomen. The inoculated *C. curvignathus* were dried for 2 min. *C. curvignathus* treated with 0.05% (v/v) Tween 80 were served as negative control. Ten termites per treatment with 10 replications were accomplished. All treated and untreated *C. curvignathus* were then placed on wet Whatman No.1 filter paper inside the 90 mm × 15 mm Petri dish at room temperature for 24 h in dark. Mortality rate was recorded daily until 100% mortality achieved, and the mortality data were corrected using Abbott's formula (Abbott 1925).

Statistical analysis

Means of treatment were compared using Tukey's test at $\alpha < 0.05$ using Statistical Analysis Software (SAS Institute Inc., USA) version 9.4. The percentage of mycoses *C. curvignathus* was calculated by comparing the mortality in the control experiment. The lethal concentration (LC_{50}) value was determined by using Probit analysis method in SPSS software version 27.0 (SPSS Inc., Chicago, IL, USA).

Table 1 Virulent score assessment for UPM EPF

Virulent score	Criteria			Virulent level
	Mean % mortality	LT_{50} (days)	Mean % mycelia formation	
0.0–1.0	< 15.0	> 12.0	< 20.1	Not virulent
1.1–2.0	16.0–30.0	9.1–12.0	20.1–40.0	Mild virulent
2.1–3.0	31.0–44.0	6.1–9.0	40.1–60.0	Moderate virulent
3.1–4.0	45.0–60.0	3.1–6.0	60.1–80.0	Highly virulent
4.1–5.0	> 60.0	< 3.1	> 80.0	Very virulent

Results

Isolation of EPF

Eleven EPF were successfully isolated from the soil in the oil palm plantation in UPM using insect bait method. Larvae of *T. molitor* showed different types of mycoses symptoms (Fig. 1).

Morphological and molecular identification of EPF

EPF can be differentiated by colony texture, colony shape and colony colour at upper and lower surfaces on PDA, and conidia size and shape (Tables 2, 3). The EPF were molecularly analysed via the amplification and sequencing of their ITS, TEF1-α and βTub2 regions to confirm the morphological identification. The nucleotide sequence of *ITS, TEF1-α* and *βTub2* genes were 500–650, 600–900 and 300–700 bp, respectively. *ITS, TEF1-α* and *βTub2* nucleotide sequences were submitted to NCBI GenBank and accession numbers were obtained.

Isolate UPM-A1C-1 *Aspergillus auricomus* (Visagie et al. 2014)

Colony growing round, yellow, raised and floccose, reverse cream. cylindrical globose, conidia with dimension 2.48 ± 0.05 µm long and 2.41 ± 0.04 µm wide, width/length ratio of 1.0. Sequence of *ITS, TEF1-α* and *βTub2* genes of UPM-A1C-1 was obtained but only ITS nucleotide sequences in GenBank were available for sequence comparison. The ITS nucleotide sequence of UPM-A1C-1 showed 100% sequence homology to *A.*

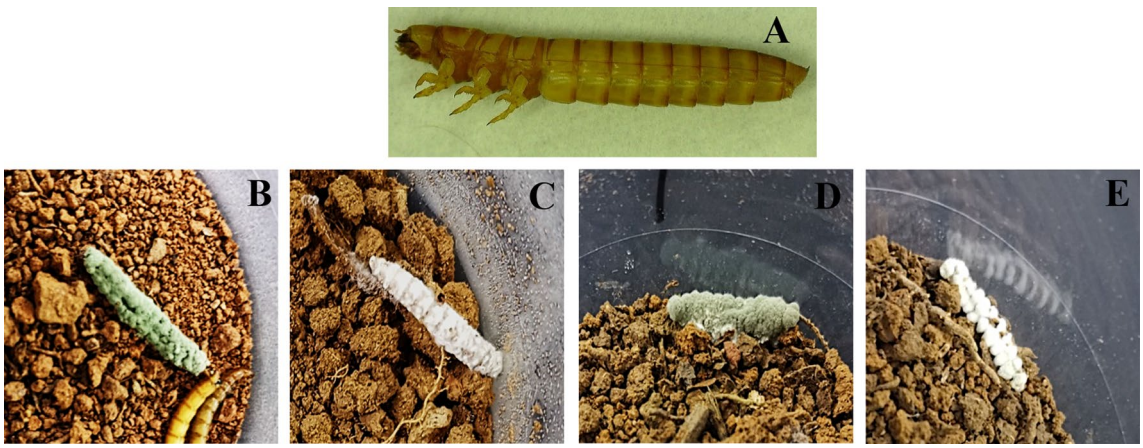

Fig. 1 Larvae of *Tenebrio molitor* with mycoses symptom. **A** Healthy larvae, **B**–**E** Larvae with mycoses symptom during baiting of EPF in the soil sample

Table 2 Cultural characteristics of the EPF isolated from oil palm plantation in UPM

No	Isolate code	Colony colour		Colony texture	Colony shape
		Upper	Reverse		
1	UPM-A1C-1	Yellow	Cream	Raised and floccose	Round
2	UPM-A1C-2	Greenish with ring yellow at centre of the plate	Cream	Flat and floccose	Round
3	UPM-A2C-1	Creamy white with green conidia	Light yellow	Cottony, thick, raised and floccose	Round
4	UPM-A2C-3	Vinaceous brown	Brownish	Floccose	Round
5	UPM-A2C-5	Greyish with brown colour at centre of the plate	White	Flat and floccose	Round
6	UPM-A3C-1	Creamy white with green conidia	Brownish	Cottony and flat	Irregular
7	UPM-A3C-2	Creamy white with green conidia	Brownish	Cottony, flat and floccose	Irregular
8	UPM-A5C-1	Creamy white with green conidia	Brownish	Cottony, thick, raised and floccose	Irregular
9	UPM-A7C-1	Vinaceous brown	Creamy white	Cottony and floccose	Round
10	UPM-A10C-1	White	Light yellow	Raised and convex	Round
11	UPM-A13C-2	White	Brownish	Raised and thick	Round

Table 3 Morphological characteristics of EPF isolated from UPM oil palm plantation

No	Isolate code	Conidial shape	Conidial size (µm) * Mean ± S. E		Length/width ratio	Genus identification
			Length	Width		
1	UPM-A1C-1	Globose	$2.48^{H} \pm 0.05$	$2.41^{F} \pm 0.04$	1.0	*Aspergillus* spp.
2	UPM-A1C-2	Globose	$3.82^{GF} \pm 0.07$	$3.79^{BA} \pm 0.06$	1.0	*Aspergillus* spp.
3	UPM-A2C-1	Cylindrical	$6.81^{E} \pm 0.10$	$3.80^{BA} \pm 0.07$	1.0	*Metarhizium* spp.
4	UPM-A2C-3	Globose	$3.54^{GF} \pm 0.06$	$1.83^{GH} \pm 0.10$	1.8	*Purpureocillium* spp.
5	UPM-A2C-5	Long ovoid	$1.56^{I} \pm 0.06$	$1.46^{H} \pm 0.04$	1.0	*Cordyceps* sp.
6	UPM-A3C-1	Cylindrical	$9.27^{A} \pm 0.16$	$3.71^{BAC} \pm .06$	1.9	*Metarhizium* spp.
7	UPM-A3C-2	Cylindrical	$6.59^{E} \pm 0.12$	$2.59^{F} \pm 0.07$	2.5	*Metarhizium* spp.
8	UPM-A5C-1	Cylindrical	$9.99^{BA} \pm 0.15$	$3.77^{BA} \pm 0.12$	2.7	*Metarhizium* spp.
9	UPM-A7C-1	Ellipsoid to fusiform	$4.27^{F} \pm 0.04$	$1.61^{H} \pm 0.06$	2.5	*Purpureocillium* spp.
10	UPM-A10C-1	Cylindrical	$9.31^{BDAC} \pm 0.31$	$3.41^{EBDAC} \pm 0.09$	2.7	*Metarhizium* spp.
11	UPM-A13C-2	Ellipsoidal-cylindrical	$9.68^{BAC} \pm 0.16$	$3.48^{EBDAC} \pm 0.06$	2.8	*Metarhizium* spp.

