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GENETIC VARIATION OF ENTOMOPATHOGENIC FUNGI, *Metarhizium anisopliae* AND *Isaria amoenerosea* AND THEIR PATHOGENICITY AGAINST SUBTERRANEAN TERMITE, *Coptotermes curvignathus*

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This study determined the genetic similarity of 22 isolates of Metarhizium anisopliae *and 51 isolates of* Isaria amoenerosea *isolated from mineral and peat soils, involving random amplified polymorphic DNA (RAPD) and using polymerase chain reaction (PCR) technology. The pathogenicity of the selected isolates from both genera of fungi were then tested against the subterranean termites,* Coptotermes curvignathus. *The RAPD-PCR analysis revealed high genetic similarity within the isolates of* M. anisopliae *and* I. amoenerosea, *at the similarity ranging from 52% to 100%. For* M. anisopliae, *the fungal isolates were grouped into two main clusters and the isolates of* I. amoenerosea *were separated into five main clusters. Within clusters in each species, there was little evidence on high similarity within isolates from the same soil type, but most of fungal isolates were well distributed within the clusters. Based on the bioassay data, the isolates of* M. anisopliae *were more pathogenic on termites, as compared to the isolates of* I. amoenerosea. *The isolates of* M. anisopliae *caused high percentage of infections, ranging from 71% to 84% at 15 days after treatment (DAT), and the isolate of M11H was the most pathogenic against termites with the* LT_{50} *value of 6.36 days. For isolates of* I. amoenerosea, *the percentage of infection were low, ranging from only 5% to 31%. This laboratory study showed that* M. anisopliae *has a potential to be used to control termites, as it has rapid infecting capability. However, further studies should be conducted to confirm the effectiveness of the* M. anisopliae *to control termites, especially in the field.*

Keywords: *Isaria amoenerosea, Metarhizium anisopliae,* random amplified polymorphic DNA (RAPD), termites, *Coptotermes curvignathus.*

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INTRODUCTION

Entomopathogenic fungi (EPF) are well-known as environmental-friendly biological control agent for numerous insect pests of important crops, domestic and urban industry. The EPF can be found by isolating them from soil, as soil provides a good environmental shelter for fungi to grow (Vega *et al*., 2012). The most common genera of EPF include *Metarhizium* and *Isaria* (Charnley and Collins, 2007; Inglis *et al*., 2001). These fungi are widespread in various habitats such as forest, hedgerow and agricultural soils (Quesada-Moraga *et al*., 2007; Sun *et al*., 2008; Medo and Cagáň, 2011).

Various methods have been used to identify the fungi such as morphological characteristics

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(Hoog, 1972; Rombach *et al*., 1987; Barnett and Hunter, 1998), biochemical properties (St Leger *et al*., 1996; Zibaee *et al*., 2011; Samar and Arash, 2014), isoenzyme analysis (St Leger *et al*., 1992) and molecular techniques (Entz *et al*., 2008; Islam *et al*., 2014; Esparza Mora *et al*., 2016). A random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) is a powerful molecular technique which has the ability to randomly amplify specific regions of DNA (Kumar and Gurusubramanian, 2011). This technique is popular as it has the ability to amplify many DNA samples at one time, cheap and quick, and it only requires substantially low quantities of DNA (Hadrys *et al*., 1992). Therefore, this technique has been widely used for confirmation of species of EPF, estimation diversity within species, characterisation of species from different hosts and locations, and infectivity of EPF against insects (Velásquez *et al*., 2007; Thakur and Sandhu, 2010; Golshan *et al*., 2014).

Termite is an insect which feeds on cellulosic materials, destroying wooden parts in buildings and also agricultural crops including oil palm. In Malaysia and Indonesia, species of termite that attack oil palm is known as *Coptotermes curvignathus* (Lim and Silek, 2001; Zulkifli, 2006). This species has the ability to attack intact woods in soils and infesting live or dead trees (Tho, 1992). Termite attack caused severe damage on young and matured oil palms especially those palms planted on peat soil (Zulkifli, 2006). In peat soil, termite infestation was 53.5%, slightly higher than those palms planted on alluvial soil, which was 33.6%. High infestation of *C. curvignathus* on peat is because of the abundance of rotten tree stumps left over in the soil, making them sources of food and conducive habitats for the termite to breed (Cheng *et al*., 2008). The species of *C. curvignathus* was also recorded attacking coconut and rubber trees (Khoo *et al*., 1991).

The infestation of termites was commonly managed by spraying or drenching of organophosphate insecticides at the base of the infested oil palms (Zulkifli, 2006). However, these chemicals are hazardous to environments, non-target organisms as well as human. Therefore, more environmental-friendly methods to control termites were studied by various workers and this included the use of biological control agents such as the EPF (Rosengaus *et al*., 2000; Ahmad *et al*., 2008; Ramle *et al*., 2013). The fungi that have been used in previous studies were mostly isolated from the infected insects and few of them were from soils. In our study, two major species of EPF were isolated from mineral and peat soil in the oil palm plantations and identified as *Metarhizium anisopliae* and *Isaria amoenerosea* (Pong *et al*., 2015). The conidia size of *M. anisopliae* was 6.0-7.0 μm long x 2.0-2.8 μm wide, while the conidia size of *I. amoenerosea* was 2.0-3.0 μm long x 1.7-2.0 μm wide. Using the RAPD-PCR analysis, those isolates that have high genetic similarity were grouped into the same cluster. Thus, the aims of this study were to determine the genetic similarity of the isolates of *M. anisopliae* and *I. amoenerosea* using the RAPD-PCR technique and to evaluate the infectivity of isolates representing each group against the subterranean termites, *C. curvignathus.*

MATERIALS AND METHODS

Experiment 1: Genetic Variation of Entomopathogenic Fungi Analysed Using Random Amplified Polymorphic DNA-polymerase Chain Reaction Technique (RAPD-PCR)

Source of fungal isolates. All isolates of EFF used in this study were obtained from our previous study (Pong *et al*., 2015). For *M. anisopliae*, there were 15 isolates (M1H-M15H) from mineral soil and seven isolates (M1T-M7T) from peat soil. While for *I. amoenerosea*, there were 25 isolates (P1H-P25H) from mineral soil and 26 isolates (P1T-P26T) from peat soil. Samples of mineral soil were collected from the Malaysian Palm Oil Board (MPOB) Research Station Hulu Paka in Terengganu, Malaysia whereas samples of peat soil were collected from the MPOB Research Station Teluk Intan in Perak, Malaysia. The fungal isolates were cultured on potato dextrose agar (PDA) and incubated at 28°C for two weeks.

