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Morphological and molecular characterization of fungus isolated from tropical bed bugs in Northern Peninsular Malaysia, *Cimex hemipterus* (Hemiptera: Cimicidae)

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ABSTRACT

Objective: To investigate some morphological and molecular characteristics of fungal parasites isolated from wild tropical bed bug, *Cimex hemipterus*.
Methods: A series of culture methods were used to obtain fungal isolates from field-collected bed bugs. Characteristics of the isolates such as colony appearance, mycelial texture and pigmentation were studied to explore their morphology. Isolates were also subjected to a PCR-based genotyping test.
Results: There were noticeable differences in morphological characteristics among the four isolates. Conidial areas of one isolate were dark green, whereas those of the remaining colonies were olive-green, black or dark brown. Conidia of the dark green isolate were globose, while those of olive-green, black and dark brown isolates were globose to subglobose, globose to spherical and globose to subglobose/finely roughened, respectively. These morphological specificities and the molecular analyses showed that the fungal internal transcribed spacer ribosomal region and *β-tubulin* gene sequences of the isolates shared clade with *Trichoderma* and *Aspergillus* sequences.
Conclusions: Overall, the new discovery of common pathogens in agricultural field developed in live bed bugs storage tank may initiate the use of biological agents in later years.

1. Introduction

Bed bugs are considered to be household insect pests for more than 3 300 years back in those early years in ancient Egypt. They have been said to tag along with the colonists in their belongings on the ship and the dispersal occurs in America. However, the indication of bed bugs origin has not been recorded according to the interview done on Louis Sorkin, an insect expert of the American Museum of Natural History [1]. Generally, bed bugs infestation is known to be a nuisance to humans as they have been battling the insect pests for millennia. As some factors can cause widespread population increase of bed bugs, the number of cases that related to the insect arose drastically in the late 1990s especially in United States [2].

Trichoderma and *Aspergillus* are very cosmopolitan spore-forming genus of fungi. The existence of these genera have been studied and reported as both have the ability to produce some of the most important mycotoxins in the world. Abbot has listed more than 15 types of mycotoxins produced by

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Aspergillus while three mycotoxins were produced by *Trichoderma* [3]. The presence of mycotoxins produced by *Aspergillus* such as aflatoxins, ochratoxins, citrinin and sterigmatocystin can contaminate food commodities and store products such as rice [4,5], barley [6], nuts [7], flour [8] and maize [9]. In insects, the impact or function of *Aspergillus* are varied, from carrier in transmitting microorganism to acting as pathogen to the host. However, reports on the occurrence of this fungi from insect are still limited and rarely reported.

About the endophytic fungus, *Trichoderma* can be commonly found both in cultivated and non-cultivated soils, and is used in biological control of a variety of plant-pathogenic fungi such as *Fusarium verticillioides* and *Rhizoctonia solani* [10–13]. *Trichoderma* species have been demonstrated to produce protease and chitinase that degrade the cell-wall during the parasitic interaction [14–17]. Protease produced by pathogenic insects possesses similar properties to those from *Trichoderma*, augmenting the possibility that proteinases are involved in entomopathogenicity. Previous report regarding larvicidal activity of *Trichoderma harzianum* (*T. harzianum*) against the cotton leaf worm has suggested that this species is pathogenic towards the insect [18].

However, the application of inaccurate and confusing species names has been one of the major hindrances to the study of *Trichoderma* and *Aspergillus* genera simultaneously has impeded their true potential. A meticulous study of their true diversity is mandatory since different species might produce a different array of potential metabolites to be exploited. Prior to the utilization of molecular approaches, the identification of *Trichoderma* and *Aspergillus* is historically based on the application of morphological species recognition concept [19]. Due to the few morphological differences and variations in culture, particularly those in anamorphic forms, many species including *T. harzianum* are inaccurately identified [20,21]. Samson *et al.* also suggested that misidentifications and incorrect names are always occurred in *Aspergillus* nomenclature [22].

