



Isolating and identifying fungi to determine whether their biological properties have the potential to control the population density of mosquitoes



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ABSTRACT

Mosquitoes transmit diseases such as dengue, chikungunya, Zika, and yellow fever to humans. Biological control methods are required for these insects because they can be environmentally friendlier, safer, and more cost-effective than chemical or physical methods currently available. The aim of this research is to identify fungi found in mosquito breeding containers that have the potential to control the population density of mosquitoes.

For the identification, water samples were taken from mosquito breeding containers situated in seven districts of Bangkok to obtain pure cultures. Deoxyribonucleic acid (DNA) was extracted from the cultures then sent for sequencing and analyzing. The results show that fourteen strains of fungi were isolated. The most common strain found was *Aspergillus* spp., which was present in 31 of the 78 fungi samples. The strains *Metarhizium anisopliae* and *Penicillium citrinum* were found to be interesting because they may have the potential to act as entomopathogenic fungi. The biological properties of these strains should be further investigated because they could help in the fight against mosquito-borne diseases.

1. Introduction

Mosquitoes are small insects that carry arboviruses belonging to the following three families: Flaviviridae, which has been associated with dengue fever; Bunyaviridae, which has been linked to chikungunya; and Zika Togaviridae, which has been related to arthritis, encephalitis, and rubella [1, 2]. The *Anopheles*, *Aedes* and *Culex* genera, have been responsible for the majority of arbovirus transmission. *Aedes aegypti* is a highly anthropophilic species that has impacted significantly on public health by transmitting arboviruses such as dengue, Zika, chikungunya and yellow fever [3].

A reduction in dengue fever transmission could be achieved by controlling the population density of *Aedes aegypti* and ensuring it is maintained at a level below the critical threshold that signals an epidemic [4]. A variety of chemical, physical, and biological methods have been used to decrease the incidence of vector-borne diseases transmitted by mosquitoes [5]. However, the use of chemical insecticides has resulted in mosquitoes becoming resistant to treatment and beneficial non-target

animals being harmed [6]. Despite this, new nanobiotechnology materials such as selenium nanowires (Cr-SeNWs) [7, 8] and phytofabricated zinc oxide (ZnO) nanoparticles are still being developed [9].

In recent years, incidences of mosquito larvae becoming resistant to larvicide have been reported [10]. Therefore, attempts to exploit novel biocontrol agents and new strategies to control mosquito larvae are still necessary because they can minimize the use of synthetic chemical materials which often have negative effects on humans, animals, and the environment.

Biological control methods are environmentally friendlier, safer, and more cost-effective than chemical or physical methods that are time-consuming to implement at mosquito breeding sites [3, 4, 5]. Natural control techniques have utilized the larvae of *Taxorhynchites*, predators such as fish, copepods or parasitic organisms such as *Bacillus thuringiensis israelensis*, and *Lysinibacillus sphaericus* to target disease vectors [3]. As entomopathogenic fungi are mostly targeted towards adult mosquitoes, and because several different toxins produced during fungal infection are lethal to mosquitoes [11], selection pressure for resistance is likely to be