Production of fungal mycelium for extraction of DNA. Fungal mycelia were propagated in a liquid medium containing 2.0% dextrose, 1.0% peptone, 0.25% yeast extract and 0.02% chloramphenicol (Ramle *et al*., 2011). All ingredients were mixed and dissolved in a conical flask containing 500 ml distilled water. Then, 75 ml of the prepared medium was poured into 100 ml conical flask and sterilised at 121°C for 15 min. Two weeks old fungal culture was harvested by adding 10 ml of 0.02% Tween 80 into the plate and with the aid of a sterilised inoculation needle, the conidia were scrapped off from the media. The conidia mixtures were filtered via glass wool into a new 20 ml universal bottle. Then, a total of 2 ml of conidia suspensions was pipetted into flasks with sterilised liquid medium as prepared previously. The inoculated fungal flask was placed in an orbital shaker e at 166 rpm at 25°C for four consecutive days.

The growing mycelia were harvested by vacuum filtration method using Whatman filter paper. The wet mycelia were scrapped off from the filter paper and kept in a sterilised bottle. The bottles were then kept inside a freezer at -30°C for overnight, then placed in a freeze dryer (Christ Beta 2-8 LD) at -80°C for a day or until the mycelia were completely dried. The dried mycelia were ground to powder using a

mortar and pestle, then stored at -30°C for further use.

Extraction of fungal DNA. Extraction of fungal DNA was conducted by adding approximately 20 mg of the dried mycelia into a 2.0 ml Eppendorf microtube. The extraction of DNA was carried out using a DNeasy Plant Mini Kit (Qiagen) and following the kit protocol. The samples of fungal DNA were stored in a deep freezer at -30°C for further use. The purity and yield of DNA were estimated from the reading of 1 µl DNA using a Nanodrop 1000 spectrometer (Thermo Scientific).

RAPD-PCR amplification. The RAPD-PCR reactions were performed in 25 µl volumes of PCR mix consisting of 2.50 µl of 10 x PCR buffer (200 mM Tris-HCl and 500 mM KCl), 1.25 µl of 2.5 mM MgCl2, 1.00 µl of 0.1 mM of dNTP's, 0.20 µl of 1 unit Taq DNA polymerase, 1.00 µl of 0.4 µM of primer, 1.00 µl DNA template, 1.00 µl bovine serum albumin (BSA) and 16.05 µl sterilised Milli-Q water. For negative control, the amplification was conducted using sterile Milli-Q water. The amplifications were performed using a Thermal Cycler machine (Mastercycler® Pro, Eppendorf) and the machine was programmed for one cycle at 94°C for 5 min, 35°C for 1 min, 72°C for 2 min, followed by 45 cycles at 94°C for 1 min, 35°C for 1 min, 72°C for 2 min and final cycle of extension at 35°C for 1 min and 72°C for 5 min.

A 10-mer's oligonucleotide random primer (OPA-09) (Operon Technologies, USA) with sequence of 5'-GGGTAACGCC-3' was used in this study. This primer was selected from 20 primers as it produced strong, thick and nicest DNA bands in the screen tests. The PCR products were loaded into 1.8% agarose gel containing 3.0 µl ethidium bromide prepared using 1X TAE buffer. The PCR products were then electrophoresed using a horizontal electrophoresis tray and run at 90 V for about 1.5 hr. The gel was visualised under an UV transillumination and the image was captured using VisionWork@LS Image Acquisition and Analysis software (UVP, LLC).

Scoring of DNA band. The DNA band patterns generated from the gel image were scored by VisionWork@LS Image Acquisition and Analysis software (UVP, LLC). The gel lane which produced DNA from the base pair band was considered as presence (1); no visible DNA was produced from the base pair band was considered as absence (0). The binary data matrix was generated and analysed using Numerical Taxonomy System of Multivariate Program (NT-SYS) software Version 2.1 (Rohlf, 1994). The similarity index data was used to estimate the genetic similarity values and to create the dendrogram. Then, dendrogram was constructed by sequential agglomerative hierarchical non-overlapping (SAHN) clustering method using unweighted pair group method with arithmetic averages (UPGMA).

Experiment 2: Infectivity of Entomopathogenic Fungi against Subterranean Termites, *Coptotermes curvignathus*

Collection of termites. The termite workers of *Coptotermes curvignathus* were collected using a baiting technique of custom-made baiting device developed by Ramle *et al*. (2011). The device was made from cylindrical PVC with holes at the bottom part of the device. The device was filled with wet corrugated cardboard and then inserted into soil near oil palm infested by termite *C. curvignathus.* After four days, those baiting devices with termites were collected and placed into a big plastic box containing peat soil with some rotten oil palm tissues. The termites were kept in a laboratory at 28°C for at least a week for habituation.

Source of fungal isolates used for bioassay. There were six isolates of *M. anisopliae* and eight isolates of *I. amoenerosea* selected and used in the bioassay (*Table 1*). Three isolates of *M. anisopliae* from mineral (M11H, M13H and M14H) and peat soil (M1T, M2T and M3T). For *I. amoenerosea*, four isolates were from each mineral (P3H, P5H, P8H and P14H) and peat soil (P3T, P12T, P21T and P24T).