Recent developments in genomics have provided better opportunities for researchers worldwide to obscure the true diversity and classification of species in the genus *Trichoderma* and *Aspergillus*. Multiple genes have been demonstrated and translation elongation factor 1-α, internal transcribed spacer (ITS) and β-tubulin have successfully delimited between the closely related species in *Trichoderma* [23]. In genetic characterization of *Aspergillus*, ITS-restriction fragment length polymorphism technique, random amplified polymorphic DNA microsatellite polymorphism analysis, random amplified polymorphic DNA and sequence analysis of intergenic spacer were used among molecular methods [24–32]. In 2007, Balajee *et al.* [33] proposed the use of *β-tubulin* gene for species identification within the section while Hong *et al.* [34] used phylogenetic analysis of β-tubulin sequence as one of the methods to describe new species in *Aspergillus* section *Fumigati*. Pildain *et al.* used a combination of phenotypic (morphology and extrolite profile) and molecular (*β-tubulin* and *calmodulin* gene sequence) to describe new species in section *Flavi* [35].

Thus, prior to the study of possible entomopathogenic role of *Trichoderma* and *Aspergillus* against tropical bed bugs, *Cimex hemipterus*, we therefore primarily examined both molecular and morphological characterizations to accurately identify the species as well as its descriptions.

2. Materials and methods

2.1. Isolation of fungal strains

Samples of eggs, nymphs and live adult bed bugs were collected and reared for chemical testing. Samples were separated and labelled according to its location. They were placed in the plastic containers (8 cm diameter × 8 cm height), which were tightly covered with net cloth (13 cm × 13 cm) and rubber band. Folded papers were provided in the container as their harbourage. The containers then were kept in the plastic tank (30 cm × 18 cm × 18 cm) in the laboratory temperature and relative humidity maintained between (26 ± 2) °C and 60% ± 10% respectively. After two weeks of rearing, two bed bugs accidentally died and possible entomopathogenic fungi that caused the death were isolated.

2.2. Single spore isolation

All fungal isolates from dead bed bugs were purified through single spore isolation technique [36]. This technique was carried out by streaking the mycelia on water agar in a zigzag manner. Once germinated, a single conidium was transferred onto potato dextrose agar (PDA) or malt extract agar (MEA) plates for further studies. For preservation purpose, the single conidial isolates were maintained in distilled water with mild agar broth.

2.3. Morphological analysis of Trichoderma species

Cultural characteristics such as colony appearances, mycelial textures and pigmentations on both obverse and reverse on PDA plates were observed after 3–7 days of incubation under the standard incubation conditions. Growth rate via colony diameter also on PDA was measured, initially standardized at 6 mm using a cork borer and incubated for 3 days in a total darkness. Trials were repeated three times and in triplicates. For microscopic observation, mycelial plugs of *Trichoderma* species were transferred onto MEA plates and were incubated in a total darkness at 28 °C for 3–7 days. The species characteristics such as conidia, conidiophores, branching patterns and chlamydospores were observed. Morphological species characterization was determined following the key characters by Samuels *et al.* [23].

2.4. Morphological analysis of Aspergillus species

For morphological observations, Czapek yeast extract agar (CYA), oatmeal agar (OA), creatine sucrose agar (CREA) and MEA were used. The isolates were inoculated at three points on each plate of each medium and incubated at 25 °C in the dark for 7 days. For microscopic observations, microscopic mounts were made in sterile distilled water from PDA or MEA colonies. The species characteristics such as colony colours, the structure of conidial heads and the shapes of conidia were observed. The fungi were used to identify the species levels [37].

2.5. DNA extraction

Conidia of fungal isolates were inoculated in MEA broth and incubated at 25 °C in the dark. The fungal mycelium were harvested after 36 h of incubation and transferred into sterile freeze dry bottles. After 2 days of freeze dried, the dried mycelium was ground using liquid nitrogen into fine powders. The genomic DNA

was extracted by using DNA extraction kit and DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions.