Key; Mean in columns with the same letter are not significantly different at $\alpha < 0.05$, Tukey's test for width and length *Mean of 30 conidia

auricomus (GenBank accession no.: MK952334.1). It is grouped with the Clade *A. auricomus.*

Isolate UPM-A1C-2 *Aspergillus caelatus* (Visagie et al. 2014)

Colony growing round, greenish with ring yellow at centre of the plate, flat and floccose, reverse cream. Cylindrical globose conidia, with dimension 3.82 ± 0.07 µm long and 3.79 ± 0.06 µm wide, width/length ratio of 1.0. Sequences of *ITS, TEF1-α* and *βTub2* genes of UPM-A1C-2 were obtained but only *ITS* and *βTub2* nucleotide sequences in GenBank were available for sequence comparison. Both *ITS* and *βTub2* nucleotide sequences of UPM-A1C-2 showed 100% sequence homology to *ITS* gene of *A. caelatus* (GenBank accession no.: MH862672.1) and *βTub2* gene of *A. caelatus* (GenBank accession no.: MN993914.1). It is grouped with the Clade *A. caelatus.*

Isolate UPM-A2C-3 *Purpureocillium lilacinum* (Sun et al. 2021)

Colony growing round, vinaceous brown, floccose, reverse brownish. Ellipsoidal-cylindrical conidia, vinaceous brown, with dimension 3.54 ± 0.06 µm long and 1.83 ± 0.10 µm wide, width/length ratio of 1.8. Sequences of *ITS, TEF1-α* and *βTub2* genes of UPM-A2C-1 were obtained. Analysis of BLASTn algorithm showed 100% sequence homology to *ITS* gene of *P. lilacinum* (GenBank accession no.: LC416799.1), *TEF1-α* gene of *P. lilacinum* (GenBank accession no.: MK550671.1), and *βTub2* gene of *P. lilacinum* (GenBank accession no.: MK503783.1), respectively. It is grouped with the Clade *P. lilacinum.*

Isolate UPM-A7C-1 *Purpureocillium lilacinum* (Sun et al. 2021)

Colony growing round, vinaceous brown, floccose, reverse creamy white. Ellipsoid to fusiform conidia, vinaceous brown, with dimension 4.27 ± 0.04 µm long and 1.61 ± 0.06 µm wide, width/length ratio of 2.5. Sequences of *ITS, TEF1-α* and *β-Tub2* genes of UPM-A7C-1 were obtained. Analysis of BLASTn algorithm showed 100, 100 and 99.6% sequence homology to *ITS* gene of *P. lilacinum* (GenBank accession no.: MT453285.1), *TEF1-α* gene of *P. lilacinum* (GenBank accession no.: MK550669.1), and *βTub2* gene of *P. lilacinum* (GenBank accession no.: MZ190339.1), respectively. It is grouped with the Clade *P. lilacinum.*

Isolate UPM-A2C-5 *Cordyceps javanica* (Ou et al. 2019)

Colony growing round, greyish with brown colour at centre of the plate, flat and floccose, reverse white. Long ovoid conidia, grey, with dimension 1.56 ± 0.06 µm long and 1.46 ± 0.04 µm wide, width/length ratio of 1.0. Sequences of *ITS, TEF1-α* and *βTub2* genes of UPM-A2C-1 were obtained. Analysis of BLASTn algorithm showed 100% sequence homology to *ITS* gene of *C. javanica* (GenBank accession no.: MT801895.1), *TEF1-α* gene of *C. javanica* (GenBank accession no.: MG659313.1), and *βTub2* gene of *C. javanica* (GenBank accession no.: MN576993.1), respectively. It is grouped with the Clade *C. javanica.*

Isolate UPM-A2C-1 *Metarhizium anisopliae* var *anisopliae* (Mayerhofer et al. 2019)

Colony growing round, creamy white, raised and floccose, reverse light yellow. Cylindrical conidia, dark green, with dimension 6.81 ± 0.10 µm long and 3.80 ± 0.07 µm

wide, width/length ratio of 1.0. Sequences of *ITS*, *TEF1-α* and *βTub2* genes of UPM-A2C-1 were obtained. Analysis of BLASTn algorithm showed 100% of sequence homology to *ITS* gene of *M. anisopliae* (GenBank accession no.: KP739826.1), *TEF1-α* gene of *M. anisopliae* (GenBank accession no.: MH048540.1), and *βTub2* gene of *M. anisopliae* (GenBank accession no.: KR706492.1), respectively. It is grouped with the Clade *M. anisopliae*. The conidia size had further confirmed isolate UPM-A2C-1 as *M. anisopliae* var *anisopliae*.

Isolate UPM-A3C-1 *Metarhizium anisopliae* var *anisopliae* (Mayerhofer et al. 2019)

Colony growing irregular, creamy white, cottony and flat, reverse brownish. Cylindrical conidia, dark green, with dimension 6.59 ± 0.12 µm long and 2.59 ± 0.07 µm wide, width/length ratio of 2.5. Sequences of *ITS*, *TEF1-α* and *βTub2* genes of UPM-A3C-1 were obtained. Analysis of BLASTn algorithm showed 100% sequence homology to *ITS* gene of *M. anisopliae* (GenBank accession no.: EU530677.1), *TEF1-α* gene of *M. anisopliae* (GenBank accession no.: EU248822.1), and *βTub2* gene of *M. anisopliae* (GenBank accession no.: KR706492.1), respectively. It is grouped with the Clade *M. anisopliae*. The conidia size has further confirmed UPM-A3C-1 as *M. anisopliae* var *anisopliae*.

Isolate UPM-A3C-2 *Metarhizium anisopliae* var *anisopliae* (Mayerhofer et al. 2019)

Colony growing irregular, creamy white, cottony, flat and floccose, reverse brownish. Cylindrical conidia, dark green, with dimension 6.59 ± 0.12 µm long and 2.59 ± 0.07 µm wide, width/length ratio of 2.5. Sequences of *ITS*, *TEF1-α* and *βTub2* genes of UPM-A3C-2 were obtained. Analysis of BLASTn algorithm showed 100% sequence homology to *ITS* gene of *M. anisopliae* (GenBank accession no.: KX255642.1), *TEF1-α* gene of *M. anisopliae* (GenBank accession no.: EU248823.1), *and βTub2* gene of *M. anisopliae* (GenBank accession no.: DQ463996.2), respectively. It is grouped with the Clade *M. anisopliae*. The conidia size has further confirmed UPM-A3C-2 as *M. anisopliae* var *anisopliae*.