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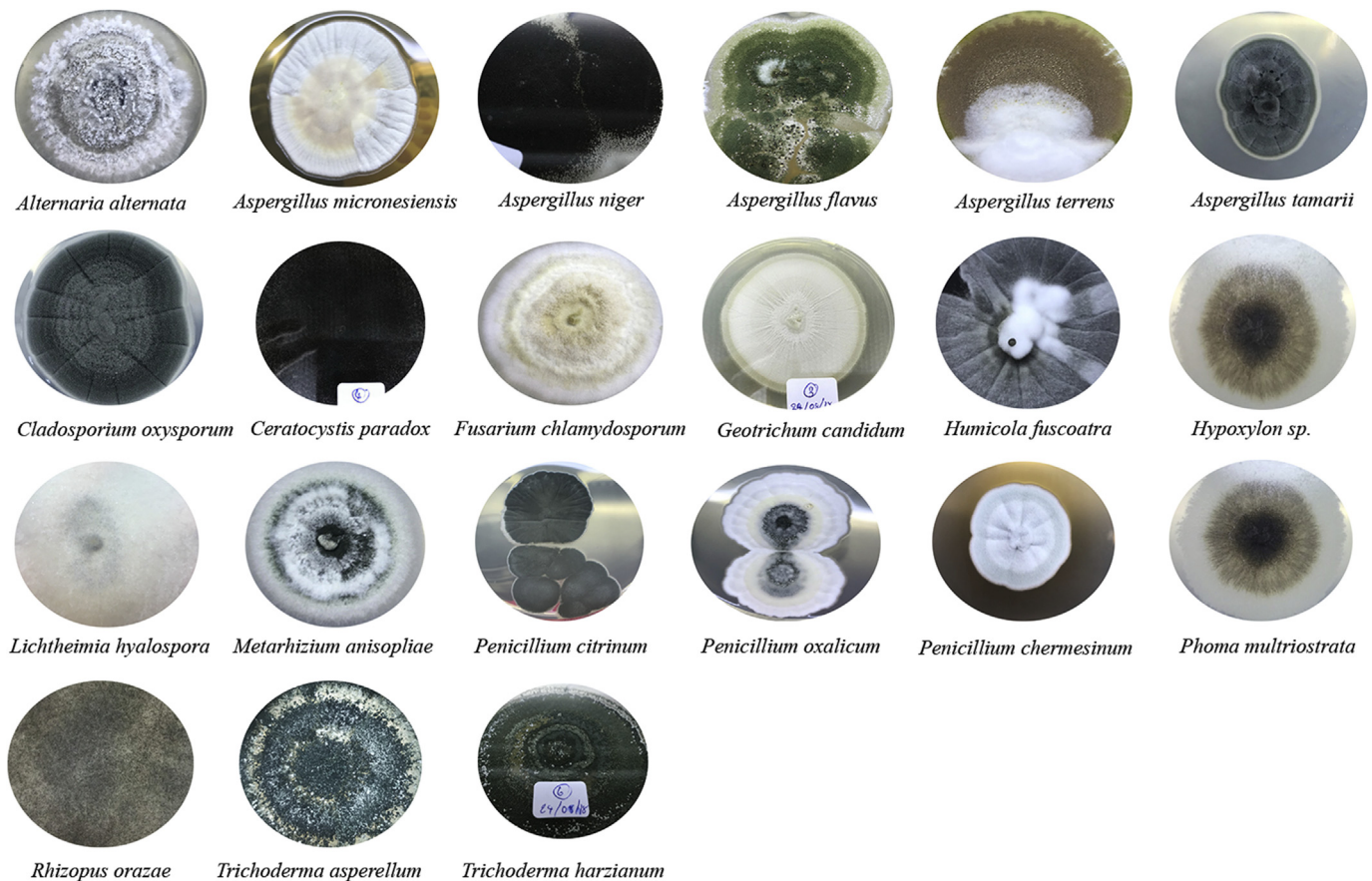


Fig. 1. Colony and conidia of isolated fungi on PDA agar medium.

less intense when compared to rapid-killing insecticides. Therefore, the evolution of fungus resistance is predicted to be much slower than the evolution of insecticide resistance [12]. The paucity of studies describing the effects of fungi on mosquito populations indicates further research is needed to determine the viability, infectivity, and persistence of fungal spores in the mosquito population [13].

Water collecting containers that serve as larval breeding habitats for mosquitoes, such as old tires, plastic cups, vases, flowerpots, water tanks, bowls pots, and coconut shells can be found throughout Bangkok. From the end of the monsoon season until the start of winter (collection period), these containers harbor still water that is suitable for egg-laying mosquitoes [14]. In addition, heterotrophic fungi and pluricellular organisms that play an ecological role in degrading organic matter are important parasites that can occur, especially, in environments that have soil debris, insect remains, or dead leaves and plants [15]. Often, fungi share the same breeding habitat as mosquitoes.

The aim of this research is to identify fungi found in mosquito breeding containers from seven area of Bangkok that have the potential to control the population density of mosquitoes.