2.6. PCR amplification

Primer pairs ITS1 and ITS4 and Bt2a and Bt2b were used to amplify ribosomal ITS regions and *β-tubulin* genes, respectively [38]. The 25 μL PCR mix contained 6 ng of genomic DNA, 25 mmol/L $MgCl_2$, 40 mmol/L of dNTP mix, 5 IU of *Taq* DNA polymerase and 1 μmol/L concentrations of each primer. Amplification with the primers pairs ITS1 and ITS4 was performed using a denaturation step of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. A final extension step at 72 °C for 5 min was then employed. Amplification with primer pairs Bt2a and Bt2b included denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 58.6 °C for 30 s and 72 °C for 1 min. A final extension step at 72 °C for 5 min was then used.

2.7. DNA sequencing

2.7.1. Analysis of sequence data

The alignment of ITS region and *β-tubulin* gene sequence data were performed using the MEGA 4 software. The consensus sequences were basic local alignment search tool (BLAST) against Genbank database (http://www.ncbi.nlm.nih.gov) to infer the identification of the isolates.

2.7.2. Nucleotide sequence accession numbers

The fungal ITS ribosomal region and *β-tubulin* gene sequences determined in this study were deposited in GenBank. The BLAST search result and sequence similarities were listed in Table 1 using the GenBank database along with assigned accession numbers.

3. Results

3.1. Morphological analysis of isolate TM1

Dark green conidia were densely formed in concentric rings toward the edge of Petri dish after 7 days incubation (Figure 1A–1B). Mycelia were scarcely found in the culture due to abundance formation of conidia, hence, suppressing the mycelial growth. While, the growth rate of *T. harzianum* was measured on PDA at 28 °C after 3 days incubation in a total darkness. The mean value for growth rate was 21–25 mm/day. Primary branches of conidiophores were in paired and held in whorls of 2–3 phialides (Figure 1C–1D). Phialides were typically flask-

Table 1
Identification of fungal isolates from bed bug based on ITS region and β-tubulin sequences.

Culture no.	Species	[a]ITS	%	Species	[b]β-tubulin	%
TM1	*T. harzianum*	KP418577	99	*T. harzianum*	KP418581	99
AM1	*A. nomius*	KP418574	99	*A. nomius*	KP418578	99
AM2	*A. tubingensis*	KP418575	99	*A. tubingensis*	KP418579	100
AM3	*A. aculeatus*	KP418576	100	*A. aculeatus*	KP418580	100

[a] GenBank accession numbers for ITS regions. [b] GenBank accession numbers for β-tubulin partial sequences; %: Percentage similarity of sequence with GenBank; *A. tubingensis*: *Aspergillus tubingensis*; *A. aculeatus*: *Aspergillus aculeatus*; *A. nomius*: *Aspergillus nomius*.

