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Entomopathogenic Fungi from South Sumatra (Indonesia) Pathogenicity to Egg, Larvae, and Adult of *Aedes aegypti*

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ABSTRACT

Fungi from South Sumatra (Indonesia) were identified morphologically and molecularly, and their pathogenicity to egg, larvae, and adult *Aedes aegypti* was evaluated. The fungal isolates used for bioassay were 11 isolates from this study and 4 isolates from the laboratory collection. Fifteen isolates of five fungal species (*Metarhizium anisopliae, Penicillium citrinum, Talaromyces diversus, Beauveria bassiana*, and *Purpureocillium lilacinum*) from South Sumatra, Indonesia, were pathogenic to the egg, larvae, and adult of *Ae. aegypti.* Egg mortality caused by *M. anisopliae* isolate MSwTp3 was the highest (38.31%). A novel finding of this study was that the eggs exposed to the fungus not only killed the eggs but could continue to kill the emerging larvae, pupae, and adults. The five fungal species induced larval mortality between 52.22−94.44% and adult mortality between 50.00−92.22%. Fungal strains belonging to *M. anisopliae, P. citrinum, T. diversus,* and *B. bassiana* from South Sumatra seem to possess remarkable ovicidal, larvicidal and adulticidal activity against *Ae. aegypti. M. anisopliae, P. citrinum, T. diversus,* and *B. bassiana* had the potential as entomopathogens to be developed into ovicides, larvicides, and adulticides for controlling *Ae. aegypti.*

1. Introduction

Indonesia has the second-highest species diversity of mosquitoes in the world after Brazil (Nugroho *et al.* 2019). *Aedes aegypti* is the most important of the many mosquito species because it acts as a primary vector of dengue, chikungunya, and yellow fever viruses that have spread worldwide (Nugroho *et al.* 2019). In Indonesia, the spread of mosquitoes has occurred, including in Kendari (Aulya and Idris 2020), Central Java (Khariri 2018), Banjarmasin (Hamid *et al.* 2018), Jakarta (Hamid *et al.* 2017), and South Sumatra (Pratiwi *et al.* 2019). These mosquito outbreaks have rapidly transmitted dengue, chikungunya, and yellow fever viruses and become endemic (Lozano-Fuentes *et al.* 2012). These diseases are major public health problems in tropical countries (Weaver 2014). The losses caused by dengue alone reach several billion dollars annually (Guzman and Harris 2015). For this reason, the chain of transmission of the dengue, chikungunya, and yellow fever must be broken by reducing or controlling the vector mosquito population, *Ae. aegypti*.

Population control of *Ae. aegypti* has been widely carried out and commonly used synthetic insecticides because of its fast action and easy application (Vontas *et al.* 2012). However, several synthetic insecticides have caused *Ae. aegypti* resistant, for example, bendiocarb, permethrin (Hamid *et al.* 2018), pyrethroid (Hamid *et al.* 2017), and temephos (Grisales *et al.* 2013). In addition, residues of synthetic insecticides can cause human health problems and water, air, and soil pollution (Hamid *et al.* 2017). Eco-friendly alternate control uses botanical insecticides from plant extracts, attractants, and entomopathogens (pathogens that cause insect disease) (Nur Athen *et al.* 2020; Raveen *et al.* 2017). There are two Entomopathogens, bacteria, such as *Bacillus thuringiensis* (Pruszynski *et al.* 2017) and fungi, such as *Metarhizium anisopliae* (Butt *et al.* 2013; de Paula *et al.* 2021; Leles *et al.* 2012) and *Beauveria bassiana* (Lee *et al.* 2019).

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Entomopathogenic fungi are one of the most widely used groups of entomopathogen agents for controlling *Ae. aegypti*, for instance, *M. anisopliae* (Leles *et al.* 2012), *Metarhizium brunneum* (Alkhaibari *et al.* 2017), and *B. bassiana* (Lee *et al.* 2019). Blastospores and conidia of *M. brunneum* proved to be effective in killing larvae of *Ae. aegypti* (Alkhaibari *et al.* 2017) and the blastospores kill faster (only 12-24 hours) compared to the conidia (Alkhaibari *et al.* 2016). *M. anisopliae* (Butt *et al.* 2013; de Paula *et al.* 2021; Leles *et al.* 2012) and *B. bassiana* also effectively kill larvae of *Ae. aegypti* (Lee *et al.* 2019). The entomopathogenic fungi have the advantage of being able to infect and kill eggs, larvae, and adults of mosquitoes (Greenfield *et al.* 2015). There is no information on Indonesia's pathogenicity of entomopathogenic fungi to kill the *Ae. aegypti* eggs, larvae, and adults. The results of previous studies have proven that the species of entomopathogenic fungi from Indonesia could kill (80−100% mortality) several insect species of agricultural pests are *B. bassiana* (Sumikarsih *et al.* 2019), *M. anisopliae* (Herlinda *et al.* 2020b), *Curvularia lunata* (Herlinda *et al.* 2021), *Penicillium citrinum*, and *Talaromyces diversus* (Herlinda *et al.* 2020a). In this study, fungi from South Sumatra (Indonesia) were identified morphologically and molecularly, and their pathogenicity to egg, larvae, and adult *Ae. aegypti* was evaluated.

2. Materials and Methods

Fungal exploration was carried out by collecting fungal inoculum from the soil and infected insect host cadavers in South Sumatra, Indonesia. Purification and identification of the fungi were carried out from January to March 2021. The entomopathogenic fungus species were identified based on the molecular analysis at a laboratory accredited according to the ISO 17025 standard of Agricultural Biotechnology, Department of Plant Protection, Faculty of Agriculture, Universitas Lampung, Indonesia.

2.1. Exploration, Isolation, and Purification of Fungi

Isolation of fungi from soil was performed following the method of Anwar *et al.* (2015), *Tenebrio bait* method by using larvae of *Tenebrio molitor* (yellow mealworm beetle), while the characterization of fungi from infected insects followed the method of Ab Majid *et al.* (2015) by collecting sick insects or cadaver infected with the fungi in the fields. Fungal exploration was carried out from the lowlands to the highlands of South Sumatra, namely in Ogan Ilir Regency (3.43186°S 104.6727°E), Palembang City (2°59′27.99″S 104°45′24.24″E), Pagar Alam City (3°52′43.8″S 103°21′30″E), Lahat District (3.78639°S 103.54278°E), Muara Enim District (4.2327°S 103.6141°E), and Banyuasin District (2.8833°S 104.3831°E). The cadaver insects infected by the fungus were surface sterilized using the method of Elfita *et al.* (2019) with 70% EtOH (Ethyl alcohol) and 1% NaOCl, then rinsed 3 times. Then, the cadavers were cultured onto Sabouraud Dextrose Agar (SDA) in a room with 26 ± 1°C temperature and 85±10% RH (Russo *et al.* 2020). Fungal culture on SDA media was purified to make an isolate per sample. The isolate was observed for the macroscopic and microscopic characteristics and continued by molecular identification. The morphological characteristics observed were the colonial color and shape, the conidial shape and size, and the conidiophores according to the method of Herlinda *et al.* (2020a).