Isolate UPM-A5C-1 *Metarhizium anisopliae* var *anisopliae* (Mayerhofer et al. 2019)

Colony growing round, creamy white, raised and floccose, reverse brownish. Cylindrical conidia, dark green, with dimension 9.99 ± 0.15 µm long and 3.77 ± 0.12 µm wide, width/length ratio of 2.7. Sequences of *ITS*, *TEF1-α* and *βTub2* genes of UPM-A5C-1 were obtained. Analysis of BLASTn algorithm showed 99.7, 99.7 and 100% sequence homology to *ITS* gene of *M. anisopliae* (GenBank accession no.: GU909512.1), *TEF1-α* gene of *M. anisopliae* (GenBank accession no.: MG893933.1), and *βTub2* gene of *M. anisopliae* (GenBank accession no.: KR706492.1), respectively. It is grouped with the Clade *M. anisopliae*. The conidia size has further confirmed UPM-A5C-1 as *M. anisopliae* var *anisopliae*.

Isolate UPM-A10C-1 *Metarhizium anisopliae* var *anisopliae* (Mayerhofer et al. 2019)

Colony growing round, white, raised and convex, reverse light yellow. Cylindrical conidia, dark green, with dimension 9.31 ± 0.31 µm long and 3.41 ± 0.09 µm wide, width/length ratio of 2.7. Sequences of *ITS*, *TEF1-α* and *β-Tub2* genes of UPM-A10C-1 were obtained. Analysis of BLASTn algorithm showed 99.8, 99.8 and 100% sequence homology to *ITS* gene of *M. anisopliae* (GenBank accession no.: FJ589649.1), *TEF1-α* gene of *M. anisopliae* (GenBank accession no.: MH698453.1), and *βTub2* gene of *M. anisopliae* (GenBank accession no.: KR706492.1), respectively. It is grouped with the Clade *M. anisopliae*. The conidia size has further confirmed UPM-A10C-1 as *M. anisopliae* var *anisopliae*.

Isolate UPM-A13C-2 *Metarhizium pinghaense* (Bischoff et al. 2006)

Colony growing round, white, raised and thick, reverse brownish. Ellipsoidal-cylindrical conidia, dark green, with dimension 9.68 ± 0.16 µm long and 3.48 ± 0.06 µm wide, width/length ratio of 2.7. Sequences of *ITS*, *TEF1-α* and *βTub2* genes of UPM-A13C-2 were obtained. Analysis of BLASTn algorithm showed 100% sequence homology to *ITS* gene of *M. pinghaense* (GenBank accession no.: LR792764.1), *TEF1-α* gene of *M. pinghaense* (GenBank accession no.: EU248821.1), and *βTub2* gene of *M. pinghaense* (GenBank accession no.: KJ588065.1), respectively. It is grouped with the Clade *M. pinghaense*. The conidia size has further confirmed UPM-A13C-2 as *M. pinghaense.*

Phylogenetic tree analysis

The isolates of UPM EPF were successfully identified by *ITS*, *TEF1-α* and *βTub2* genes (Table 4). The phylogenetic tree of *ITS* gene confirmed the UPM EPF isolates into 4 clades (Fig. 2). Clade 1 consists of *M. anisopliae* var *anisopliae* (UPM-A2C-1, UPM-A3C-1, UPM-A3C-2, UPM-A5C-1, and UPM-A10C-1) and *M. pinghaense* (UPM-A13C-2) with 90 and 96% bootstraps value, respectively. Low sequence variation was observed between *M. anisopliae* and *M. pinghaense.* Clade 2 consists of *P. lilacinum* isolates (UPM-A2C-3 and UPM-A7C-1). Both UPM-A1C-1 *A. auricomus* and UPM-A1C-2 *A. caelatus* were grouped into Clade 3 and

Table 4 Percentage of sequence similarity based on *ITS*, *βTub2* and *TEF1-α* genes of EPF morphospecies isolated from UPM

No	Isolate Code	ITS GAN	ITS (%)	β-Tub2 GAN	β-Tub2 (%)	TEF1-α GAN	TEF1-α (%)	Identity
1	UPM-A1C-1	MK952334.1	100.0	–	100.0	–	–	*Aspergillus auricomus*
2	UPM-A1C-2	MH862672.1	100.0	MN993914.1	100.0	–	–	*Aspergillus caelatus*
3	UPM-A2C-1	KP739826.1	100.0	MH048540.1	100.0	KR706492.1	100.0	*Metarhizium anisopliae* var *anisopliae*
4	UPM-A2C-3	LC416799.1	100.0	MK550671.1	100.0	MK503783.1	99.8	*Purpureocillium lilacinum*
5	UPM-A2C-5	MT801895.1	100.0	MG659313.1	100.0	MN576993.1	100.0	*Cordyceps javanica*
6	UPM-A3C-1	EU530677.1	100.0	EU248822.1	100.0	KR706492.1	100.0	*M. anisopliae* var *anisopliae*
7	UPM-A3C-2	KX255642.1	100.0	EU248823.1	100.0	DQ463996.2	100.0	*M. anisopliae* var *anisopliae*
8	UPM-A5C-1	GU909512.1	99.7	MG893933.1	99.7	KR706492.1	100.0	*M. anisopliae* var *anisopliae*
9	UPM-A7C-1	MT453285.1	100.0	MK550669.1	100.0	MZ190339.1	99.6	*P. lilacinum*
10	UPM-A10C-1	FJ589649.1	99.8	MH698453.1	99.8	KR706492.1	100.0	*M. anisopliae* var *anisopliae*
11	UPM-A13C-2	LR792764.1	100.0	EU248821.1	100.0	KJ588065.1	100.0	*Metarhizium pinghaense*

GAN GenBank Accession Number

completely separated by 93% bootstrap values. *Aspergillus tamarii* and *A. caelatus* were grouped into *Aspergillus* group section Flavi, however, *A. tamarii* has larger tuberculate spores than *A. caelatus*. UPM-A2C-5 *C. javanica* fall in Clade 4 with 90% bootstrap values. *Lecanicillium fungicola* was used as an out group in the ITS phylogenetic tree.

The phylogenetic tree of *TEF1-α* gene of EPF was divided into 3 clades, which were *Purpureocillium, Cordyceps* and *Metarhizium* (Fig. 3). Members of each clade showed monophyletic group. The *βTub2* nucleotide sequences of UPM EPF were grouped into 5 major clades (Fig. 4). Clade 1 consists of *M. anisopliae* var *anisopliae* (UPM-A2C-1, UPM-A3C-1, UPM-A3C-2, UPM-A5C-1, and UPM-A10C-1) with 100% bootstraps value. *P. lilacinum* isolates (UPM-A2C-3 and UPM-A7C-1) were grouped into Clade 2 with 55% bootstrap values. The UPM-A1C-2 *A. caelatus* was grouped into Clade 3 with 100% bootstrap values. Clade 4 consists of UPM-13C-2 *M. pinghaense* with 100% bootstraps value while Clade 5 consists of UPM-A2C-5 *C. javanica* with 100% bootstrap values. *S. litura* was used as an-out-group in the phylogenetic tree of TEF1-α regions and βTub2 regions.