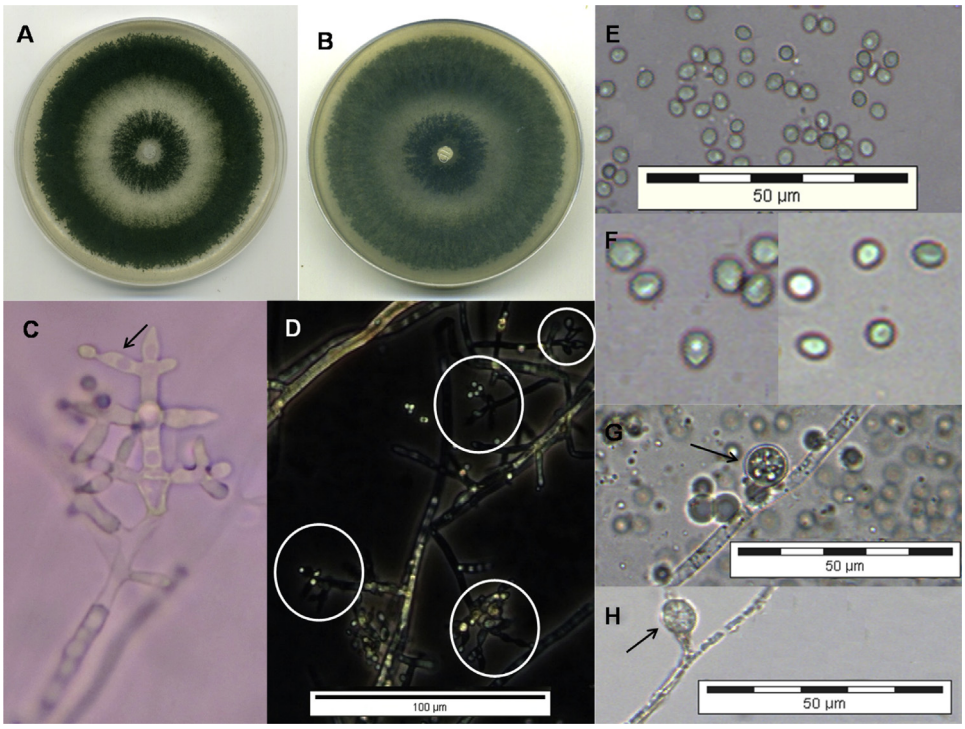

Figure 1. *T. harzianum*.
A–B: Colony appearances; C–D: Conidiophores bearing phialides (arrowed and circled); E–F: Conidia; G–H: Chlamydospores (arrowed).

shaped, swollen in the middle with pointed tip and slightly narrowed base. Green conidia were profusely produced, one-celled, smooth surface and mostly globose. Only a few conidia were found in slightly ovoidal shaped (Figure 1E–1F). Chlamydospores were rarely found in the culture, thick and rough-walled, globose to subglobose and formed at the terminal or intercalary in hyphae (Figure 1G–1H). Macroscopic and microscopic characters of *T. harzianum* were presented in Figure 1.

3.2. Morphological analysis of isolate AM1

Colony colours on MEA were olive green and sclerotia when presented were brown to black. On CYA, conidial area was yellow and sometimes overlaid with white to olive yellow areas. Mycelium on both MEA and CYA was white. Reverse colonies were orangish to light yellow. *A. nomius* produced uniseriate phialides, radiate to columnar conidial heads, smooth-walled stipes and globose to subglobose vesicles. The stipes average length was 200–500 µm while the width ranged between 5 and 9 µm. The vesicle diameter was 20–40 µm in range. Conidia diameter was 3.5–4.5 µm in range with very rough to finely roughened surface. Macroscopic and microscopic characters of *A. nomius* are presented in Figure 2.

3.3. Morphological analysis of isolate AM2

Colony colours on MEA and CYA were black to dark black and the colony was densely packed. Reverse colonies were colourless to light yellow. *A. tubingensis* produced uniseriate and biseriate phialides, radiate conidial heads, smooth-walled stipes and globose to spherical vesicles. The stipes average length was 500–550 µm while the width ranged between 12 and 15 µm. The vesicle diameter was 50–70 µm in range. Conidia diameter was 3.0–4.5 µm in range with finely roughened surface. Macroscopic and microscopic characters of *A. tubingensis* are presented in Figure 3.

3.4. Morphological analysis of isolate AM3

Colony colours on MEA and CYA were black to dark brown and the colony was densely packed. Reverse colonies were brownish to light yellow. *A. aculeatus* produced uniseriate phialides, radiate conidial heads, smooth-walled stipes and globose to subglobose vesicles. The stipes average length was 500–700 µm while the width ranged between 10 and 16 µm. The vesicle diameter was 55–70 µm in range. Conidia diameter was 2.0–3.5 µm in range with finely roughened surface. Macroscopic and microscopic characters of *A. aculeatus* are presented in Figure 4.