Preparation of the conidia suspensions. Conidia from two weeks old fungal culture were harvested by adding 10 ml of sterilised 0.02% Tween 80 into the culture plate. Then, the conidia were scrapped off from the surface of the culture using a sterilised L-shaped inoculation needle. The conidia suspensions were filtered via glass wool into a small

TABLE 1. LIST OF FUNGAL ISOLATES USED IN THE BIOASSAY OF TERMITE

No.	Fungal isolate	Isolate identity	Origin	Soil type
1	*M. anisopliae*	M11H	Terengganu	Mineral
2	*M. anisopliae*	M13H	Terengganu	Mineral
3	*M. anisopliae*	M14H	Terengganu	Mineral
4	*M. anisopliae*	M1TH	Perak	Peat
5	*M. anisopliae*	M2T	Perak	Peat
6	*M. anisopliae*	M3T	Perak	Peat
1	*I. amoenerosea*	P3H	Terengganu	Mineral
2	*I. amoenerosea*	P5H	Terengganu	Mineral
3	*I. amoenerosea*	P8H	Terengganu	Mineral
4	*I. amoenerosea*	P14H	Terengganu	Mineral
5	*I. amoenerosea*	P3T	Perak	Peat
6	*I. amoenerosea*	P12T	Perak	Peat
7	*I. amoenerosea*	21T	Perak	Peat
8	*I. amoenerosea*	24T	Perak	Peat

bottle and then the bottle was shaken for a few seconds to obtain homogeneous suspensions. The concentration of the conidia was then determined using an improved Neubauer heamocytometer. The concentration of conidia for each tested fungal isolate was then standardised to 1 x 10^7 conidia ml^{-1}.

Bioassay of entomopathogenic fungi on termites. Termite workers used in this study were obtained from the reared colony in laboratory as previously described. Only the active termites were chosen and placed in a group of 20 termites into a clean petri dish. For each fungal isolate, the bioassay tests were replicated five times using 20 workers in each replicate. Treatment was carried out by inoculating each individual by directly dropping 20 μl conidia suspensions onto the abdominal segment of the termite (Ramle *et al.*, 2011). In control, the termites were only inoculated with 0.02% Tween 80 solution. The mortality of termites was recorded every three days until 15 days after treatment (DAT). Confirmation of infected termites was done by placing the termite cadavers into a clean petri dish which was filled with a wet filter paper. Infected termite developed mycoses where it hardened and later would be fully covered by the fungi.

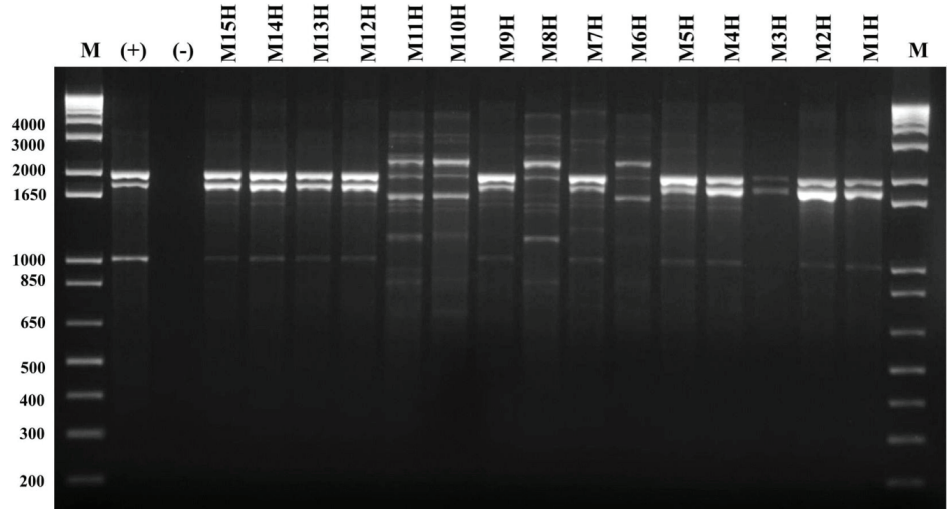
Statistical analysis. The percentage of cumulative mortality and infection of termites treated with the conidia suspension at 1 x 10^7 conidia ml^{-1} were calculated. The percentage of cumulative mortality and infection of termites were arcsine transformed before performing the analysis of variance (ANOVA). The means were separated by Duncan's Multiple Range Test (DMRT) at P=0.05 (SAS, 2013). Probit analysis was performed to calculate the lethal time 50% (LT_{50}) and to rank the virulence of the isolates against the termites. The LT_{50} was calculated manually following the protocol by Finney (1971) and Wigley and Kalmakoff (1977).

RESULTS AND DISCUSSION

Genetic Variation of Entomopathogenic Fungi Analysed by RAPD-PCR

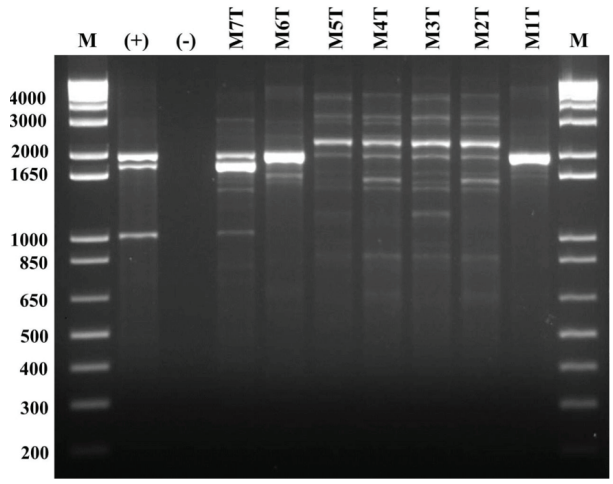
The RAPD band patterns for 15 isolates of *M. anisopliae* from mineral soil produced 77 scorable bands ranging from 900 to 10 000 base pairs and with 93.75% polymorphism (*Figure 1*). While, the RAPD band patterns for seven isolates of *M. anisopliae* from peat soil produced 46 scorable bands, ranging from 600 to 6500 base pairs and resulted in 95.24% polymorphism (*Figure 2*). Isolates of *M. anisopliae* from both mineral and peat soils showed monomorphisms at 2000 base pair, in which clear DNA bands were produced by all isolates. The RAPD band patterns of 51 isolates of *I. amoenerosea* produced a total of 258 scorable bands. A total of 117 scorable bands were produced by *I. amoenerosea* isolated from mineral soil with size ranging from 700 to 7000 base pairs (*Figure 3*). While, for *I. amoenerosea* isolated from peat soil have produced a total of 141 scorable bands, ranging from 600 to 8000 base pairs (*Figure 4*). For the isolates of *I. amoenerosea*, none of the DNA produced at the same base pair band.