2. Materials and methods

2.1. Water sample collection

Water samples were collected from mosquito breeding containers situated in seven districts of Bangkok, Thailand (Bang Khen, Lat Krabang, Min Buri, Phra Khanong, Rat Burana, Taling Chan, and Thung Khru). The collection period was from the end of the monsoon season until the start of winter, when the temperature and relative humidity conditions were favorable for Aedes mosquitoes to breed. The samples were kept in 250

ml screw-capped sterilized bottles that were sealed and then stored at 4 °C prior to the isolation procedure [16, 17].

2.2. Isolation of filamentous fungi

First, the water samples were filtered through a 47 mm diameter sterile 0.45 µm membrane cellulose nitrate filter (Whatman, 7141-104; Whatman International Ltd, UK) using Millipore vacuum apparatus in a laminated flow chamber. Then, the filter was thoroughly washed for 30 seconds in a petri dish containing 4 ml of sterile water. Next, 1 ml of sterile water was cultured on Potato Dextrose Agar (PDA, Difco, BBL/USA) supplemented with 50 mg/l of Chloramphenicol and 25 mg/l of Gentamycine. Three replicates were used for each water sample to ensure the accuracy of the results. The agar plates were incubated at 28 °C ± 2 °C for 7–10 days and then monitored daily for the appearance of fungal colonies. Finally, the fungal isolates were subcultured separately to obtain pure cultures on PDA for identification [18, 19, 20, 21].

2.3. Identification of filamentous fungi

Colony descriptions were based on PDA observations under ambient daylight conditions. Microscopic observations and measurements were logged from preparations that were mounted in lactic acid. The morphological characteristics of the fungi were septation of hypha, formation, morphology, conidiophores, and the branching frequency of the fruiting bodies. Characteristics of the colony such as structure, size, and color were also observed and recorded. Filamentous fungi were identified at the generic level according to morphological characteristics described in fungal atlases.

Table 1

The collection district, the genus of the isolated fungi and the number of isolated fungi identified.

District	Genus	Isolate
Lat Krabang (total 22 isolates)	<i>Aspergillus</i> spp.	5 isolates
	<i>Penicillium</i> spp.	4 isolates
	<i>Rhizopus</i> spp.	1 isolate
	<i>Trichoderma</i> spp.	5 isolates
	<i>Geotrichum</i> spp.	1 isolate
	<i>Cladosporium</i> spp.	3 isolates
	<i>Fusarium</i> spp.	1 isolate
	<i>Metarhizium</i> spp.	1 isolate
	<i>Alternaria</i> spp.	1 isolate
	<i>Aspergillus</i> spp.	1 isolate
	<i>Penicillium</i> spp.	3 isolates
Rat Burana (total 6 isolates)	<i>Trichoderma</i> spp.	1 isolate
	<i>Cladosporium</i> spp.	1 isolate
	<i>Aspergillus</i> spp.	10 isolates
	<i>Penicillium</i> spp.	3 isolates
Thung Khru (total 20 isolates)	<i>Trichoderma</i> spp.	1 isolate
	<i>Cladosporium</i> spp.	3 isolates
	<i>Ceratocystis</i> spp.	1 isolate
	<i>Phoma</i> spp.	1 isolate
	<i>Hypoxyton</i> spp.	1 isolate
	<i>Aspergillus</i> spp.	10 isolates
	<i>Penicillium</i> spp.	1 isolate
	<i>Rhizopus</i> spp.	2 isolates
Phra Khanong (total 16 isolates)	<i>Trichoderma</i> spp.	1 isolate
	<i>Humicola</i> spp.	1 isolate
	<i>Lichtheimia</i> spp.	1 isolate
	<i>Aspergillus</i> spp.	2 isolates
	<i>Penicillium</i> spp.	3 isolates
Taling Chan (total 8 isolates)	<i>Trichoderma</i> spp.	2 isolates
	<i>Cladosporium</i> spp.	1 isolate
	<i>Aspergillus</i> spp.	1 isolate
	<i>Rhizopus</i> spp.	1 isolate
Min Buri (total 3 isolates)	<i>Trichoderma</i> spp.	1 isolate
	<i>Aspergillus</i> spp.	2 isolates
	<i>Penicillium</i> spp.	1 isolate