3.5. Molecular analysis and DNA sequencing

From PCR amplification of ITS region and *β-tubulin* gene, four fungal isolates obtained from the bed bug yielded a single fragment of an approximately 500 and 600 bp, respectively. BLAST search using Genbank database showed that isolate TM1 shared 99% similarities with *T. harzianum*, isolate AM1 shared 99% similarity with *A. nomius* and isolate AM2 shared 99%–100% similarity with *A. tubingensis*. Meanwhile, sequence

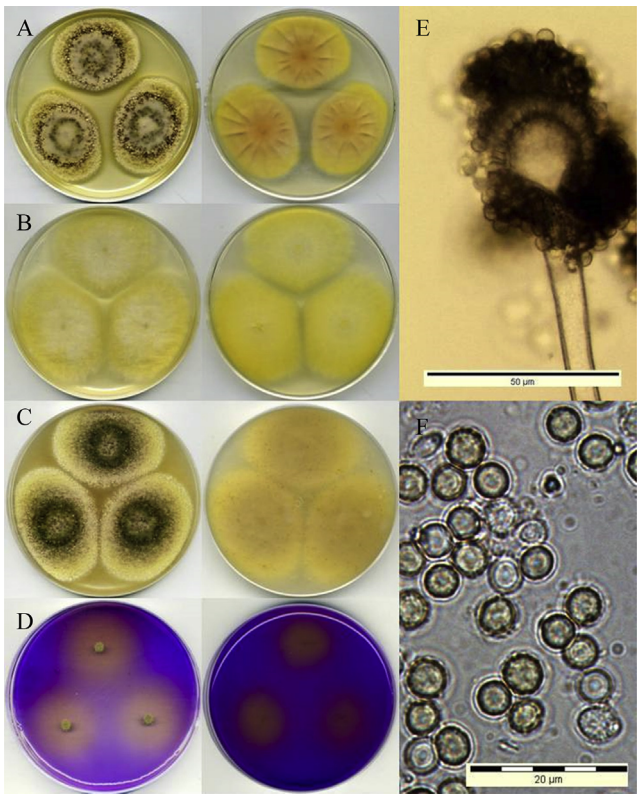

Figure 2. Colony appearance and pigmentation of *A. nomius* on different agar media.
A: MEA; B: CYA; C: OA; D: CREA; E: Uniseriate phialides; F: Globose to subglobose, finely roughened conidia.

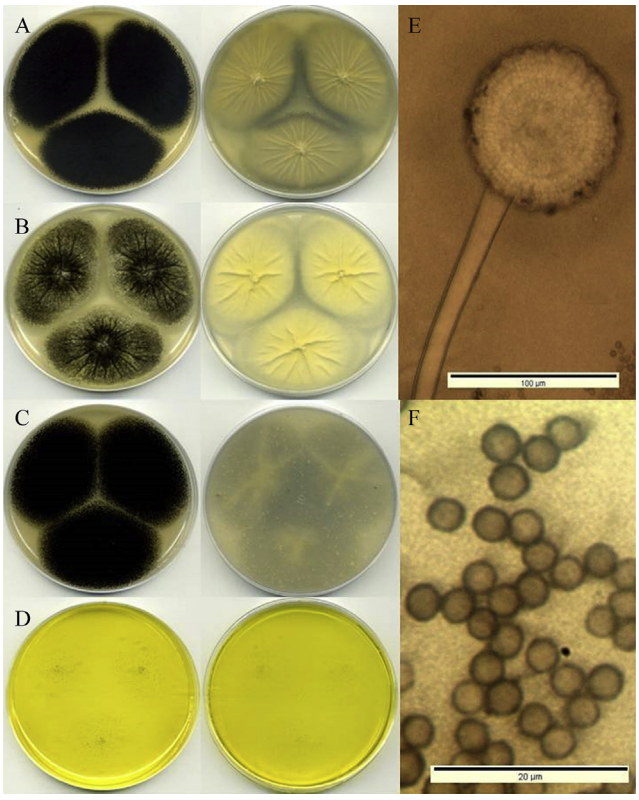

Figure 3. Colony appearance and pigmentation of *A. tubingensis* on different agar media.
A: MEA; B: CYA; C: OA; D: CREA; E: Globose vesicle with biseriate phialides; F: Finely roughened, globose conidia.