2.2. DNA Extraction, PCR Amplification, and Sequencing

The fungal DNA extraction method used refers to the method of Swibawa *et al.* (2020). DNA extraction was carried out on 7 days old fungal conidia. PCR amplification was carried out using the Sensoquest Thermal Cycler (Germany) PCR machine in the Internal Transcribed Spacer (ITS) region using ITS1 and ITS4 primers (White *et al.* 1990). The PCR was carried out with a total volume of 25 µl consisting of a mixture of Master Mix (Red Mix) (bioline) as much as 12.5 µl, 10 µM of primer ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3') and ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3') 1 µl each, 1 L of template DNA and 9.5 µl of sterile water. The PCR results were then electrophoresed and then visualized using a DigiDoc UV transilluminator (UVP, USA).

The results of the sequencing were analyzed, using Bio Edit ver. 7.2.6 for windows and submitted to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine species that had the greatest homology or similarity molecularly. The phylogeny tree was designed using the Mega 7 for Windows program (Kumar *et al.* 2016), using the UPGMA (jukes and cantor model) method. The ITS region sequences for several strains used as a reference were obtained from NCBI (https://www.ncbi.nlm.nih.gov/).

2.3. Mass Rearing of *Ae. aegypti*

Eggs of *Ae. aegypti* were obtained from P2B2 Research and Development Loka, the Health Research and Development Center (the Balitbangkes), the Ministry of Health of Indonesia in Baturaja, South Sumatra. They have been identified molecularly and mass-rearing since June 2013. Furthermore, the cultures were incubated in a sterile room and the lighting was set to photoperiod 12:12 (L:D) h., 26±1°C temperature, and 85±10% RH following the method of Kauffman *et al.* (2017) at the Laboratory of Entomology, Faculty of Agriculture, Universitas Sriwijaya. The emerging larvae from the eggs were put into a disinfected transparent plastic cup (Ø 7 cm, height 9 cm) containing 50 ml of water and fed with dog biscuits according to the method of Vivekanandhan *et al.* (2018). The plastic cup containing the larvae was then put into a disinfected transparent plastic cage (50 x 50 x 50 cm) so that when the adults were emerging, they have remained in the the cage. For adult diet, the 10% sucrose solution impregnated on cotton wool was placed on the top of the cage. The newly emerged adult mosquitoes were still kept in the plastic cage, containing an ovitrap. The ovitrap was created following the method of Wu *et al.* (2013) that was a disinfected transparent plastic cup (Ø 9 cm, height 13 cm) whose wall was dark or black and filled with water as much as 3/4 of the height of the cup. Every day eggs were harvested for bioassay test.

2.4. Entomopathogenic Fungal Pathogenicity to the Egg, Larvae, and Adult of *Ae. aegypti*

The bioassay was carried out at the Laboratory of Entomology, Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Sriwijaya at the average temperature and the relative humidity, 28.77°C and 82.82%, respectively. The isolates used for the pathogenicity test were 11 isolates from the exploration of this study, and 4 isolates were taken from the collection of Siti Herlinda (Herlinda *et al.* 2020a) consisting of *P. citrinum* isolate BKbTp (GenBank acc. no. MT448730), *T. diversus* isolate MSwTp1 (GenBank acc. no. MT448731), *B. bassiana* isolate BSwTd4 (GenBank acc. no. MT448732), and *M. anisopliae* isolate MSwTp3 (GenBank acc. no. MT448733) (Table 1). All the isolates were grown in SDA medium, after the fungal culture was 14 days old, then the culture was transferred to the liquid medium, SDB (Sabouraud Dextrose Broth) following the method of Gustianingtyas *et al.* (2020) and the fungal cultured were carried out in a sterile laminar air flow room (Ayudya *et al.* 2019). When the fungus was cultured in SDB, it was shaken for 7 days and incubated at rest (not shaken) for 7 days, then the conidial density was calculated for testing pathogenicity to the egg, larvae, or adult of *Ae. aegypti*.

The bioassay of the entomopathogenic fungi against eggs of *Ae. aegypti* followed the method of Luz *et al.* (2011). The pathogenicity was assessed by

Table 1. Origin of the isolates of entomophatogenic fungi from South Sumatra, Indonesia

Location (village, district/city)	Isolate origin	Altitude (m)	Fungal species	Fungal isolate code	GenBank Acc. No.
Tanjung Pering. Ogan Ilir	Insect	36.0	*Beauveria bassiana*	LtTpOi	OM791684
Tanjung Steko. Ogan Ilir	Soil	36.0	*Beauveria bassiana*	TaTsOi	OM791686
Alang-alang Lebar, Palembang	Soil	23.0	*Beauveria bassiana*	TaAIPa	OM791688
Sukarami. Palembang	Soil	32.0	*Purpureocillium lilacinum*	TaSkPA	OM780287
Bangun Rejo. Pagar Alam	Soil	789.5	*Beauveria bassiana*	TaBrPGA	OM791682
Curup Jare. Pagaralam	Soil	806.0	*Beauveria bassiana*	TaCjPGA	OM791681
Air Perikan. Pagaralam	Insect	625.9	*Beauveria bassiana*	LtApPGA	OM791685
Kota Raya. Lahat	Insect	369.9	*Beauveria bassiana*	LtKrLH	OM791680
Tanjung Tebat. Lahat	Soil	377.0	*Beauveria bassiana*	TaTtLH	OM791683
Lebak. Muara Enim	Soil	33.5	*Beauveria bassiana*	TaLmME	OM791687
Purwosari. Banyuasin	Soil	19.0	*Beauveria bassiana*	TaPsBA	OM791689
Talang Patai. Pagar Alam	Soil	175.0	*Penicillium citrinum*	BKbTp	MT448730
Talang Patai. Pagar Alam	Soil	193.0	*Talaromyces diversus*	MSwTp1	MT448731
Talang Dabok. Ogan Komering Ilir	Soil	24.0	*Beauveria bassiana*	BSwTd4	MT448732
Talang Patai. Pagar Alam	Soil	193.0	*Metarhizium anisopliae*	MSwTp3	MT448733

pouring 10 ml of a suspension of entomopathogenic fungal isolate with a concentration of 1 x 10^{10} conidia/ml into the ovitrap containing 100 ml of water, while for the control only 10 ml of sterile distilled water was exposed. The treatments in this experiment were isolates/species of entomopathogenic fungi (15 isolates) and control (water), and repeated three times using a completely randomized design. Thirty gravid female adults that have copulated were put in a plastic cage in which there was an ovitrap for adults laying eggs. When exposed to the fungi, the mosquito gravid female were provided 10% sucrose solution for their diet and were allowed to lay eggs for 4 x 24 hours. Then, the ovitrap containing eggs was removed from the cage and the number of eggs laid and the hatched eggs were counted and recorded. The dead larvae and pupae were also recorded daily until adult stage, followed the method of Blanford *et al.* (2012). In addition, changes in egg morphology were observed every day. Unhatched eggs were grown in SDA medium to confirm the viability of microorganism that caused unhatched.