2.4. The sequencing and analyzing of fungal strains by ITS

The DNA was extracted from the isolated fungi using a fungal DNA extraction kit (Presto™ Mini gDNA Yeast kit; Geneaid, New Taipei City, Taiwan). Purified DNA specimens were amplified in a PCR reaction with universal primers of ITS1: 5'-TCCGTAGGTGAACCTGCGC-3' and ITS4: 5'-TCCTCGCTTATTGATATGC-3' [21]. The PCR reaction contained 0.4 μM of ITS1 and ITS4 primers, 0.2 mM dNTPs, 1.5 mM MgCl₂, 1xPCR buffer (50 mM KCl, 10 mM Tris-HCl), and 1.25 units of *Taq* DNA polymerase (New England Biolabs). The reaction was carried out in a BIO-RAD MJ Mini Personal Thermal Cycler. The cycle conditions consisted of a single initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The PCR product size was about 550 bp. It was sent to Solutions for Genetic Technologies in South Korea for sequencing.

First, the resulting sequences were checked and aligned using the BioEdit 7.0 sequence alignment editor (Isis Pharmaceuticals, Inc., Carlsbad, CA, USA). Then, they were compared with a homologous sequence stored on the GenBank database. Finally, the BLAST program, downloaded from the National Center for Biotechnology Information (NCBI) website, was used to evaluate the sequences.

3. Results

Seventy-eight fungal isolates were found in water samples collected from seven districts around Bangkok. The results of the rDNA-ITS sequencing show that 550 bp of the PCR product was sequenced from the isolated strains. The sequences were compared with the 18S rDNA BLAST sequence stored on the GenBank database using the BLAST tool. Twenty-one strains of fungi were isolated, as shown in Fig. 1.

Aspergillus spp. was the most dominant of the isolated strains, it was

found in all the districts of Bangkok that were sampled for this research, as shown in Table 1. The following six species of *Aspergillus* were identified: *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus alterata*, *Aspergillus flavus*, and *Aspergillus tamarii*. These species of *Aspergillus* consisted of 31 isolates. Six isolates of this strain accounted for 40 % of the total population, as shown in Fig. 2. *Penicillium* spp. was found in all the districts, except Min Buri. The following three species of *Penicillium* were identified: *P. oxalicum*, *P. citrinum*, and *P. chermesinum*. These species of *Penicillium* consisted of 15 isolates, which accounted for 19% of the total population. The following two species of *Trichoderma* spp. were identified: *T. asperellum* and *T. harzianum*. These species of *Trichoderma* consisted of 11 isolates, which accounted for 14 % of the total population. Four isolates of *Cladosporium oxysporum* were identified, which accounted for about 5 % of the total population. In addition, the following minor species with only one or two isolates were identified: *Geotrichum candidum*, *Ceratocystis paradox*, *Fusarium chlamydosporum*, *Metarhizium anisopliae*, *Phoma multirostrata*, *Hypoxyton sp*, *Humicola fuscoatra*, and *Lichtheimia hyalospora*.

4. Discussion

Female *Aedes* mosquitoes breed in various artificial containers such as discarded tires and old flower pots. Uniquely, they lay their eggs in a variety of habitats to spread them around and increase their offsprings' survival probability. This leads to an abundance of mosquitoes that have the potential to transmit dengue, chikungunya, Zika and other viral infections [22]. In their immature stages, they are non-selective filter-feeders of organic particles such as bacteria, protozoa, and fungi that are suspended in water [23]. Generally, fungi live in the alimentary canal of insects as symbiotic microorganisms, however, some fungi can attack and kill their hosts.

Monitoring the diversity of filamentous fungi as part of mosquito vector management could considerably benefit public health. This is the first study to isolate and identify fungi gathered from mosquito breeding containers situated in Bangkok. The results of the BLAST tests show that two strains of the entomopathogenic fungi *Metarhizium anisopliae* and *Penicillium citrinum* may have the potential to biologically control mosquitoes.