Mortality test of EPF against *C. curvignathus*

UPM-A1C-1 *A. auricomus* and UPM-A1C-2 *A. caelatus* were slow in killing, with initial mortality recorded after 4 DAI and only 28–29% mortality recorded after 6 DAI (Table 5). Other EPF such as UPM-A2C-5 *C. javanica* recorded 84% mortality rate, followed by UPM-A2C-1 *M. anisopliae* var *anisopliae* (82%), UPM-A13C-2 *M. pinghaense* (81%), UPM-A7C-1 *P. lilacinum* (74%), UPM-A2C-3 *P. lilacinum* (72%), UPM-A5C-1 *M. anisopliae* var *anisopliae* (71%), and UPM-A3C-1 *M. anisopliae* var *anisopliae* (64%). However, these EPF showed non- significant different in their mean mortality result. Other isolates of *M. anisopliae* var *anisopliae* such as UPM-A10C-1 (57%) and UPM-A3C-2 (54%) showed moderate mortality to *C. curvignathus*. No mortality of *C. curvignathus* was observed in the control. The lethal time (LT_{50}) of all 11 EPF isolates varied from 3.90 to 7.75 days (Table 5). Among the EPF isolates tested, UPM-A2C-1 *M. anisopliae* var *anisopliae* showed the lowest LT_{50} value at 3.90 days, followed by UPM-A13C-2 *M. pinghaense* (3.94 days), UPM-A2C-5 *C. javanica* (3.95 days), UPM-A7C-1 *P. lilacinum* (4.00 days), UPM-A2C-3 *P. lilacinum* (4.35 days), UPM-A5C-1 *M. anisopliae* var *anisopliae* (4.36 days), UPM-A3C-1 *M. anisopliae* var *anisopliae* (4.80 days), UPM-A3C-2 *M.anisopliae* var *anisopliae* (4.93 days), UPM-A10C-1 *M. anisopliae* var *anisopliae* (5.37 days), UPM-A1C-2 *A. caelatus* (6.83 days), UPM-A1C-1 *A. auricomus* (7.75 days), respectively.

Assessment of *C. curvignathus* with mycoses

The percentage of *C. curvignathus* with mycoses was correlated to those of mortality test (Table 5). Among the EPF tested, UPM-A1C-1 *A. auricomus* and UPM-A1C-2 *A. caelatus* were the slowest EPF in mycelia formation on the carcass, while the UPM-A2C-5 *C. javanica*, UPM-A2C-1 *M. anisopliae* var *anisopliae* and UPM-A13C-2 *M. pinghaense* scored the highest percentage of *C. curvignathus* with mycoses after 6 DAI. UPM-A2C-3 *P. lilacinum* and UPM-A5C-1 *M. anisopliae* var *anisopliae* did not show promising mycelia formation on *C. curvignathus* with their high percentage of mortality after 6 DAI.

Symptom of *C. curvignathus* with mycoses

The *C. curvignathus* infected with the EPF became inactive and showed abnormal behaviour such as avoiding others caste, changing in dietary and less feeding as early as 1DAI. The dead *C. curvignathus* become harden and reduced in body size. A thin layer of mycelia slowly

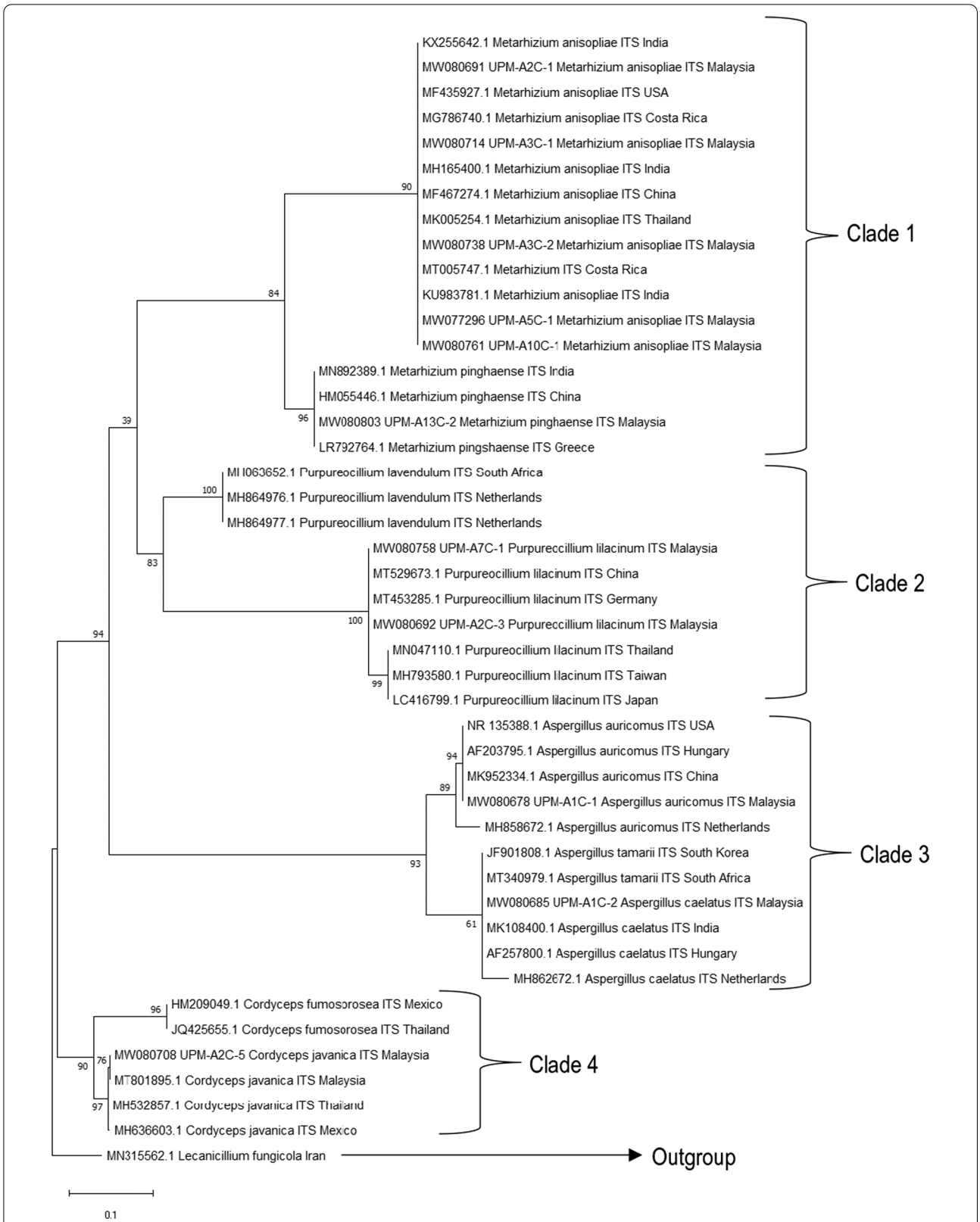

Fig. 2 Phylogenetic tree of *ITS* gene of EPF. Maximum Likelihood Tree generated based on the combined dataset of *ITS* nucleotide sequences of UPM EPF isolates developed using MEGA X. Only data with bootstrap value > 30% are shown. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best, Kimura 3 parameter model + Gamma Distributed