Pathogenicity of entomopathogenic fungi to larvae of *Ae. aegypti* was carried out by modifying method of Alkhaibari *et al.* (2017). The third-instar larvae (n = 30) of each isolate were exposed to 10 ml suspension of 1 x 10^{10} conidia/ml in a disinfected transparent plastic cups (Ø 7 cm, height 9 cm) containing 100 ml of water, while for control treatment, the larvae were exposed to 10 ml of sterile water, in triplicate. After 1 x 24 hours of exposure to the fungus, the dead larvae were observed and counted every day for 8 days. The variables considered were the number of larval deaths and the time of larval death for determining of LT_{50} and LT_{95}, the morphology of malformed larvae, and the behavior of unhealthy larvae. The dead larvae were grown in SDA medium to confirm the fungal infection.

Pathogenicity of entomopathogenic fungi to adults of *Ae. aegypti* was assessed by following method of Blanford *et al.* (2012) and Shoukat *et al.* (2020). Thirty adults (15 female and 15 male adults) per replication of 3-d-old *Ae. aegypti* were exposed to 1 x 10^{10} conidia/ml fungal suspension. Disinfected transparent plastic cage (50 × 50 × 50 cm) were sprayed with the 10 ml of the fungal suspension from inside and were air-dried for 2 h (Mnyone *et al.* 2011), while for control treatment, the cage was sprayed with 10 ml of sterile water and this experiment was repeated three times. For the adult diet, 10% sucrose solution was placed and hang on the cage. After fungal exposure for 24 hours, the adult mortality was monitored and recorded daily for 7 days. The adults with no movement were considered as dead (Shoukat *et al.* 2020). The other variables were the time of adults dying for determining of LT_{50} and LT_{95}, the morphology of malformed adults. The dead adults were grown in SDA medium to confirm the fungal infection and to determine whether the fungus emerged from the cadavers.

2.5. Data Analysis

The eggs laid data and the egg, larvae, and adult mortality data were analyzed using analysis of variance (ANOVA) and were statistically compared with Tukey's Honestly Significant (HSD) at a 5% level of significance. LT_{50} and LT_{95} were estimated for mortality time of larvae and adults and subjected to probit analysis. Differences in LT_{50} and LT_{95} were compared by ANOVA and were statistically compared with HSD at a 5% level of significance. All statistical analyses were calculated using software of SAS University Edition 2.7 9.4 M5. The morphology or malformation of eggs, larvae, pupae, and adults infected by the fungus were presented in photograph.

3. Results

3.1. Identification Results of the Entomopathogenic Fungal Species

The isolates of LtTpOi, TaTsOi, TaAIPa, TaBrPGA, TaCjPGA, LtApPGA, LtKrLH, TaTtLH, TaLmME, and TaPsBA had a white colony (Figure 1), the non-septate and globose conidia and the hyaline hyphae and mycelia (Figure 2). The result of BLAST search revealed that the isolates of LtTpOi, TaTsOi, TaAIPa, TaBrPGA, TaCjPGA, LtApPGA, LtKrLH, TaTtLH, TaLmME, and TaPsBA showed 99.38% of similarity to *B. bassiana* isolate GZMS-28 (Acc. No KT715480.1), strain TF6-1B (Acc No. JX122736.1) and isolate BSwTd4 (Acc. No. MT448732.1). Based on the phylogenetic tree, the 10 isolates were placed within group of *B. bassiana* isolate GZMS-28 (Acc. No KT715480.1), strain TF6-1B (Acc No. JX122736.1) and isolate BSwTd4 (Acc. No. MT448732.1) (Figure 3). The 10 isolates were deposited in the GenBank with the accession number OM791684 (LtTpOi), OM791686 (TaTsOi), OM791688 (TaAIPa), OM791682 (TaBrPGA), OM791681 (TaCjPGA), OM791685 (LtApPGA), OM791680 (LtKrLH), OM791683 (TaTtLH), OM791687 (TaLmME), and OM791689 (TaPsBA).

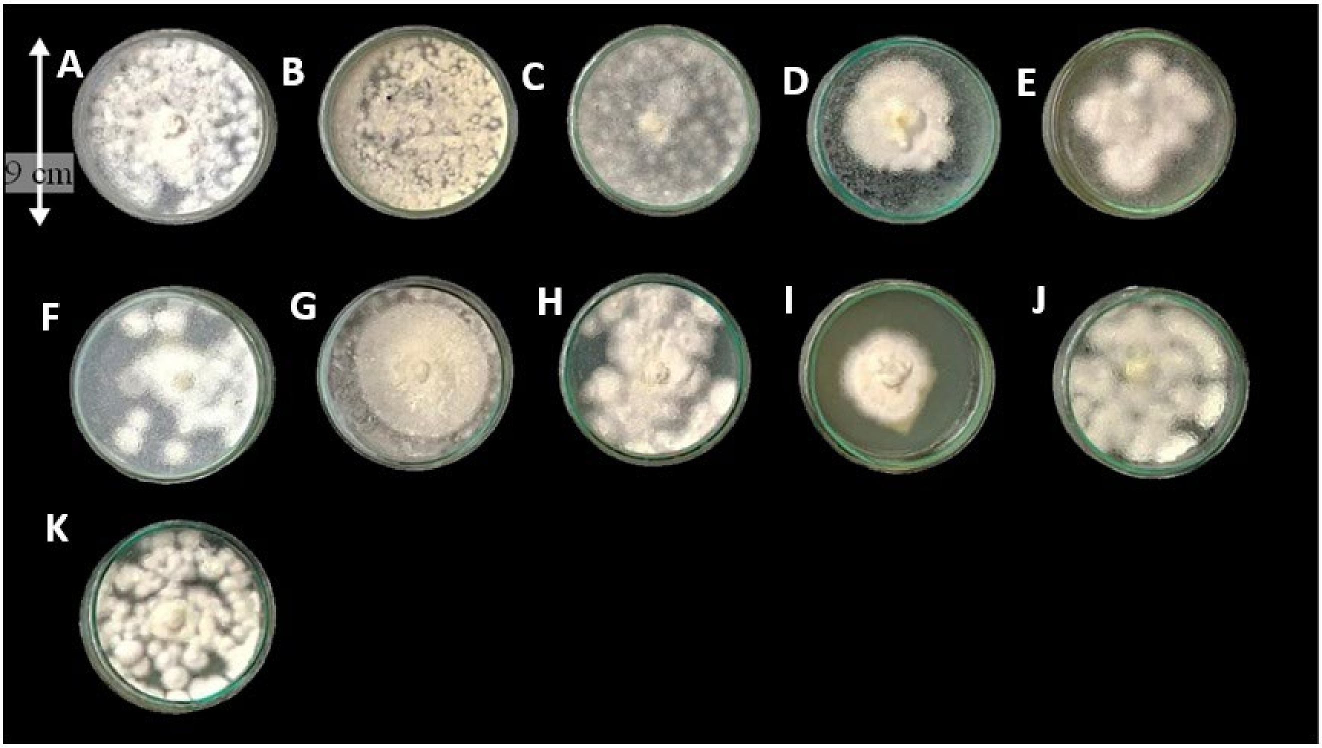

Figure 1. Colonial morphology of entomopathogenic fungal species: *Beauveria bassiana* isolates of LtTpOI (A), TaTsOI (B), TaAlPA (C), TaBrPGA (E), TaCjPGA (F), LtApPGA (G), LtKrLH (H), TaTtLH (I), TaLmME (J), TaPsBA (K), and *Purpureocillium lilacinum* isolate of TaSkPA (D)