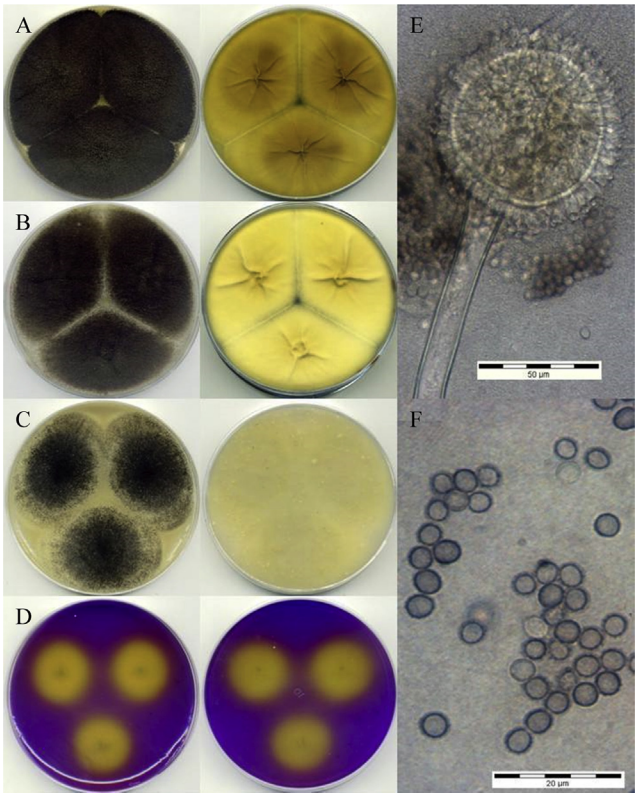

Figure 4. Colony appearance and pigmentation of *A. aculeatus* on different agar media.
A: MEA; B: CYA; C: OA; D: CREA; E: Subglobose vesicle with uniseriate phialides; F: Globose to subglobose conidia.

similarity of isolate AM3 showed 100% similarity to *A. aculeatus*. Sequence results of all four fungal isolates are summarized in Table 1.

4. Discussion

Fungus is an opportunistic organism that is able to develop rapidly depending on favourable temperature and moisture [39]. Despite of their main roles in contaminating large amount of agricultural crops in storage or field, they also had potential in becoming natural enemy of insect [40,41]. A form of defense mechanism produced by fungus is known as mycotoxin, secondary metabolites that may also affect human health and insect which however, relied on the amount of toxicity produced [42].

In this study, bed bugs (*Cimex hemipterus*) are dominantly affected by *T. harzianum* as well as *Aspergillus* sp. namely *A. nomius*, *A. tubingensis* and *A. aculeatus*. *Trichoderma* sp. are commonly found in enclosed environment such as in dust and water-damaged buildings, while a study conducted by Docampo *et al.* showed that spores of *Aspergillus* are predominantly found in both indoor and outdoor environment [43–45]. A ten years study performed by Falvey and Streifel also reported that it is almost impossible that any environment is completely devoid of *Aspergillus* spores thus making the exposure to *Aspergillus* is difficult to avoid [46]. In Malaysia's warm climate, *Aspergillus* sp. are proven to be widespread in indoor environments [47]. However, the occurrence of *Trichoderma* sp. in enclosed building has never been reported thus far. Apart of being mycoparasitic towards other fungi, *Trichoderma* sp. also can be pathogenic towards insects [48]. Production of enzymes and antibiotics in *Trichoderma* sp. causes a decrease in other plant pathogens as well as insect cuticle when they are exposed to the fungal strain. This includes studies revolving peptaibols production in *T. harzianum* strain that represent insecticidal activity against plant pathogens and insect [49]. *T. harzianum*, for instance, has been tested in previous studies in controlling termites through their feeding behaviour by growing fungal strain along with the bait but the outcomes showed fluctuated responses [31]. *Aspergillus* sp. on the other hand, has been discovered majorly contaminating early harvest crops including corn, barley, wheat and many others. Some of the species in the genus *Aspergillus* are also known to produce secondary metabolites called mycotoxins which have been reported to be carcinogenic, teratogenic, tremorgenic, haemorrhagic, nephrogenic, hepatogenic and dermatitis to both humans and animals [50–52].