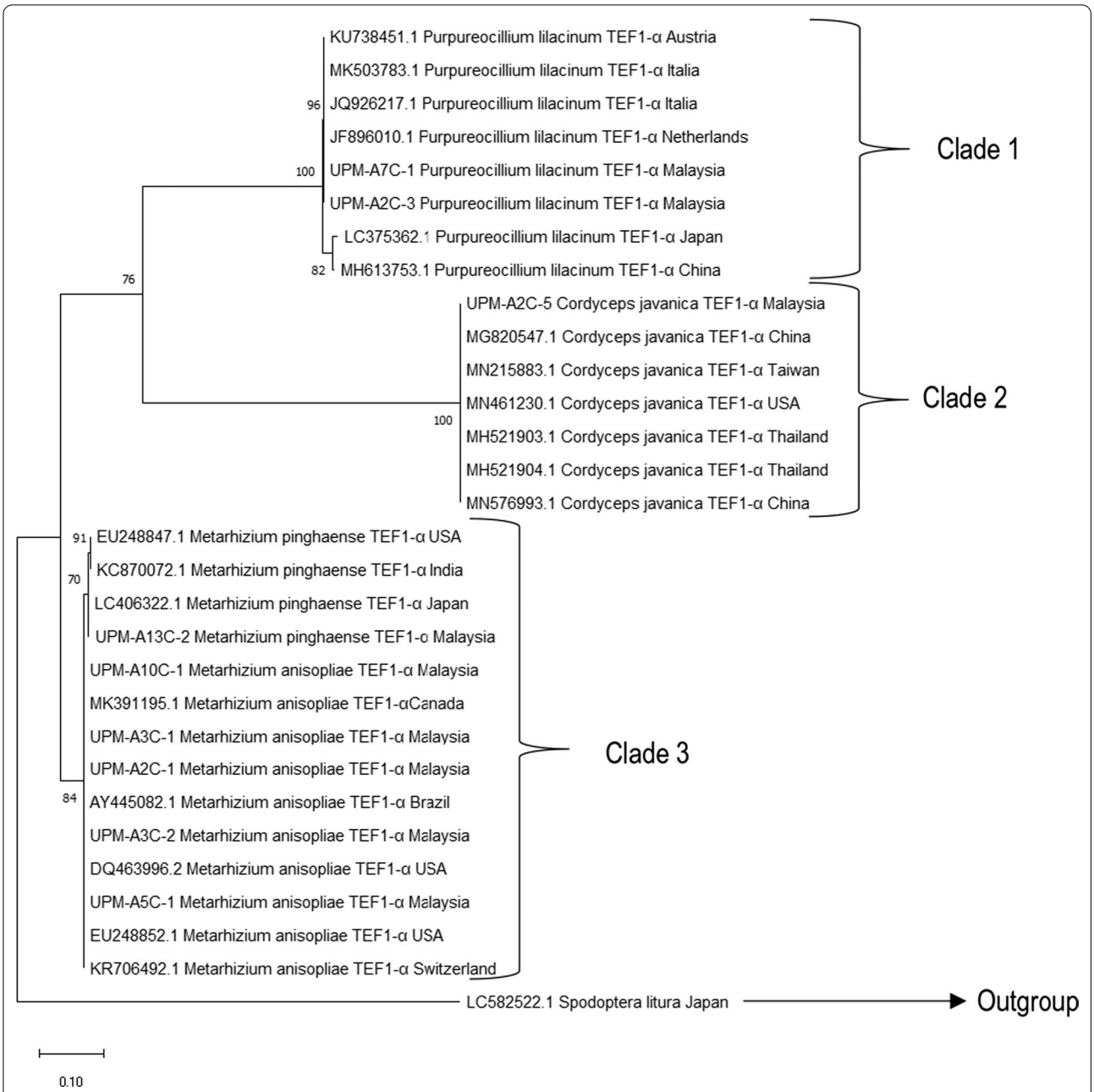

Fig. 3 Phylogenetic tree of *TEF1-α* gene of EPF. Maximum Likelihood Tree generated based on the combined dataset of *TEF1-α* nucleotide sequence of UPM EPF isolates developed using MEGA X. Only data with bootstrap value > 30% are shown. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best, Tamura 2-parameter model

appeared on the carcass. Different EPF showed different colours of conidia formed on the dead *C. curvignathus*. *Metarhizium* spp. produced white conidia on the dorsal abdomen of dead *C. curvignathus* and gradually covered the whole body of the carcass. The white conidia turned to green colour after 3DAI (Fig. 5A–F). UPM-A2C-3 *P. lilacinum* and UPM-A7C-1 *P. lilacinum* produced vinaceous-brown conidia on the carcass of *C. curvignathus* (Fig. 5G, H). *C. curvignathus* infected with UPM-A2C-5 *C. javanica* were covered with greyish conidia starting from the dorsal abdomen and then spread to the whole body of the infected *C. curvignathus* (Fig. 5I). *C. curvignathus* infected with *Aspergillus* spp. had a dark integument after death before formation of conidia on their body. The conidia were scattered all over the body. The UPM-A1C-2 *A. caelatus* produced yellowish green