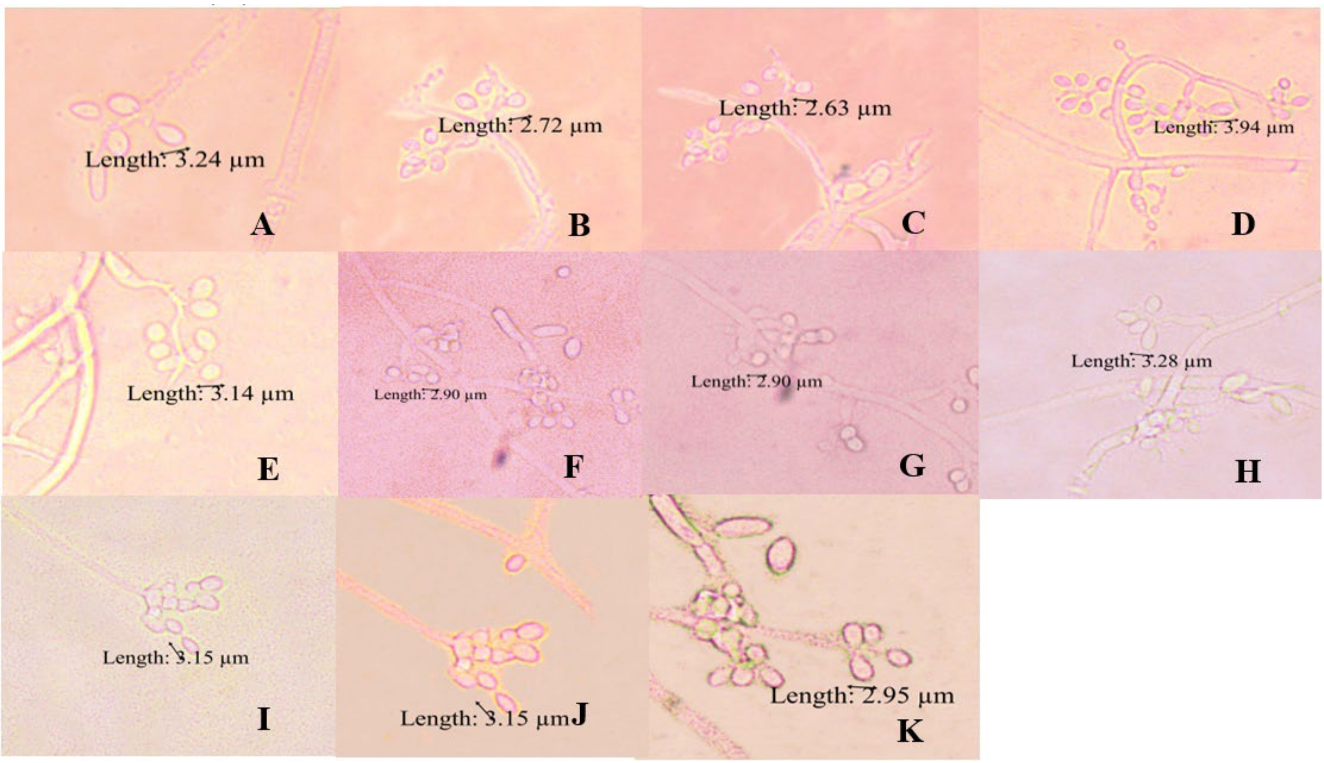

Figure 2. Conidial and hyphal morphology of entomopathogenic fungal species: *Beauveria bassiana* isolates of LtTpOI (A), TaTsOI (B), TaAlPA (C), TaBrPGA (E), TaCjPGA (F), LtApPGA (G), LtKrLH (H), TaTtLH (I), TaLmME (J), TaPsBA (K), and *Purpureocillium lilacinum* isolate of TaSkPA (D)