Figure 5 shows the dendrogram constructed based on DNA bands of all 22 isolates of *M. anisopliae* isolated from mineral and peat soils. The similarity index between all isolates of *M. anisopliae* ranged from 0.517 to 1.00 (52% to 100%). Obviously, the dendrogram generated two distinguishable main clusters. Cluster I consisted of 15 isolates from mineral soil and three isolates from peat soil. In this cluster, the fungal isolates could not be separated



Note: The lane labelled as M is 1 Kb plus DNA marker. Each lane represents one isolate.

Figure 1. The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) band patterns of 15 isolates M. anisopliae *isolated from mineral soil at the MPOB Research Station, Hulu Paka in Terengganu.*

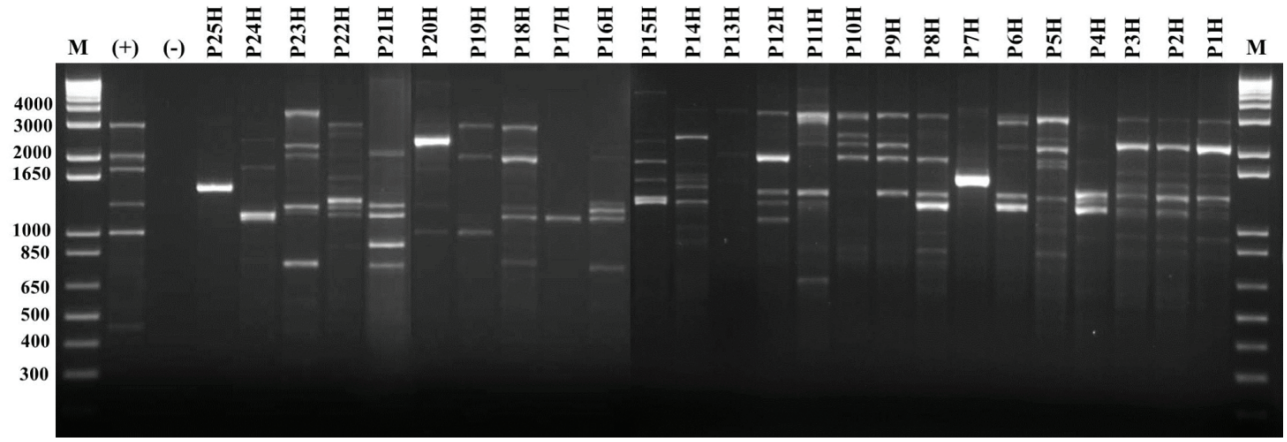


Note: The lane labelled as M is 1 Kb plus DNA marker. Each lane represents one isolate.

Figure 2. The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) band patterns of seven isolates M. anisopliae *isolated from peat soil at the MPOB Research Station, Teluk Intan, Perak.*

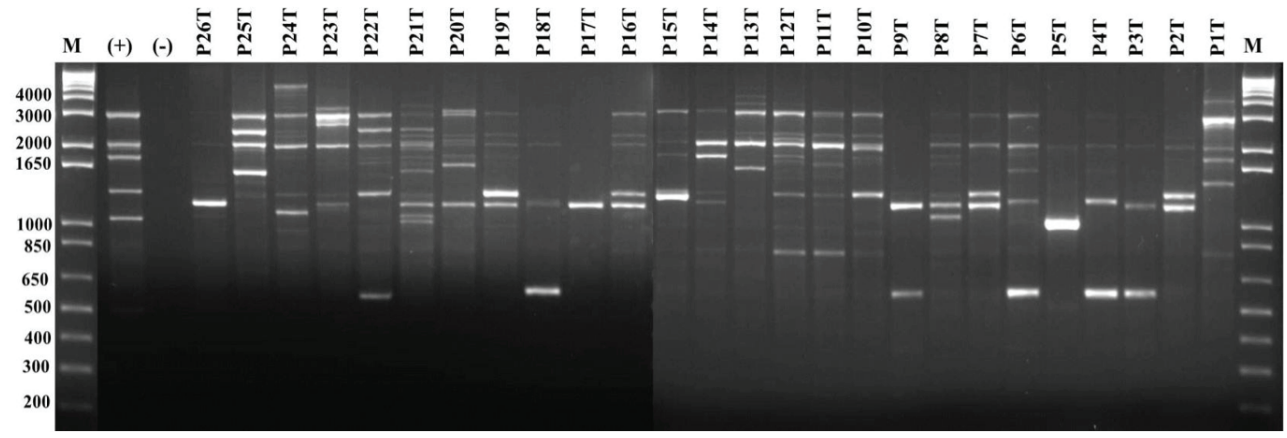
based on their origin, either from mineral or peat soils. Of seven isolates of *M. anisopliae* originated from peat soil, three of them, namely the M1T, M6T and M7T were grouped in cluster I, together with other isolates from mineral soil. These three isolates produced similar DNA bands with other isolates of *M. anisopliae* from mineral soil at 1000 and 1600 base pairs (*Figure 2*). This result suggests that those three isolates from peat have close genetic similarity with isolates of *M. anisopliae* from the mineral soil. Cluster II comprised of four isolates, the M2T, M3T, M4T and M5T, in which all of them were isolated from peat soil. The isolates of *M. anisopliae*, M2T, M3T, M4T and M5T produced distinct DNA band from other isolates at 800 base pairs (*Figure 2*). This shows that some isolates of *M. anisopliae* from the peat soil were genetically distinct from those isolates from the mineral soil at 74% similarity.