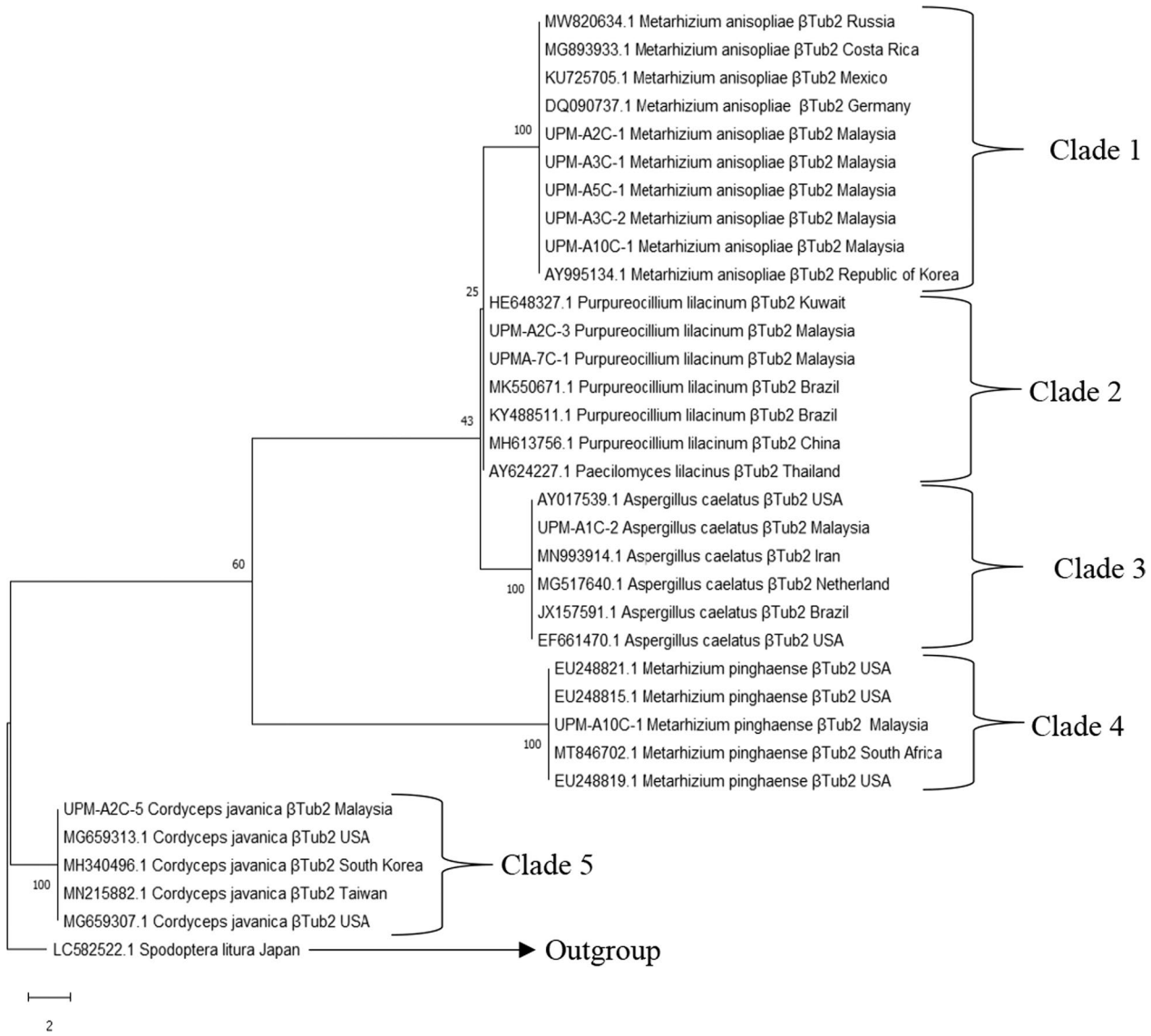

Fig. 4 Phylogenetic tree of *βTub2* gene of EPF. Maximum Likelihood Tree generated based on the combined dataset of *βTub2* nucleotide sequence of UPM EPF isolates developed using MEGA X. Only data with bootstrap value > 30% are shown. Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best, Jukes–Cantor model

conidia (Fig. 5J), while the UPM-A1C-1 *A. auricomus* produced yellow conidia (Fig. 5K).

Virulent score

The virulent score of EPF is shown in Table 5. There are grouped into 3 groups: group 1 consisted of highly virulent EPF (Mean virulent score = 3.3) such as UPM-A2C-1 *M. anisopliae* var *anisopliae*, UPM-A13C-2 *M. pinghaense* and UPM-A2C-5 *C. javanica*, group 2 consisted of severe virulent EPF (Mean virulent score 2.7–3.0), which comprised of UPM-A7C-1 *P. lilacinum,* UPM-A2C-3 *P. lilacinum*, UPM-A5C-1 *M. anisopliae* var *anisopliae,* UPM-A3C-1 *M. anisopliae* var *anisopliae*, UPM-A3C-2 *M. anisopliae* var *anisopliae* and UPM-A10C-1 *M. anisopliae* var *anisopliae*. UPM-A1C-2 *A. caelatus* and UPM-A1C-1 *A. auricomus* had mean virulent score 1.0 and therefore both were grouped in group 3. The mean mortality and mycoses were standardized at 6 DAI for all isolates. The group 1 EPF showed similar response against subterranean termite *C. curvignathus* with the highest mean mortality, highest percentage of mycoses and shorter LT_{50} compared to group 2 and group 3 EPF. Therefore, group 1 EPF were used for concentration dependent bioassay study.

Table 5 Performances of UPM EPF isolates against *Coptotermes curvignathus* under laboratory assays at 6 DAI using 1×10^7 conidia ml^{-1} inoculum

No	Isolates	Species	Mean mortality (%) (Vr1)	LT_{50} (days) (Vr2)	Mean mycoses (%) (Vr3)	Virulent score			Mean virulent score	Virulent level	Grouping
						Vr1	Vr2	Vr3			
1	UPM-A2C-1	*M. anisopliae* var *anisopliae*	82.0^{a}	3.90	67.0^{ba}	4.0	3.0	3.0	3.3	Highly virulent	Group 1
2	UPM-A13C-2	*M. pinghaense*	81.0^{a}	3.94	71.5^{a}	4.0	3.0	3.0	3.3	Highly virulent	Group 1
3	UPM-A2C-5	*C. javanica*	84.0^{a}	3.95	69.0^{ba}	4.0	3.0	3.0	3.3	Highly virulent	Group 1
4	UPM-A7C-1	*P. lilacinum*	74.0^{ab}	4.00	54.0^{bc}	4.0	3.0	2.0	3.0	Moderate virulent	Group 2
5	UPM-A2C-3	*P. lilacinum*	72.0^{ab}	4.35	43.5^{fe}	4.0	3.0	2.0	3.0	Moderate virulent	Group 2
6	UPM-A5C-1	*M. anisopliae* var *anisopliae*	71.0^{ab}	4.36	36.5^{f}	4.0	3.0	1.0	2.7	Moderate virulent	Group 2
7	UPM-A3C-1	*M. anisopliae* var *anisopliae*	64.0^{ab}	4.80	45.0^{dc}	4.0	3.0	2.0	3.0	Moderate virulent	Group 2
8	UPM-A3C-2	*M. anisopliae* var *anisopliae*	54.0^{b}	4.93	44.5^{de}	3.0	3.0	2.0	2.7	Moderate virulent	Group 2
9	UPM-A10C-1	*M. anisopliae* var *anisopliae*	57.0^{b}	5.37	50.5^{dc}	3.0	3.0	2.0	2.7	Moderate virulent	Group 2
10	UPM-A1C-2	*A. caelatus*	29.0^{c}	6.83	2.4^{g}	1.0	2.0	0.0	1.0	Mild virulent	Group 3
11	UPM-A1C-1	*A. auricomus*	28.0^{c}	7.75	2.9^{g}	1.0	2.0	0.0	1.0	Mild virulent	Group 3
12	Control	0.05% Tween 80 + double autoclave distilled water	0.00^{c}	–	0.0^{g}	0.0	0.0	0.0	0.0	Not virulent	Group 4

Mean in columns with the same letter are not significantly different at $\alpha < 0.05$, Tukey's Test

DAI day after inoculation; *Vr* Virulent score, Mean virulent score = (Vr1 + Vr2 + Vr3/3)