Prior research [24, 25] has shown that *Aspiragilus* spp., *Rhizopus* spp., *Geotrichum* spp., and *Saccharomyces* were present in the water of breeding containers and larval food. The results demonstrated that all the mosquito larvae were exposed to either oral or cuticle fungal infection [24]. Some fungi have developed symbiotic relationships or can be pathogenic due to toxin production [25]. There may be naturally occurring fungi at mosquito breeding sites that have the potential to kill insects. Research has been published that investigated the interactions between fungi and Culicidae larvae [26, 27]. *Smittium morbosum* were found to be lethal to mosquito larvae, and they have been known to invade ovaries and produce cysts which are "oviposited" by females instead of eggs, thus reducing the fertility in populations of those kinds of aquatic insects. The results suggested that mosquitoes might have the potential to serve as a type of biological control [28, 29, 30]. Entomopathogenic fungi have shown promise as biological control agents of mosquito vectors of tropical diseases [31].

M. anisopliae or *Entomophthora anisopliae* is a fungus found in nature throughout the world which has caused disease in various insects by acting as a parasitoid [13]. *M. anisopliae* has infected mosquitoes in the genera of *Aedes* and *Culex* [32, 33]. A histological study has reported that the ingestion of conidia eventually leads to blockage of the anatomic structure [34]. Conidia attach to the cuticle, germinate, and penetrate the cuticle. Once in the hemocoel, the mycelium grows throughout the host, forming hyphal bodies called blastospores. Death of the insect is often due to a combination of the action of fungal toxins, physical obstruction of blood circulation, nutrient depletion, and invasion of the organs. After the host has died, hyphae usually emerge from the cadaver and, under suitable abiotic conditions, conidia are produced on the exterior of the