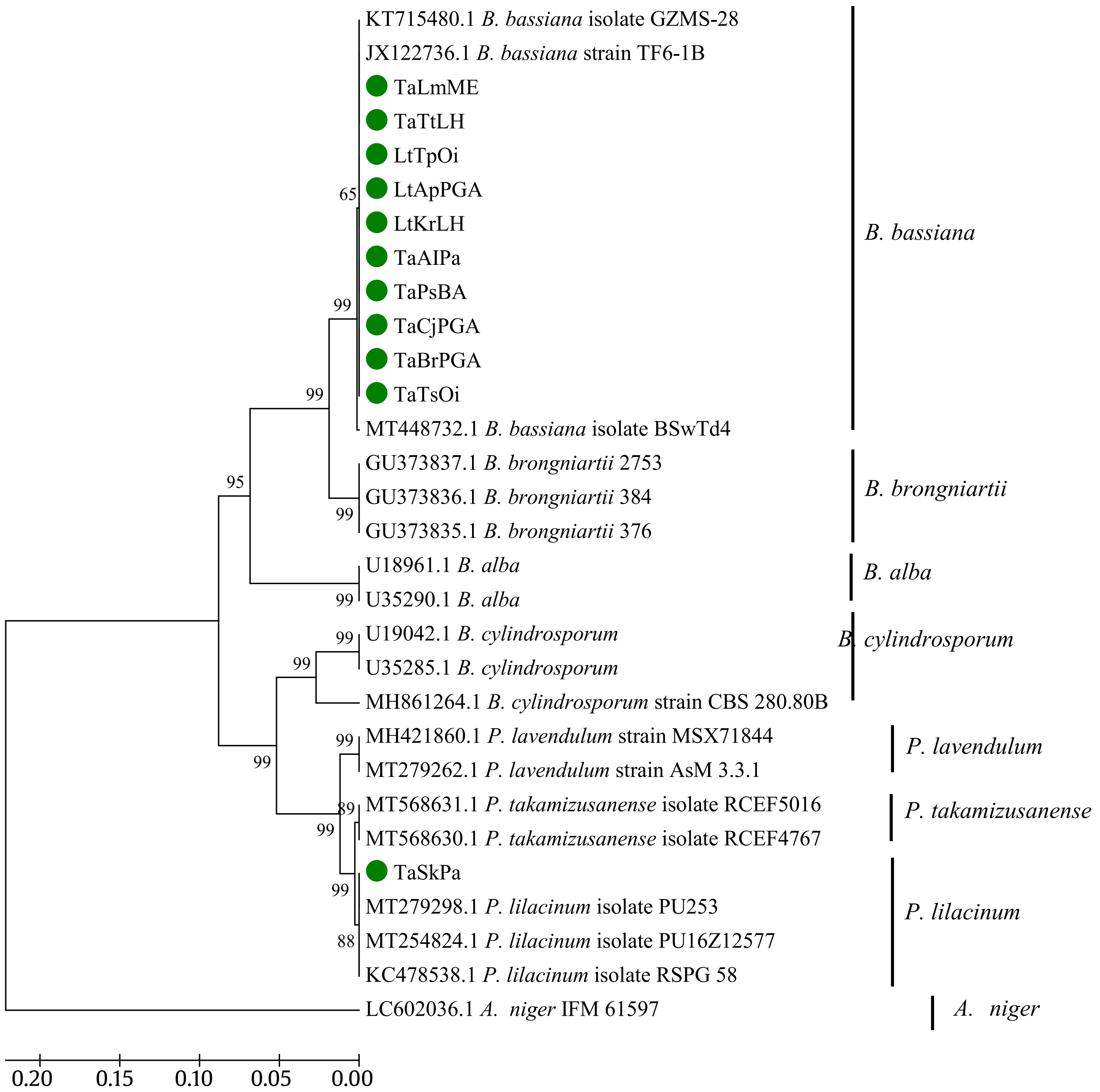

Figure 3. Phylogenetic tree based on Phylogenetic tree developed based on Internal Transcribed Spacer (ITS) region by UPGMA method (jukes and cantor model) using Mega7 for windows. Totally, 10 isolates were placed within group of *Beauveria bassiana* isolate GZMS-28 (Acc. No KT715480.1), strain TF6-1B (Acc No. JX122736.1) and isolate BSwTd4 (Acc. No. MT448732.1) and 1 isolate was in the group of *Purpureocillium lilacinum* isolate PU16Z12577 (Acc. No MT254824.1), isolate RSPG 58 (Acc No. KC478538.1) and isolate PU253 (Acc. No. MT279298.1). *Aspergillus niger* IFM61597 (Acc. No. LC602036.1) was used as out group

The TaSkPa isolate had a white to violet colony (Figure 1) the ellipsoidal fusiform conidia, and the hyaline hyphae and mycelia (Figure 2). The result of BLAST search revealed that the TaSkPa isolate had 100% of similarity to *Purpureocillium lilacinum* isolate PU16Z12577 (Acc. No MT254824.1), isolate RSPG 58 (Acc No. KC478538.1) and isolate PU253 (Acc. No. MT279298.1). Based on the phylogenetic tree, the TaSkPa isolate was in the group of *P. lilacinum* isolate PU16Z12577 (Acc. No MT254824.1), isolate RSPG 58 (Acc No. KC478538.1) and isolate PU253 (Acc. No. MT279298.1) (Figure 3). The TaSkPa isolate were deposited in the GenBank with the accession number OM780287.

3.2. Entomopathogenic Fungal Pathogenicity to the Egg of *Aedes aegypti*

Out of the two isolates (TaLmMe and TaPsBA) of the 11 isolates of the entomopathogenic fungi found in this study and four fungal isolates (BKbTp, MSwTp1, BSwTd4, and MSwTp3) from laboratory collection were the most pathogenic fungal isolates against *Ae. aegypti* eggs but, all fungal isolates caused higher egg mortality rate and were statistically significant differences from the untreated entomopathogenic fungi (control). Untreated or control eggs showed 22.51% mortality or 77.49% hatchability. Egg mortality of *Ae. aegypti* caused by *M. anisopliae* isolate MSwTp3 was the highest (38.31%) and was not significantly different from the egg mortality caused by *B. bassiana* isolate BSwTd4 (36.77%) and *T. diversus* isolate MSwTp1 (35.64%) (Table 2). However, the egg mortality of *Ae. aegypti* resulted by the *T. diversus* isolate MSwTp1 was not significantly different from the mortality by the *P. citrinum* isolate BKbTp (34.69%), the *B. bassiana* isolate TaPsBA (33.99%), and the *B. bassiana* isolate TaLmMe (34.93%). Thus, the most pathogenic fungal species against eggs of *Ae. aegypti* were *M. anisopliae* (MSwTp3 isolate), *B. bassiana* (the BSwTd4 and TaPsBA isolates), *T. diversus* (MSwTp1 isolate), and *P. citrinum* (BKbTp isolate). This is the first record that the four species of fungi from Indonesia have been pathogenic to the eggs of *Ae. Aegypti*. The *Ae. aegypti* eggs infected with the entomopathogenic fungi had specific characteristics and differences from the healthy eggs. The infected eggs had an eggshell covered with the white or greenish white mycelia (Figure 4) depending on the fungal species that infected them, whereas the healthy eggs were not covered by the mycellia. The infected eggs were shriveled and dry and generally empty inside, whereas the unhatched healthy eggs were still filled with fluid.

After the treated and untreated eggs hatching into larvae, then the emerging larvae were observed and the results showed that the highest mortality of the larvae was 33.68% by *M. anisopliae* (MSwTp3 isolate) and was not significantly different from the mortality caused by *B. bassiana* (BSwTd4 and TaPsBA isolates), *P. citrinum* (BKbTp isolate), *T. diversus* (MSwTp1 isolate) (Table 3). In contrast, the control eggs induced 1.61% larval mortality. After finishing the larval stage, the

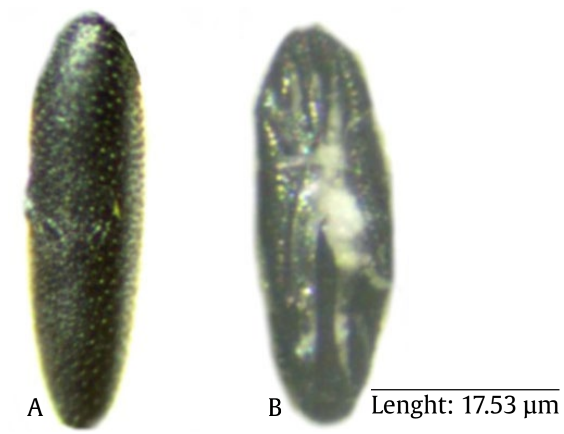

Figure 4. Morphology of the *Aedes aegypti* eggs: a healthy egg of control (A) and an infected treated egg (B)

Table 2. Effect of eggs treated with entomopathogenic fungi (1 x 10^{10} conidia/ml) on egg, larval, and pupal mortality