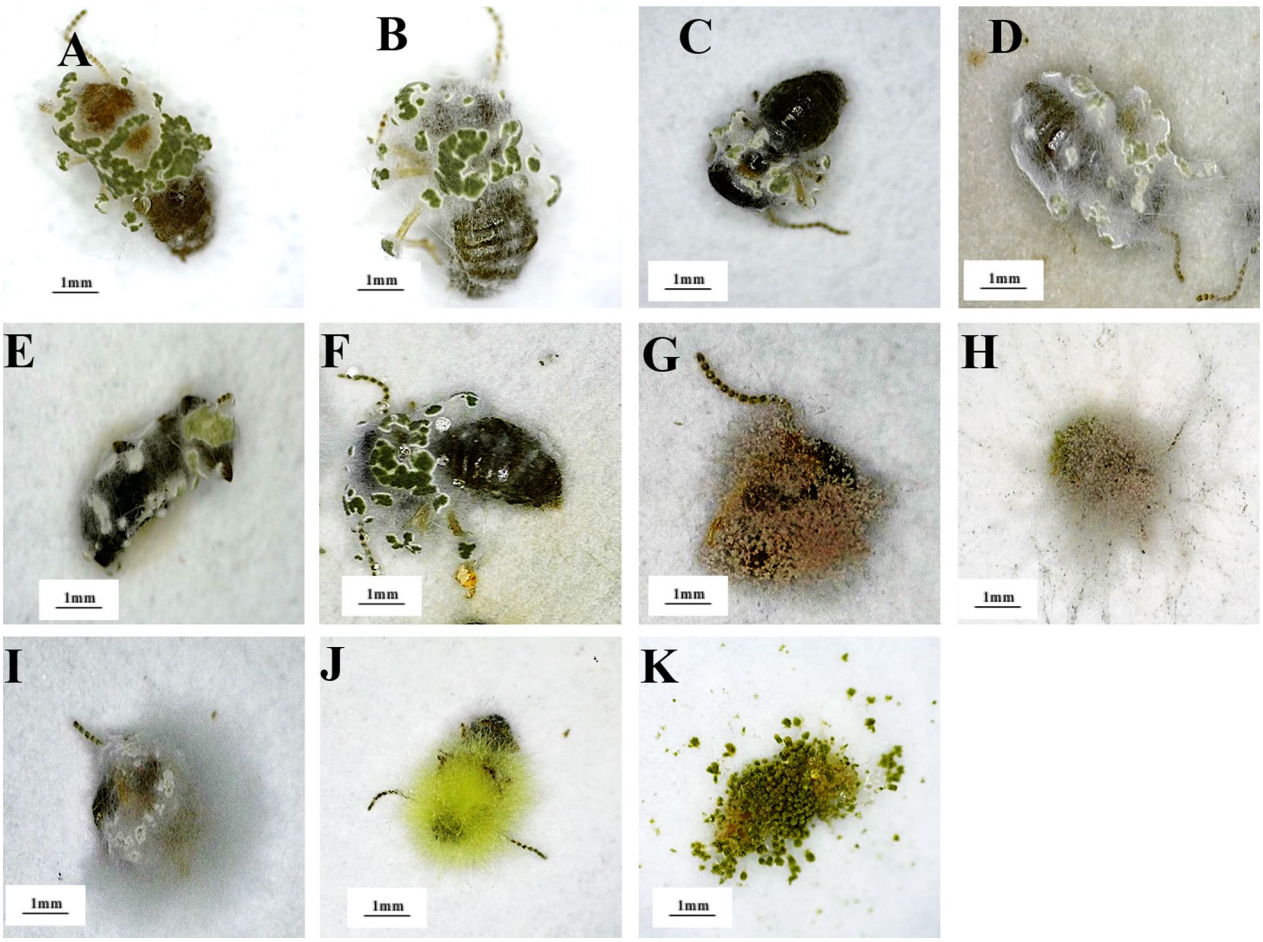

Fig. 5 Mycelia grown on the carcass of *Coptotermes curvignathus*. **A** UPM-A2C-1 *Metarhizium anisopliae* var *anisopliae*, **B** UPM-A3C-1 *M. anisopliae* var *anisopliae*, **C** UPM-A3C-2 *M. anisopliae* var *anisopliae*, **D** UPM-A5C-1 *M. anisopliae* var *anisopliae*, **E** UPM-10C-1 *M. anisopliae* var *anisopliae*, **F** UPM-A13C-2 *M. pinghaense*, **G** UPM-A7C-1 *Purpureocillium lilacinum*, **H** UPM-A2C-3 *P. lilacinum*, **I** UPM-A2C-5 *Cordyceps javanica*, **J** UPM-A1C-1 *Aspergillus auricomus*, **K** UPM-A1C-2 *A. caelatus*. These images were observed using Dino-Lite Digital Microscope and the image were taken using the DinoXcope 2.0 software

Concentration dependent bioassay of group 1 EPF against *C. curvignathus*

The lethal concentration (LC_{50}) of group 1 EPF isolates varied from 1.49×10^5 to 7.08×10^5 conidia ml^{-1} (Table 6). UPM-A2C-1 *M. anisopliae* var *anisopliae* showed the lowest LC_{50} value (1.49×10^5 conidia ml^{-1}), followed by UPM-A13C-2 *M. pinghaense* (3.90×10^5 conidia ml^{-1}), and UPM-A2C-5 *C. javanica* (7.08×10^5 conidia ml^{-1}). The confidence limit at 95% of isolate UPM A2C-1 *M. anisopliae* var *anisopliae* was the lowest with lower and upper limits of conidia concentration at 7.42×10^4–2.54×10^6 conidia ml^{-1}, and slope

Table 6 Lethal concentration (LC_{50}) of Group 1 EPF isolates against *Coptotermes curvignathus*

No	Isolate code	Slope ± SE	X^2	LC_{50} (conidia ml^{-1})	95% limit (conidia ml^{-1})	
					Lower	Upper
1	UPM-A2C-1	3.0 ± 0.242	4.19	1.49×10^5	7.42×10^4	2.54×10^6
2	UPM-A13C-2	3.0 ± 0.364	3.184	3.90×10^5	2.09×10^5	6.52×10^5
3	UPM-A2C-5	3.0 ± 0.084	6.649	7.08×10^5	4.62×10^5	1.04×10^6

UPM-A2C-1 *Metarhizium anisopliae* var *anisopliae*, UPM-A13C-2 *M. pinghaense* and UPM-A2C-5 *Cordyceps javanica*

at 3.0 ± 0.242, followed by UPM A13C-2 *M. pinghaense* (lower and upper limits of conidia concentration: 2.09×10^5– 6.52×10^5 conidia ml^{-1}; slope at 3.0 ± 0.364) and the highest was UPM A2C-5 *C. javanica* (lower and upper limits of conidia concentration: 4.62×10^6–1.04×10^6 conidia ml^{-1}; slope at 3.0 ± 0.084).

Discussion

Soil is a natural habitat for microorganisms such as entomopathogenic fungi and other beneficial microorganisms and pathogens. It protects the soil-borne microorganisms from the environmental stress such as UV radiation and humidity, as well as providing micro- and macro-nutrients to them (Ignoffo and García 1995). The baiting method used in the present study was able to isolate 11 EPF of 4 different fungi genera. Several studies reported that insect baiting method by using *T. molitor* to isolate soil borne EPF have been shown to acquire many EPF from various genus (Kim et al. 2018). Morphological identification confirmed the identity of EPF in 6 species in the present study. A total of 5 isolates of *M. anisopliae* var *anisopliae,* 1 isolate of *M. pinghaense,* 1 isolate of *Cordyceps javanica,* 2 isolates of *Purpureocillium lilacinum,* 1 isolate of *Aspergillus auricomus* and 1 isolate of *A. caelatus* were identified. The UPM *Metarhizium* isolates showed similar morphological features to those *Metarhizium* spp. reported by Sánchez-Peña et al. (2011) in the southern Thailand. *M. anisopliae* var *anisopliae* had similar mycelia colour and conidia shape with *M. anisopliae* var *majus*, except the *M. anisopliae* var *anisopliae* had shorter conidia. Therefore, the identity of the UPM *M. anisopliae* isolates was assigned as *M. anisopliae* var *anisopliae*. The UPM *C. javanica* was in line with the study conducted by Ou et al. (2019), which demonstrated *C. javanica* consisted of long ovoid conidia with grey mycelia. *Aspergillus auricomus* and *A. caelatus* can be differentiated based on the colour of their mycelia. The mycelia of *Aspergillus auricomus* was yellow in colour while *A. caelatus* had greenish mycelia. Both were having similar size and shape of conidia which is in line with the study of Visagie et al. (2014). Mongkolsamrit et al. (2020) concluded that specimens with 100% sequence homology can be identified as the same strain, 99% sequence homology as the same species, and 89–99% sequence homology as the same genus. The UPM EPF showed more than 99% sequence homology to those published sequences in GenBank. Both morphological and molecular results have confirmed the UPM EPF into species level.