In this study, *M. anisopliae* showed high genetic similarity among the isolates either from peat or mineral soils at 66% to 100% similarities. This finding



Note: The lane labelled as M is 1 Kb plus DNA marker. Each lane represents one isolate.

Figure 3. The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) band patterns of 25 isolates I. amoenerosea *isolated from mineral soil at the MPOB Research Station Hulu Paka in Terengganu.*



Note: The lane labelled as M is 1 Kb plus DNA marker. Each lane represents one isolate.

Figure 4. The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) band patterns of 26 isolates I. amoenerosea *isolated from peat soil at the MPOB Research Station, Teluk Intan, Perak.*

contradicted with many studies which reported that there were high levels of genetic variation among the genus of *Metarhizium* (Bidochka *et al*., 1994; Tigano-Milani, Gomes, *et al*., 1995a; Velásquez *et al*., 2007). However, those studies found insufficient evidence on close association between geographical origin, types of soils and insect hosts with genetic variation among isolates. Velásquez *et al*. (2007) demonstrated high variability of *Metarhizium* isolated from soils, but isolates from the same soil type were not grouped in the same cluster, but they clustered together with those *Metarhizium* isolated from insects. Freed *et al*. (2014) also found that isolates of *Metarhizium* obtained from the same type of soils were not grouped into the same cluster. In this study, *M. anisopliae* isolated from mineral and peat soils were grouped in the same cluster. The result shows that type of soil is not a factor influencing the genetic variation of *M. anisopliae*.

The dendrogram in *Figure 6* showed that 51 isolates of *I. amoenerosea* from mineral and peat soils produced four main distinguishable clusters with similarity index ranging from 0.514 to 1.00 (52% to 100%). Cluster I consisted all 25 isolates of *I. amoenerosea* from mineral soil and 22 isolates of *I. amoenerosea* from peat soil. The level of genetic distant separating cluster IA from cluster IB was at 76.8 % similarity. Eleven isolates of *I. amoenerosea* from mineral soil were grouped into cluster IA as they produced similar DNA bands at 1300, 1400 and 3000 base pairs (*Figure 3*). Clusters II, III and IV consisted of isolates from peat soil. Two isolates, P13T and P20T, produced similar DNA bands at 1700, 2100, 3300 and 3800 base pairs, so they were grouped into cluster II. Cluster III consisted of only one isolate P24T, as only this isolate produced distinct DNA bands from the others at 4800 and 8000 base pairs (*Figure 4*). The isolate of P21T was the most distinct among others in cluster IV, at 67% similarity. The DNA profiles isolate of P21T was fragmented into 11 bands which were the highest number of DNA bands produced compared to other isolates.

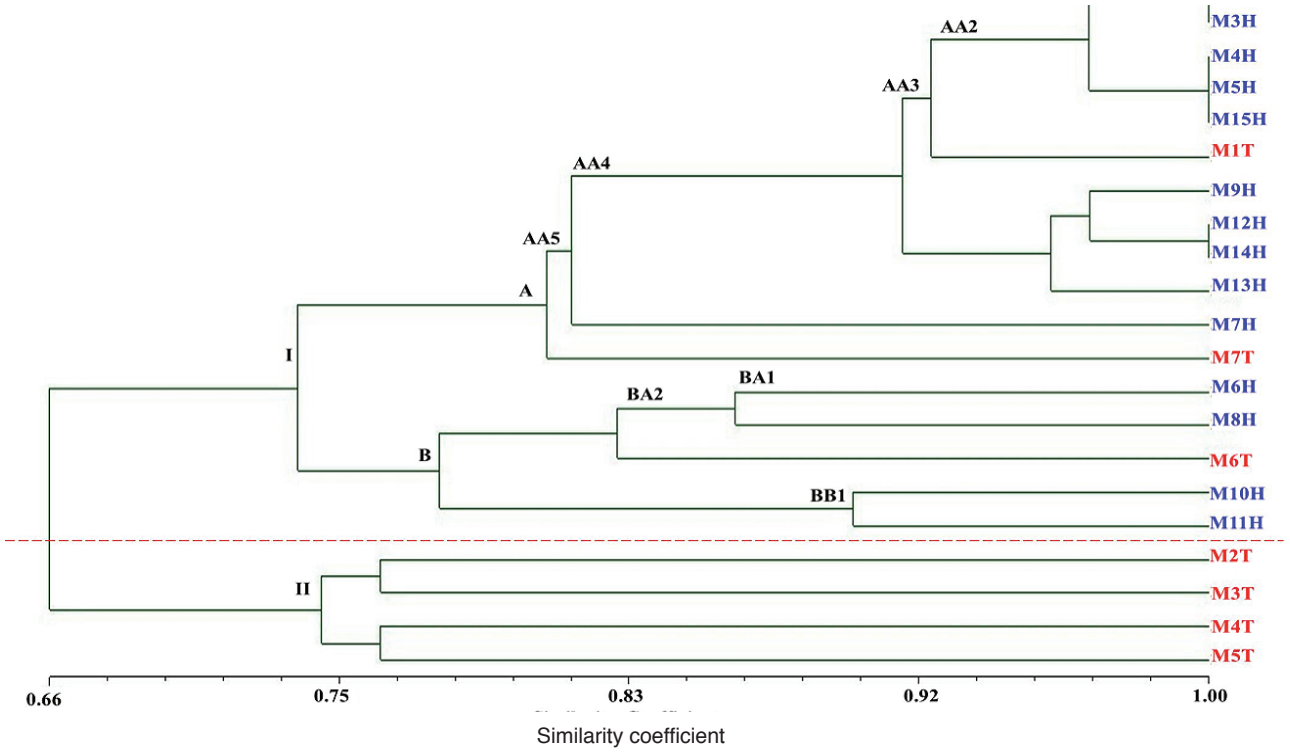
The dendrogram showed that of 26 isolates of *I. amoenerosea* from peat soil, only four isolates, P13T, P20T, P24T and P21T had clearly high genetic variation, thus separated them from other isolates. In cluster I, there were three sub-clusters that have a group of isolates either from mineral or peat soils. The first was the sub-cluster IA, consisting of 11 isolates from mineral soils. Then, the sub-cluster IBB4, consisted of seven isolates from peat soil. Lastly, the sub-cluster IBB9, consisted of a group of eight isolates from the peat soil.