Fungal species	Fungal isolate code	Eggs laid	Egg mortality (%)	Larval mortality (%)	Pupal mortality (%)
Control	-	1232.00a	22.51e	1.61j	0.68h
Beauveria bassiana	LtTpOi	969.33bc	30.44cd	21.80gh	1.70fgh
Beauveria bassiana	TaTsOi	955.66bc	31.45bcd	27.38de	3.87cdef
Beauveria bassiana	TaAIPa	953.00bc	32.80bcd	28.16de	4.64bcde
Purpureocillium lilacinum	TaSkPA	997.66b	30.30cd	16.26i	1.48gh
Beauveria bassiana	TaBrPGA	978.66bc	30.67cd	20.94h	2.37efg
Beauveria bassiana	TaCjPGA	980.33bc	29.48d	17.27i	1.34gh
Beauveria bassiana	LtApPGA	965.33bc	31.2cd	24.36fg	3.21defg
Beauveria bassiana	LtKrLH	971.00bc	33.69abcd	28.83cd	5.68abcd
Beauveria bassiana	TaTtLH	982.66bc	31.72bcd	25.34ef	3.27defg
Beauveria bassiana	TaLmME	970.33bc	34.93abc	29.98bcd	7.26ab
Beauveria bassiana	TaPsBA	982.00bc	33.99abcd	28.74cd	5.75abcd
Penicillium citrinum	BKbTp	949.66c	34.69abcd	31.68abc	6.56abc
Talaromyces diversus	MSwTp1	989.33bc	35.64abc	30.06bcd	6.63abc
Beauveria bassiana	BSwTd4	968.66bc	36.77ab	32.62ab	8.20a
Metarhizium anisopliae	MSwTp3	981.66bc	38.31a	33.68a	9.27a
F-value		12.71*	13.28*	100.20*	27.71*
P-value		1.76 x10^{-9}	1.01 x 10^{-9}	2 x 10^{-16}	4.05 x 10^{-14}
HSD value		0.04	3.29	3.58	3.91

* = significantly different; values within a column followed by the same letters were not significantly different at P<0.05 according to Tukey's HSD test

Table 3. Effect of larvae treated with entomopathogenic fungi (1 x 10^{10} conidia/ml) on larval mortality, LT_{50}, and LT_{95}

Fungal species	Fungal isolate code	Larval mortality	LT_{50} (days)	LT_{95} (days)
Control	-	0.00e	16.53a	22.90a
Beauveria bassiana	LtTpOi	56.67d	5.66cd	12.03cd
Beauveria bassiana	TaTsOi	66.67cd	4.76fg	11.13efg
Beauveria bassiana	TaAlPa	66.67cd	4.85fg	11.22efg
Purpureocillium lilacinum	TaSkPA	52.22d	6.77b	13.14b
Beauveria bassiana	TaBrPGA	62.22cd	5.05ef	11.42efg
Beauveria bassiana	TaCjPGA	54.44d	6.17c	12.53bc
Beauveria bassiana	LtApPGA	62.22cd	5.48de	11.85cde
Beauveria bassiana	LtKrLH	74.44c	4.53g	10.89fg
Beauveria bassiana	TaTtLH	63.33cd	5.18def	11.55def
Beauveria bassiana	TaLmME	86.67b	3.59h	9.95h
Beauveria bassiana	TaPsBA	71.11c	4.41g	10.78g
Penicillium citrinum	BKbTp	92.22ab	3.16ij	9.52hi
Talaromyces diversus	MSwTp1	93.33a	2.83j	9.20i
Beauveria bassiana	BSwTd4	86.67b	3.39hi	9.75hi
Metarhizium anisopliae	MSwTp3	94.44a	2.83j	9.191i
F-value		36.95*	196.60*	114.30*
P-value		6.03 x 10^{-16}	2 x 10^{-16}	2 x10^{-16}
HSD value		15.89	0.21	0.20

* = significantly different; values within a column followed by the same letters were not significantly different at $P<0.05$ according to Tukey's HSD test

larvae turned into pupae and not all larvae were able to reach the pupae stage. The highest percentage of unemerged pupae (9.27%) was caused by *M. anisopliae* (MSwTp3 isolate) and was not significantly different from the mortality caused by *B. bassiana* (BSwTd4 and TaPsBA isolates), *P. citrinum* (BKbTp isolate), *T. diversus* (MSwTp1 isolate). From the eggs stage and the eggs developed into larvae and pupae, then pupae became adults and the adults died, the data showed a significant decreased in the individual number (also the percentage) of each stage that survived in the treatment with fungi compared to the control. For example, from 981.66 eggs treated with *M. anisopliae* (MSwTp3 isolate) became the first instar were 605.33 larvae (38.31% of the egg mortality), finally the last instar died 33.68% so that the remaining alive larvae were 401.4549 larvae, and at pupal stage, the dead pupae found 9.27% so the adults emerged only 365 individuals (Table 2). These data showed that from 981.66 eggs treated with *M. anisopliae* (MSwTp3 isolate) could became adults were 365 individuals (28.01%). So, the *M. anisopliae* could induce 71.99% cumulative mortality. A similar trend occurred in eggs treated with *B. bassiana* (BSwTd4 and TaPsBA isolates), *P. citrinum* (BKbTp isolate), *T. diversus* (MSwTp1 isolate), and the rest species/isolates. A novel finding of this study was the *Ae. aegypti* eggs exposed with the fungus not only killed the eggs but could continue to kill the emerging larvae, pupae, and adult. The contradictory result showed that from 1,232 untreated eggs (control) could become adults were 932.67 individuals (75.70%). So, the control eggs could only produce 24.30% cumulative mortality. This result clearly showed that the four fungal species confirmed to have the ovicidal activity. Further research is needed to develop these fungal species into ovicides.

3.3. Entomopathogenic Fungal Pathogenicity to the Larvae of *Aedes aegypti*

The *Ae. aegypti* larvae treated with the entomopathogenic fungi (1 x 10^{10} conidia/ml) underwent mortality between 52.22−94.44% and their mortality was significantly different from the control larvae (Table 3). The larval mortality caused by *M. anisopliae* isolate MSwTp3 (94.44% with LT_{50} 2.83 days and LT_{95} 9.19 days) was highest and not significantly different from mortality caused by *P. citrinum* isolate BKbTp (92.22% with LT_{50} 3.16 days and LT_{95} 9.52 days) and *T. diversus* isolate MSwTp1 (93.33% with LT_{50} 2.83 days and LT_{95} 9.20 days). The other fungal species that caused high mortality was *B. bassiana* isolate BSwTd4 (86.67% with LT_{50} 3.39 days and LT_{95} 9.75 days) and not significantly different from *B. bassiana* isolate TaLmMe (86.67% with LT_{50} 3.59 days and LT_{95} 9.95 days). This result clearly showed that the *M. anisopliae, P. citrinum, T. diversus*, and *B. bassiana* possessed larvicidal activity.