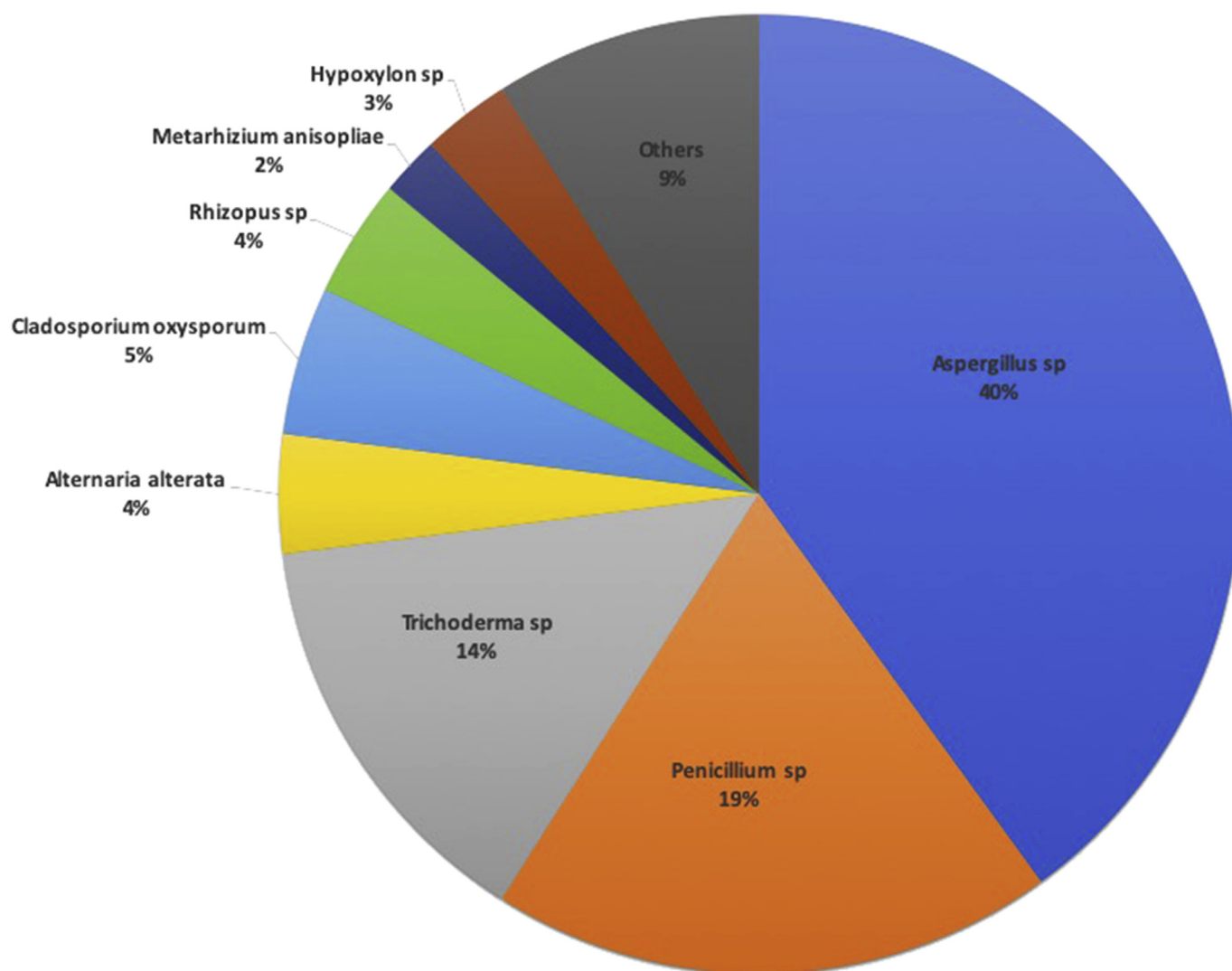


Fig. 2. The percentage of isolated strains of fungi gathered from the water samples.

host. Then, they are dispersed by wind or water [35]. The gut of mosquito larvae was found to contain high concentrations of conidia, suggesting that it is usually ingested [33]. Experiments have shown that the mortality of mosquito larvae is related to stress-induced apoptosis [36]. Blastospores produced by the *Metarhizium* species have potential for field applications because they exhibit a high degree of host specificity and cause mortalities in mosquito species rapidly after ingestion [3].

P. citrinum, which was also isolated, may have the potential to control mosquitoes biologically. This fungus has been found to cause mortalities in *Culex quinquefasciatus* or the southern house mosquito [37], which is a carrier of the West Nile and Japanese encephalitis virus [38]. Light transmission electron micrographs showed that the fungal conidia were ingested by the larvae and deposited in the gut [39].

The results of this research show that the most abundant genus found was *Aspergillus spp.* This may be because these fungi grow as saprophytes on decaying vegetation, soil debris, insect remains, dead leaves or plants, and organic compost piles that are commonly found in artificial mosquito breeding containers. Six of the thirty isolated fungi were found to be of the *P. citrinum* species. Further research should evaluate whether a combination of *M. anisopliae* and *P. citrinum* could be used to manage mosquito populations.

Some bacteria such as *Bacillus thuringiensis israelensis* and *Lysinibacillus*

sphaericus have been used to control mosquito populations. The toxins produced by *Bti* and *Ls* have the advantage of being highly effective as larvicides for various vector species of arboviruses. *Bti* has been broadly effective against mosquitoes in the genera of *Aedes*, *Culex*, and *Anopheles*, whereas the toxicity of *Ls* has been limited to *Culex* and *Anopheles* [40].

Research into the application of microorganism insecticides has found that mosquitofish or *Gambusia affinis* were compatible with the simultaneous use of other chemical or biological control tools [41]. An experiment in the rice fields of California, that mainly focused on *Culex tarsalis*, evaluated the release of *G. affinis* followed by treatment with *Bacillus thuringiensis israelensis* [42].

In the future, we hope to further investigate the isolates of *M. anisopliae* and *P. citrinum* to determine whether they have biological control properties that could be used to manage the mosquito populations.

5. Conclusions

In this research, several genera of isolated fungi were identified from water samples taken from artificial containers situated in districts around Bangkok, however, only two possible entomopathogenic fungi, *M. anisopliae* and *P. citrinum*, were identified. Our future research will

focus on these fungi to determine whether their biological control properties could be used to manage mosquito populations.

Declarations

Author contribution statement

Ladawan Wasinpiyamongkol: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Panan Kanchanaphum: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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