Results of this study clearly showed that some isolates of *I. amoenerosea* originated either from mineral or peat soils have high levels of genetic similarity among them, as they were placed in the same sub-cluster or well distributed within the clusters. This finding suggests that no association between soil and genetic variation of isolates of *I. amoenerosea*. Several studies have reported similar finding as in this study (Tigano-Milani *et al*., 1995c; Cantone and Vandenberg, 1998). There were several studies that showed *Isaria* spp. isolated from soil have high genetic diversity, however, those soils were sampled from different geographical origins (Tigano-Milani *et al*., 1995b,c). The high level of genetic variation was also commonly reported on other species of EPF such as *M. anisopliae* and *B. bassiana* (Kao *et al*., 2002; Thakur *et al*., 2005; Thakur and Sandhu, 2010).

Bioassay of Entomopathogenic Fungi against Termites *Coptotermes curvignathus*

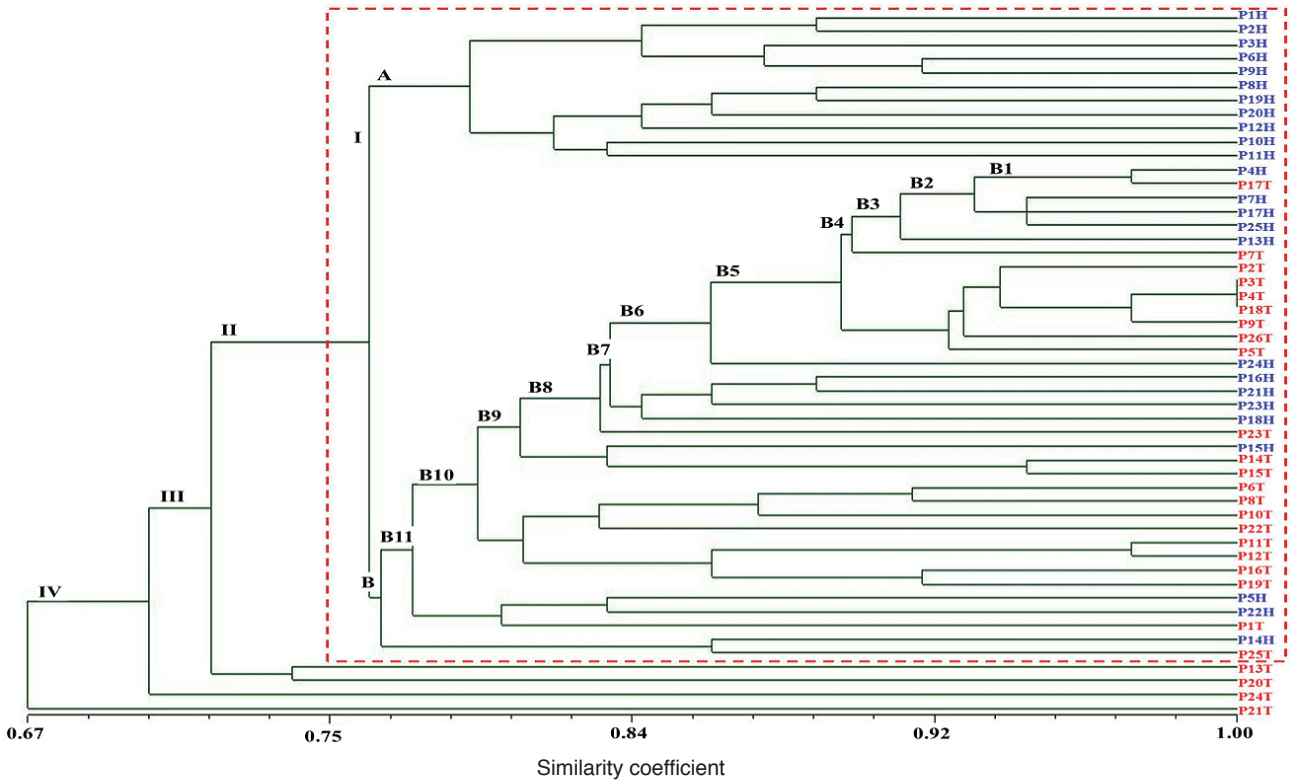
The percentage of cumulative mortality of termites treated with isolates of both EPF species (*M. anisopliae* and *I. amoenerosea*) at the same conidia concentration of 1 x 10^7 conidia ml^{-1} are shown in *Tables 2* and *3*. All tested isolates of *M. anisopliae* and one isolate of *I. amoenerosea* were able to show 100% mortality of termites. However, data on mortality of termites did not represent accurate level of pathogenicity of the tested EPF. For example, although all isolates of *M. anisopliae* were able to kill 100% termites at 15 DAT, but the highest infection level of termites was only 84% (*Table 4*). For *I. amoenerosea*, the infection levels were even lower as compared to *M. anisopliae*, ranging from only 5% to 31% (*Table 5*). This finding suggests that *I. amoenerosea* is less pathogenic than *M. anisopliae* to *C. curvignathus.* Dead termites infected by EPF would develop mycoses after five to six days after they were placed in the wet condition. For dead termite infected by *M. anisopliae*, they were covered by green conidia, while for *I. amoenerosea* the reddish pink conidia were fully grown on the termite cadavers (*Figure 7*). Some of the termite cadavers were not recovered due to natural decomposition.