The *Ae. aegypti* larvae treated with the entomopathogenic fungi that were sick and died showed typical symptoms. The sick larvae had a ruptured gut lumen and an indistinct segment of abdomen, an epithelial lining with milky color, a fractured anal segment. On the other hand, the untreated healthy larvae had a clearly visible gut lumen, a distinct segment of abdomen, a transparent epithelial lining, and an intact anal segment (Figure 5). Information on the gut lumen larvae of *Ae. aegypti* ruptured caused by the fungus is a new information. In addition, the larval cadavers grown on SDA media could covered with mycellia, while the healthy larvae were clean and not covered with the fungus.

The *Ae. aegypti* larvae treated with the entomopathogenic fungi that still survived could grow into the pupae. Most of the emerging pupae were unhealthy. The unhealty pupae became thinner and less rounded, stiff, hardened, and black head, while the healthy pupae were round, fat, bent like a comma shape, flexible and soft body, and head dark-brown in color (Figure 6). If the treated pupal cadaver was grown on SDA media, the cadaver could be covered with the fungal mycellia, while on the untreated pupal cadaver, the fungal mycellia could not be found.

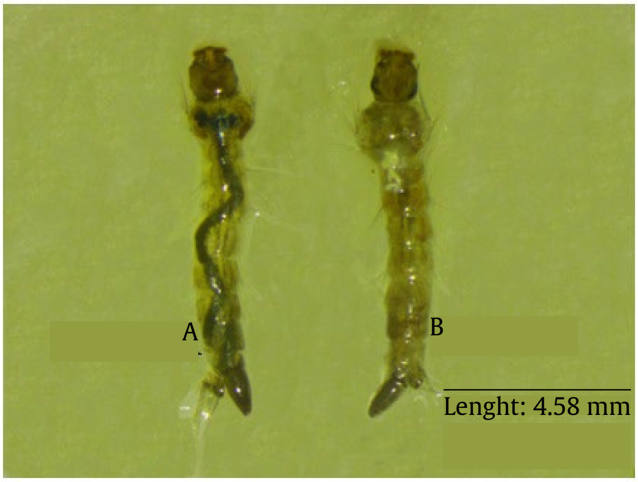

Figure 5. Morphology of the *Aedes aegypti* larvae: a healthy larvae of control (A) and an infected treated larvae (B)

3.4. Entomopathogenic Fungal Pathogenicity to the Adult of *Aedes aegypti*

The *Ae. aegypti* adults treated with the entomopathogenic fungi (1 x 10^{10} conidia/ml) induced the adult mortality of 50.00–92.22%, and was significantly different from the untreated mortality (control) (Table 4). The highest adult mortality (92.22% with LT_{50} 3.89 days and LT_{95} 7.76 days) was recorded when the adults treated with *M. anisopliae* isolate MSwTp3 and was not significantly different from the mortality caused by *P. citrinum* isolate BKbTp (91.11% with LT_{50} 4.33 days and LT_{95} 8.19 days), *T. diversus* isolate MSwTp1 (90.00% with LT_{50} 4.16 days and LT_{95} 8.02 days), *B. bassiana* isolate BSwTd4 (88.89% with LT_{50} 4.29 days and LT_{95} 8.15 days), and *B. bassiana* isolate TaLmMe (91.11% with LT_{50} 4.05 days and LT_{95} 7.91 days). This research highlighted that the four fungal species had adulticidal activity.

The sick and dead adults of *Ae. aegypti* caused by exposure of the entomopathogenic fungi showed typical symptoms. The treated adults had malformation and asymmetrical wing shapes, mycosis in abdomen and thorax, the hard and stiff abdomen and thorax, and the curled proboscis (Figure 7). If the adult cadaver was grown in SDA media, the fungal mycellia covered the cadaver's body. By contrast, the healthy adults had the symmetrical wing shapes, elongate abdomen, and no mycosis in abdomen and thorax, a black proboscis with short

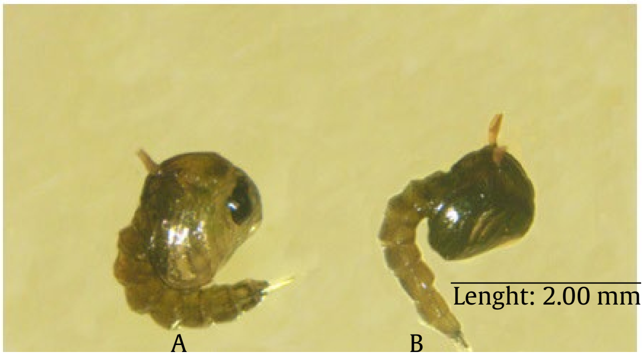

Figure 6. Morphology of the *Aedes aegypti* pupae: a healthy pupae of control (A) and an infected treated pupae (B)

Table 4. Effect of adults treated with entomopathogenic fungi (1×10^{10} conidia/ml) on adult mortality, LT_{50}, and LT_{95}

Fungal species	Fungal isolate code	Adult mortality	LT_{50} (days)	LT_{95} (days)
Control	-	0.00g	9.88a	13.74a
Beauveria bassiana	LtTpOi	54.44f	6.02bc	9.88b
Beauveria bassiana	TaTsOi	63.33def	5.56d	9.42cde
Beauveria bassiana	TaAIPa	74.44cde	5.52d	9.39def
Purpureocillium lilacinum	TaSkPA	50.00f	6.20b	10.06b
Beauveria bassiana	TaBrPGA	60.00ef	5.78cd	9.64bcd
Beauveria bassiana	TaCjPGA	54.44f	5.97bc	9.83bc
Beauveria bassiana	LtApPGA	65.56def	5.51d	9.42def
Beauveria bassiana	LtKrLH	78.89bcd	5.03e	8.89g
Beauveria bassiana	TaTtLH	67.78def	5.15e	9.01efg
Beauveria bassiana	TaLmME	91.11a	4.05gh	7.91hi
Beauveria bassiana	TaPsBA	74.44cde	5.14e	9.00fg
Penicillium citrinum	BKbTp	91.11ab	4.33f	8.19h
Talaromyces diversus	MSwTp1	90.00a	4.16fgh	8.02hi
Beauveria bassiana	BSwTd4	88.89abc	4.29fg	8.15h
Metarhizium anisopliae	MSwTp3	92.22a	3.89h	7.76i
F-value		23.97*	140.4*	83.72*
P-value		3.23×10^{-13}	2×10^{-16}	2×10^{-16}
HSD value		19.79	0.12	0.13

* = significantly different; values within a column followed by the same letters were not significantly different at $P<0.05$ according to Tukey's HSD test

Figure 7. Morphology of the *Aedes aegypti* adults: a healthy adult of control (A) and an infected treated adult (B)

palpi and long protruding. If the untreated cadaver was grown in SDA media, the fungal mycelia could not be found.