Construction of a phylogenetic tree depends on the availability of reference sequences. Reference sequences of TEF-*α* gene were lacking for *A. auricomus* and *A. caelatus* in the present study. The phylogenetic trees had grouped the UPM EPF into different monophyletic clusters. *βTub2* gene showed a better grouping of UPM EPF into cluster compared to *ITS* gene and *TEF1-α* gene.

Interaction study between termites and fungi were discussed by many researchers (Ye et al. 2019). The common reported EPF were derived from the genus *Aspergillus* spp., *Metarhizium* spp., *Beauveria* spp., *Isaria* spp. and *Paecilomyces* (former name of *Purpureocillium*) spp. (Sharma et al. 2018). The virulence potential of different isolates of UPM EPF was assessed against *C. curvignathus*. Their performance in terms of LT_{50} was as follows: *M. anisopliae* var *anisopliae* > *M. pinghaense* > *C. javanica* > *P. lilacinum* > *A. caelatus* > *A. auricomus*. UPM-A2C-1 *Metarhizium anisopliae* var *anisopliae* was proven more virulence (LT_{50}: 3.90 days, LC_{50}: 1.49×10^5 conidia ml^{-1}) than other EPF in controlling *C. curvignathus*. *M. anisopliae* has been confirmed pathogenic to other termites such as *C. formosanus*, *Odontotermes* sp. and *Reticulitermes* sp. (Denier and Bulmer 2015). Singha et al. (2011) reported that *M. anisopliae* (LC_{50}: 3.21×10^5 to 3.82×10^5 conidia ml^{-1}) performed better than *B. bassiana* isolates (LC_{50}: 4.39×10^5 to 5.08×10^5) against tea termite, *Microtermes obesi*. The mean mortality percentage of termites depends on the conidia concentration (Sileshi et al. 2013). The time taken to kill *C. curvignathus* decreased with the increase in conidia concentration of UPM EPF. The virulence of UPM EPF varied among species and hosts. Among them, *M. anisopliae* var *anisopliae, P. lilacinum, M. pinghaense* and *C. javanica* were very virulent against *C. curvignathus*. The LC_{50} value of *M. anisopliae* var *anisopliae* was much lower (less than 2.0×10^5 conidia ml^{-1}) than those reported by Singha et al. (2011).

Symptoms and signs shown by the infected *C. curvignathus* varied among the UPM EPF, however, the formation of mycelia and colour of conidia were in line with other published EPF either on termites or other insects. UPM EPF such as *C. javanica* and *M. anisopliae* var *anisopliae* induced more than 65% mycoses at 6 DAI. Other EPF were slow in mycoses formation. Beside the data on the pathogenicity study, mycelium and spore formation is also an important factor in the selection of potential candidate as a biological control agent (Ansari et al. 2004). Grooming behaviour is a defensive mechanism in termites' colony in order to protect themselves against disease (Yanagawa et al. 2008). This behaviour was observed throughout the mycoses formation on the *C. curvignathus* infected with different isolates of EPF. Dead infected *C. curvignathus* were covered with pieces of filter paper in Petri dish by other alive *C. curvignathus* to protect the spread of fungi to other members. Infected members were observed having social distancing among the alive and infected members during the

study. Rosengaus et al. (2000) reported that infected *C. curvignathus* by parasitoids could separate itself socially from other members and avoid other infected members. *C. curvignathus* would clean their surface body with their mouth after exposure to conidia, and this cleaning can prevent them from contact with the fungus (Yanagawa et al. 2008). The percentage of mycoses depended on the successful germination rate of the fungal spore. The grooming behaviour of *C. curvignathus* will be removed the spore attached on its body and thus reduce the successful germination rate of fungal spore and also the percentage of mycoses.

The 5 isolates of UPM *M. anisopliae* var *anisopliae* demonstrated different virulence potential to *C. curvignathus*. According to Altre et al. (1999), different isolates of same species do not have equal virulence potential to the same insect pest. The differences in pathogenicity to an insect host with the same fungal species was also reported by Rohrlich et al. (2018). *Aspergillus auricomus* and *A. caelatus* did not show a promising insecticidal potential to control *C. curvignathus* in the present study. The present research findings were in line with those reported by Aihetasham et al. (2015) whom reported *A. parasiticus* was slow in causing mortality to *Coptotermes hei.* Based on the virulence score, UPM-A2C-1 *M. anisopliae* var *anisopliae*, UPM-A13C-2 *M. pinghaense* and UPM-A2C-5 *C. javanica* showed promising potential in controlling *C. curvignathus*. Among them, *M. anisopliae* var *anisopliae* was the best EPF, followed by *M. pinghaense* and *C. javanica.* UPM *P. lilacinum* showed moderate virulent against *C. curvignathus* with more than 70% mean mortality score in the present study. The data reported in the present study, in particular using *P. lilacinum, M. pinghaense, A. auricomus, A. caelatus* and *C. javanica* against *C. curvignathus*, novel and would be the world's first report in the present research.

Conclusion

Entomopathogenic fungi have been known for their potential as insect control agents. In this study, 11 EPF were successfully isolated using yellow mealworm, *T. molitor* and identified as *Aspergillus* spp., *Metarhizium* spp., *Purpureocillium* spp. and *Cordyceps* sp. All tested EPF isolates were pathogenic to *C. curvignathus* with different degrees of virulence and percentage of infection. UPM-A2C-1 *M. anisopliae* var *anisopliae* was found to be the most promising considering the lowest LT_{50} and LC_{50} values. Further research is recommended to determine the viability and efficacy of this isolate for the control of *C. curvignathus* in the storage and field environment, as well as its potential use in the control of other insect pests.

Abbreviations

EPF: Entomopathogenic fungi; *C. curvignathus*: *Coptotermes curvignathus*; *T. molitor*: *Tenebrio molitor*; *M. anisopliae* var *anisopliae*: *Metarhizium anisopliae* var *anisopliae*; *P. lilacinum*: *Purpureocillium lilacinum*; *C. javanica*: *Cordyceps javanica*; *M. pinghaense*: *Metarhizium pinghaense*; *A. auricomus*: *Aspergillus auricomus*; *A. caelatus*: *Aspergillus caelatus*; MEA: Malt extract agar; PDA: Potato dextrose agar; w/v: Weight over volume; v/v: Volume over volume; ITS: Internal transcribed spacer; *β-Tub*: Beta-tubulin; rDNA: Ribosomal DNA; TEF: Transcription elongation factor; DNA: Deoxyribonucleic acid; PCR: Polymerase chain reaction; LT_{50}: The effective lethal time; LC_{50}: The effective lethal concentration; DAI: Day after inoculation; HSD: Honestly significant difference; UPM: Universiti Putra Malaysia; SE: Standard error; GAN: GenBank Accession Number; BIC: Bayesian information criterion; BLASTn: Nucleotide Basic Local Alignment Search Tool; Vr: Virulent score; *P. hypostomma*: *Psammotermes hypostoma*.

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Author contributions

All authors contributed to the creation of the manuscript. M.A.K., S.A. and W.H.L.: execution of experiments, design of work, analysis and interpretation of results, and wrote the manuscript. M.A.K. and W.H.L.: review and editing the manuscript. All authors read and approved the final manuscript.

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Declarations

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Competing interests

The authors declare that they have no competing interests.

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