Aflatoxins which is mass-produced of some *Aspergillus* sp., is the most carcinogenic mycotoxin that may cause aflatoxicosis, an infection involving vital organ like liver wihch could be useful in biological studies for further discovery in biological control [2]. *A. nomius* retrieved from this study is classified as the strain predominantly formed uniseriate conidial heads. This species has the ability to produce aflatoxin B_1 and G_1 [53]. Commonly, aflatoxin is classified as Group 1 carcinogens by the International Agency for Research on Cancer but aflatoxin B_1 has been proved to have the highest toxicity. In the meantime, ochratoxins A which is mostly found in *A. tubingensis* and *A. aculeatus* is classified as Group 2B that has less toxic effects. To this date, researches regarding their toxins were continuously carried out despite their toxicity uses. Reports using entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* in controlling hematophagous insect including biting midges, mosquitoes and even bed bugs have been recently updated [54,55]. This is due to their ability in probing throughout the cuticles of the host and eventually kills the entire population when the infected insect came in contact with other colony members. Nevertheless, investigations on developing fungal strains as biological agents especially from these two species in this research against household insect pest should be further studied to prevent harmful effects in the surrounding.

Morphological and molecular identification using ITS region and *β-tubulin* gene confirmed the identity of non-aggressive type 1 (Th 1) *T. harzianum*. Published works on morphological characteristics and phylogenetic analysis of *Trichoderma* species suggest that this genus is a species complex [23,56]. In case of *T. harzianum*, four genetically distinct biotypes, Th 1 to Th 4 have been recognized in which Th 1 and Th 3 are non-aggressive and Th 2 and Th 4 are aggressive types. Molecular data has shown that these four biotypes are in fact different species. For instance, Th 1 is *T. harzianum*, Th 2 is *Trichoderma aggressivum*, Th 3 is *Trichoderma atroviride* and Th 4 is *Trichoderma aggressivum f. europaeum*. Identifying all these species is difficult given that they are morphologically indistinguishable and their complexity has long been discussed by numerous authors [57–59]. According to Samuels *et al.*, several morphological characteristics of *T. harzianum* differed from others such as the fast growth rate on media cultures, smaller conidia, less chlamydospore formation and shorter

phialides [23]. Those are quantifiable characters that separate this species from other biotypes. However, only one strain of *T. harzianum* was isolated from bed bug sample, thus, characters comparison with other biotypes was not possible in this study. It is now widely acknowledged that morphological and colony appearance alone is insufficient to accurately identify this species. For that reason, DNA sequencing using *β-tubulin* gene and ITS region was used to support morphological identification of this species.

With respect to species identification in the genus *Aspergillus*, three species belonged to the different sections were identified, namely, *A. nomius* from section *Flavi*, *A. tubingensis* and *A. aculeatus* from section *Nigri*. The typically yellow to green conidial heads of *A. nomius* as well as brown to black colour of *A. tubingensis* and *A. Aculeatus* colonies were in agreement with the descriptions by Raper and Fennell [60] and Klich [36] who described colour of the colonies as the distinguishable characters of section *Flavi* and *Nigri*. Balajee *et al.* suggested that species in section *Nigri*, *Flavi* and *Fumigati* are referred as species complex due to the complexity and variability of phylogenetic relationship within *Aspergillus* section [33].

Additional works on possible metabolite compounds including mycotoxins produced by each of these fungi, however, should be done prior to select a potential candidate as a bed bug controlling agent.

Overall, the new discovery of common pathogens in agricultural field developed in live bed bugs storage tank may initiate the use of biological agents in later years. This however includes further researches by inventing such formulations and upgrading existing biopesticide so that it is well-adapted to the environment while effectively reduce the pest population.

Conflict of interest statement

We declare that we have no conflict of interest.

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