4. Discussion

LtTpOi, TaTsOi, TaAIPa, TaBrPGA, TaCjPGA, LtApPGA, LtKrLH, TaTtLH, TaLmME, and TaPsBA isolates of entomopathogenic fungi found in this research had the same morphological characteristics to *B. bassiana* illustrated by Herlinda *et al.* (Herlinda *et al.* 2020a). As stated by BLAST reference species, the 10 isolates of the fungi had a ribosomal DNA sequence similarity value of 99.38% (more than 99%) to *B. bassiana* isolate GZMS-28 (Acc. No KT715480.1), strain TF6-1B (Acc No. JX122736.1) and isolate BSwTd4 (Acc. No. MT448732.1), meaning that the isolates had a high phylogenetic relationship and were in the same species. Ribosomal DNA sequences were used to determine the phylogenetic relationships of organisms to taxa species (Bich *et al.* 2021). Henry *et al.* (Henry *et al.* 2000) stated that the similarity value of 99% shows that the isolates are the same species. Shenoy *et al.* (Shenoy *et al.* 2007) added that an organism is the same species if the difference in DNA sequences is between 0.2–1.0%.

The TaSkPa isolate had the same morphological characteristics as *P. lilacinum*, as illustrated by Kepenekci *et al.* (Kepenekci *et al.* 2015). According to BLAST reference indicated that the TaSkPa isolate had 100% of similarity to *P. lilacinum* isolate PU16Z12577 (Acc. No MT254824.1), isolate RSPG 58 (Acc No. KC478538.1) and isolate PU253 (Acc. No. MT279298.1). If the similarity value is 100%, the isolates are the same strain (Henry *et al.* 2000).

These results highlighted that species of the entomopathogenic fungi that were pathogenic to eggs of *Ae. aegypti* were *M. anisopliae* isolate MSwTp3, *B. bassiana* isolate BSwTd4 and TaPsBA, *T. diversus* isolate MSwTp1, and *P. citrinum* isolate

BKbTp. Although the eggs treated with the fungus that did not hatch were still low (38.31%). However, the treated and hatched eggs induced the sick and infected larvae and produced up to 71.99% cumulative mortality. In addition, mycosis on the dead larvae failed to emerge from the eggs of *Ae. Aegypti* was found in this research. The treated unhatched eggs not only contained the dead and dry larvae, but generally, the eggs had empty and dry inside. The body fluids of the host insects are dry because they are absorbed by the fungi (Gabarty *et al.* 2014). The infected eggs caused the first instar up to the last instar to continue to undergo death. Leles *et al.* (Leles *et al.* 2012) reported that *M. anisopliae* caused the eggs of *Ae. aegypti* unhatched, although they could hatch, the emerging larvae died due to infection by the fungus, and some eggs were aborted. Compared with larvae mortality, the percentage of unhatched eggs (egg mortality) caused by the fungus was lower because the eggshell cuticle was thicker and comprised of the exochorion, endochorion, and serosal cuticle (Farnesi *et al.* 2015). By contrast, the cuticle of the larvae is thinner, and the thinner the insect's cuticle, the easier it is to be infected by the fungus (Ortiz-Urquiza and Keyhani 2013). The effect of the entomopathogenic fungi continued in the pupal and adult stages. The pupae and adults are dying due to infection of the fungi. These results also showed that the ovitrap contaminated with conidia used in this study could infect the eggs, larvae, pupae, and adults of *Ae. aegypti*.

Species of the entomopathogenic fungi that were pathogenic to the *Ae. aegypti* larvae were *M. anisopliae* isolate MSwTp3, *P. citrinum* isolate BKbTp, *T. diversus* isolate MSwTp1, *B. bassiana* isolate BSwTd4 and TaLmMe. The mortality of larvae treated with the fungus was high with a short mortality time (up to 94.44% with LT_{50} 2.83 days). It is caused by the fungus cultured in the broth medium (SDB). The fungal broth culture can produce blastospores more effective at killing *Ae. aegypti* compared with aerial conidia (Alkhaibari *et al.* 2017), and the blastospores can kill faster than the aerial conidia (Alkhaibari *et al.* 2016).

The results showed that the fungi could induce the larvae to get a ruptured gut lumen, an indistinct abdomen segment, an epithelial lining with milky colour, and a fractured anal segment. The dead larvae are caused by the fungal conidia germinating. Then the hyphae penetrate the integument to the body cavity (Boomsma *et al.* 2014). The hyphae grow in the hemolymph and produce blastospores producing secondary metabolites and enzymes that disrupt normal cell metabolism (Mancillas-Paredes *et al.* 2019). The *Ae. aegypti* larvae treated with the entomopathogenic fungi could produce unhealthy or dead pupae characterized by thinner, less rounded, stiff, hardened, and blackheads.

Species of the entomopathogenic fungi that were pathogenic to the *Ae. aegypti* adults were *M. anisopliae* isolate MSwTp3, *P. citrinum* isolate BKbTp, *T. diversus* isolate MSwTp1, *B. bassiana* isolate BSwTd4 and TaLmMe. We highlighted that the fungal species pathogenic to adults were the same as those pathogenic to the eggs and larvae. The adults of *Ae. aegypti* treated with the entomopathogenic fungi (1 x 10^{10} conidia/ml) caused the adult wings to become asymmetrical, mycosis in the abdomen and thorax, the complex and stiff abdomen and thorax, and the curled proboscis. Adult mortality was also induced by the hyphae penetrating the adult body and poisoning by secondary metabolites produced by the fungus (Mancillas-Paredes *et al.* 2019). In addition, the body of adults undergoes mycosis dry body because during growth, the fungus absorbs the body fluids of insects and the fungus grows and covers the cadaver (Gabarty *et al.* 2014).

Molecular identifications recorded two species of the entomopathogenic fungi found in this study, namely *B. bassiana* (LtTpOi, TaTsOi, TaAIPa, TaBrPGA, TaCjPGA, LtApPGA, LtKrLH, TaTtLH, TaLmME, and TaPsBA isolates) and *P. lilacinum* (TaSkPa isolate). However, these results show that the 15 isolates of five species (*M. anisopliae, P. citrinum, T. diversus, B. bassiana*, and *P. lilacinum*) of the entomopathogenic fungi from South Sumatra, Indonesia are pathogenic to the egg, larvae, and adult of *Ae. aegypti.* The most pathogenic species to the eggs, larvae, pupae, and adults of *Ae. Aegypti* are *M. anisopliae* isolate MSwTp3, *P. citrinum* isolate BKbTp, *T. diversus* isolate MSwTp1, *B. bassiana* isolate BSwTd4 and TaLmM. A novel finding of this study is the *Ae. aegypti* eggs exposed to the fungus not only kill the eggs but can continue to kill the emerging larvae, pupae, and adults. The first report of *M. anisopliae, P. citrinum, T. diversus*, and *B. bassiana* from South Sumatra possess remarkable ovicidal, larvicidal and adulticidal activity against an important vector mosquito, *Ae. aegypti.* Further research is needed to develop these fungal species into ovicides, larvicides, and adulticides for controlling *Ae. Aegypti.*

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