Of the six isolates of *M. anisopliae*, five isolates, M2T, M3T, M11, M13H and M14H caused high percentage of infection as early as six DAT and up to 15 DAT. The highest infection of these isolates was recorded at 15 DAT, ranging from 71% to 84% (*Table 3*). The infection level of these isolates was significantly higher ($P<0.05$) as compared to infection caused by isolate of M1T, which was 46%. Based on the probit analysis as shown in *Table 6*, isolate of M11H was ranked first with the LT_{50} value of 6.36 days and then followed by isolate of M3T (6.38 days), M2T (6.48 days), M14H (6.73 days) and M13H (7.67 days). The isolate of M1T was the least pathogenic on termites with the LT_{50} value of 13.48 days. These results indicated that most of the isolates of *M. anisopliae*, except for M1T, took about six to seven days to infect at least 50% of the termite population.



Note: Blue colour texts represent the isolate identity of *M. anisopliae* from mineral soil; red colour texts represent the isolate identity of *M. anisopliae* from peat soil; the red dotted line separates the cluster I and II.

Figure 5. The dendogram based on the random amplified polymorphic DNA (RAPD) band patterns of M. anisopliae *isolated from mineral and peat soils generated by the OPA-09 primer..*



Note: Blue colour texts represent isolates identity of *I. amoenerosea* from mineral soil; red colour texts represent isolates identity of *I. amoenerosea* from peat soil; the red dotted lines indicate the cluster I.

Figure 6. The dendogram based on random amplified polymorphic DNA (RAPD) band patterns of I. amoenerosea *isolated from mineral and peat soils generated by the OPA-09 primer.*

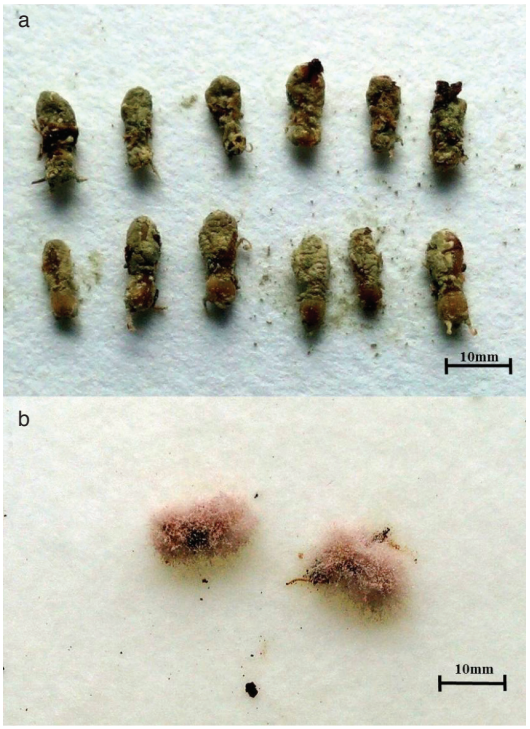


Figure 7. The dead cadavers of C. curvignathus *which a) covered by green spores of* M. anisopliae *and b) by reddish pink spores of* I. amoenerosea.

The LT_{50} values for all isolates of *I. amoenerosea* on termites were not able to be calculated as none of them caused more than 50% infection, thus suggesting that *I. amoenerosea* is less pathogenic than *M. anisopliae*. Data on percentage of infection clearly showed that the isolates *M. anisopliae* had higher percentage of infection as compared to *I. amoenerosea* (*Tables 4* and *5*). This finding was in line with findings by Hoe *et al*. (2009), who found that the *M. anisopliae* was highly virulent to *C. curvignathus*. Furthermore, various studies have reported that the *M. anisopliae* was the most pathogenic and has a potential to control subterranean termites especially the *Coptotermes* spp. (Sukartana *et al*., 2000; Sun *et al*., 2002; Ahmed *et al*., 2008).

Rapid increase on mortality of termites treated by *M. anisopliae* at six DAT was mainly due to high infection of the fungus on termites. About a week after death, the termite cadavers were then covered by the green conidia of *M. anisopliae* (*Figure 7a*) and reddish pink conidia of *I. amoenerosea* (*Figure 7b*). This result was consistent with findings by Sileshi *et al*. (2013) and Krutmuanga and Mekchay (2005), who found that whitish mycelia initially emerged from the termite cadavers at five to six days after death and then followed by the sporulation on termite cadavers. It was reported that time required by the EPF to infect and penetrate into the cuticle of termites was about a week (Krutmuanga and Mekchay, 2005).

A unique defensive behaviour of termites was observed in this study, as some of the dead infected

TABLE 2. PERCENTAGE CUMULATIVE MORTALITY OF TERMITES AFTER TREATED WITH DIFFERENT ISOLATES OF *M. anisopliae*

Fungal isolate	Percentage of cumulative mortality (%) at days after treatment (DAT)				
	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT
CTR	8^{a}	25^{d}	32^{c}	40^{b}	56^{b}
M1T	23^{a}	46^{cd}	91^{ab}	97^{a}	100^{a}
M2T	13^{a}	90^{a}	96^{ab}	100^{a}	100^{a}
M3T	10^{a}	85^{ab}	93^{ab}	96^{a}	100^{a}
M11H	21^{a}	97^{a}	100^{a}	100^{a}	100^{a}
M13H	20^{a}	81^{ab}	92^{ab}	99^{a}	100^{a}
M14H	9^{a}	68^{bc}	86^{b}	96^{a}	100^{a}

Note: Means in columns with the same letters are not significantly difference after Duncan's Multiple Range Test (DMRT) at $\alpha = 0.05$.
DAT – days after treatment.

TABLE 3. PERCENTAGE CUMULATIVE MORTALITY OF TERMITES AFTER TREATED WITH DIFFERENT ISOLATES OF *I. amoenerosea*

Fungal isolate	Percentage of cumulative mortality (%) at days after treatment (DAT)				
	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT
CTR	1^{b}	3^{c}	7^{c}	19^{d}	41^{e}
P3T	1^{b}	7^{bc}	22^{bc}	46^{cd}	77^{d}
P12T	12^{ab}	15^{abc}	40^{ab}	61^{bc}	88^{bcd}
P21T	6^{ab}	34^{ab}	50^{ab}	79^{ab}	92^{abc}
P24T	1^{b}	7^{bc}	26^{bc}	56^{bc}	78^{cd}
P3H	32^{a}	48^{a}	68^{a}	85^{a}	100^{a}
P5H	7^{ab}	47^{a}	67^{a}	84^{ab}	98^{ab}
P8H	1^{b}	17^{abc}	40^{ab}	76^{abc}	94^{abc}
P14H	5^{b}	22^{abc}	52^{ab}	71^{abc}	88^{abc}

Note: Means in columns with the same letters are not significantly difference after Duncan's Multiple Range (DMRT) at $\alpha = 0.05$.

TABLE 4. PERCENTAGE INFECTION OF TERMITES AFTER TREATED WITH DIFFERENT ISOLATES OF *M. anisopliae*

Fungal isolate	Percentage infection of termites (%) at days after treatment (DAT)				
	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT
CTR	0^{a}	0^{c}	0^{c}	0^{c}	0^{c}
M1T	1^{a}	8^{b}	44^{b}	46^{b}	46^{b}
M2T	2^{a}	68^{a}	74^{a}	76^{a}	76^{a}
M3T	6^{a}	69^{a}	73^{a}	73^{a}	76^{a}
M11H	2^{a}	72^{a}	74^{a}	74^{a}	74^{a}
M13H	2^{a}	58^{a}	68^{a}	71^{a}	71^{a}
M14H	3^{a}	55^{a}	72^{a}	80^{a}	84^{a}

Note: Means in columns with the same letters are not significantly difference after Duncan's Multiple Range (DMRT) at $\alpha = 0.05$.

TABLE 5. PERCENTAGE INFECTION OF TERMITES AFTER TREATED WITH DIFFERENT ISOLATES OF *I. amoenerosea*

Fungal isolate	Percentage infection of termites (%) at days after treatment (DAT)				
	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT
CTR	0^{a}	0^{c}	0^{b}	0^{c}	0^{c}
P3T	0^{a}	0^{c}	1^{b}	5^{bc}	12^{ab}
P12T	0^{a}	0^{c}	10^{a}	17^{a}	31^{a}
P21T	0^{a}	4^{abc}	9^{a}	17^{a}	19^{a}
P24T	0^{a}	0^{c}	5^{ab}	15^{ab}	24^{a}
P3H	0^{a}	6^{ab}	13^{a}	20^{a}	22^{a}
P5H	1^{a}	9^{a}	12^{a}	21^{a}	24^{a}
P8H	0^{a}	0^{c}	0^{b}	0^{c}	5^{bc}
P14H	0^{a}	2^{bc}	6^{ab}	11^{ab}	16^{ab}

Note: Means in columns with the same letters are not significantly difference after Duncan's Multiple Range (DMRT) at $\alpha = 0.05$.

TABLE 6. THE LT_{50} VALUES OF ISOLATES *M. anisopliae* ON TERMITES

Fungal isolate	LT_{50} value (day)	Fiducial limit (day)	Rank
M1T	13.48	10.30 - 17.64	6
M2T	6.48	5.06 - 8.29	3
M3T	6.38	4.98 - 8.18	2
M11H	6.36	4.85 - 8.33	1
M13H	7.67	6.03 - 9.75	5
M14H	6.73	5.51 - 8.23	4

termites were found covered within soil mixture. It was speculated that when the healthy termites found their mates infected by fungi, they covered them with soil to protect further spread of the fungi to other areas. Other defensive behaviours of termites included avoiding themselves from infected members and the ability to react against pathogens by digesting the conidia (Rosengaus *et al*., 1999; 2000; Rosengaus and Traniello, 2001). Thus, fungi that could cause rapid infection such as *M. anisopliae* has a potential to control termites. Rapid killing ability is extremely needed as to avoid other healthy termites to groom the infected members by removing the conidia from the body to prevent infection (Sun *et al*., 2002).

CONCLUSION

The RAPD-PCR analysis revealed homogenous populations with high genetic similarity for isolates of *M. anisopliae* isolated from mineral soil at the MPOB Research Station in Hulu Paka, Terengganu and peat soil at the MPOB Research Station in Teluk Intan, Perak. Population homogeneity was also found with the isolate of *I. amoenerosea* isolated from mineral and peat soils from both localities. Generally, no genetic variation of *M. anisopliae* and *I. amoenerosea* was found in mineral and peat soil, indicating that soil type did not influence the genetic variability of *M. anisopliae* and *I. amoenerosea*. This termite's bioassay experiment demonstrated that the isolates of *M. anisopliae* were more pathogenic to the termite C. *curvignathus* as compared to the isolates of *I. amoenerosea*. The infection level of the isolates of *M. anisopliae* either from mineral or peat soil have no significant difference against the termite except one isolate M1T. Although this study demonstrated that *M. anisopliae* was highly pathogenic, but more researches are required to evaluate the efficiency of *M. anisopliae* to control termites, particularly in the field.

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