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Effect of symbiotic bee fungus on survival of stingless bee *Heterotrigona itama* larvae

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ABSTRACT

Aims: Symbiotic bee fungus associated with the stingless bee larval food has been revealed to play a major role in performing a mutual relationship with the host. The fungus is believed capable to produce crucial nutrients that are required for larval pupation. However, detailed information on the fungus identification isolated from the larval food of our native Indo-Malaya stingless bee, *Heterotrigona itama* (Hymenoptera: Apidae), is poorly understood. Hence, this study was conducted to determine the effect of fungus isolated from the stingless bee larval food on the survival of *H. itama* larvae and identify the isolated fungus using both morphological and molecular analyses.
Methodology and results: Elisa plates designed with F-bottom type were used as artificial brood cells in a controlled condition. The eggs transferred to Elisa plates were kept in the incubator with 75%-100% humidity and 25 ± 2.5 °C of temperature. This study carried out in three different treatments: (1) microbes-free larval food, (2) fresh larval food and (3) microbes-free larval food with fungus supplement. Results showed that the survival of *H. itama* larvae depends on the presence of beneficial fungus with the highest survival rate (Treatment 2 = 85.71%). The absence of the beneficial fungus in the fluid food of *H. itama* showed the lowest survival rate (Treatment 1 = 37.14%). Microscopy analysis showed that the fungus had various forms, including unconjugated asci, round-shaped ascospore and pseudohyphae. The molecular characterisation of the isolated fungi was performed using 23S rRNA gene sequencing using universal primers ITS1 and ITS4. DNA barcoding of three isolated fungi confirmed all isolated fungi matched *Panus lecomtei* (Basidiomycetes; Poriales; Polyporaceae) with 99.70% of similarity.
Conclusion, significance and impact of study: This study provides information on the importance of whitish fungus in appearance that existed in the brood cell, which is proposed to be the crucial component of *in-vitro* stingless bee queen rearing protocol.

Keywords: Bee fungus, larval food, *Heterotrigona itama*, stingless bee, symbiotic

INTRODUCTION

Stingless bees (Hymenoptera; Apidae; Meliponini) or locally known as 'kelulut' are a group of bees that possesses unfunctional sting for defence and abundantly distributed in tropical as well as subtropical regions (Wille, 1983). Currently, there are more than 38 stingless bee species have been found in Malaysia (Mustafa *et al.*, 2018). One of them is *Heterotrigona itama* which is the most common species reared by Malaysian beekeepers (Jaapar *et al.*, 2018; Omar *et al.*, 2020).

Studies on microbes associated with stingless bees are currently being discussed at a greater length. Most of the studies focus on beneficial microbes, especially bacteria isolated from stingless bee products such as honey, propolis and bee bread. For example, five different strains of *Lactobacillus brevis* had been isolated from *H. itama* farmed in coastal areas in Kelantan and Terengganu had shown probiotic and antibacterial properties (Hasali *et al.*, 2018a). In addition, there are significant differences ($P<0.05$) of physico-chemical analysis for moisture, ash, protein, carbohydrate and

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energy of *H. itama* honey samples from different locations, except for pH and fat (Hasali *et al*., 2018b). Different seasons may have an influence on stingless bee behaviour while different flower sources may affect the properties of stingless bee honey (Mahmood *et al*., 2021).

However, little information is available about beneficial fungus associated with stingless bee's nest products since most reports focus mainly on the isolation and identification of bacteria. Early investigation of our work had shown the presence of fungi in *H. itama* honey samples during storage study at ambient temperature for six weeks (Lani *et al*., 2017). This finding showed that the competition between yeast and mold and lactic acid bacteria (LAB) was observed, where LAB counts decreased while the yeast and mold count increased throughout storage. It is believed the pH changes also affect the antagonistic effects between bacteria and fungi during prolonged storage of *H. itama* honey samples at ambient temperature. Thus, investigation on beneficial fungus needs to be further explored to understand its role and function in the stingless bee colonies.

Fungus isolated from the stingless bee's larval food has been reported to be involved during the larval stage. Recent research on Brazilian stingless bee, *Scaptotrigona depilis,* by Menezes *et al.* (2018) indicated that besides honey and pollen, the stingless bee also required fungus to survive during their developmental period. According to Menezes *et al.* (2018), when the egg is about to hatch, there is a proliferation of fungus at the internal walls of wax cells. The study has confirmed that the Brazilian bee's larvae need to consume beneficial fungi in order to survive during the larval stage.

Paludo *et al.* (2018) found that *S. depilis* were unable to pupate without the presence of fungus, which was identified as *Monascus* sp. Symbiosis relationship between *S. depilis* and *Monascus* that benefits from sterols derived from the fungus can be the most important element in completing metamorphosis throughout the process. Paludo *et al.* (2019) revealed that the specific brood fungus consumption by stingless bees, *S. depilis* during the larval stage is beneficial for steroid-derived metabolism from ergosterol that stimulates the major transformation of immature larvae into an adult.

In the study of Menezes *et al.* (2018), the researchers proposed two treatments through *in-vitro* rearing, which were larvae treated with mycelia fungal and mycelia fungal-free larvae treatment to investigate how effective the fungus in benefiting the stingless bee larvae of *S. depilis.* The results appeared to have high survival (76%) in larvae treated with mycelia fungal than the larvae treated with mycelia fungal-free (8%) which signals the necessity of fungal consumption in the larvae stage for their survival and transformation. Symbiotic bee fungus may develop secondary metabolites due to no evidence of the larval food contamination presented during the whole queen development period under *in-vitro* conditions (Paludo *et al*., 2018). A genetic study employed in the fungal symbionts field revealed the mycangial fungus enhancing the diet in the larval food by gradually increasing the nitrogen concentration that can significantly influence the higher population growth rates and increase in lipid content (Paludo *et al*., 2019). Fungus nutritional intake aids in transformation into a cocoon throughout the development for certain insects as the fungus may compensate nitrogen insufficiency in their provisioned foods (Paludo *et al*., 2019), synthesise sterol molecules and provide micro-substances necessary for insects (Ayres *et al*., 2000).

The beneficial fungus that presents in a colony may vary in each stingless bee species, particularly for our native species. Due to that, this study aimed to determine the effect of fungus isolated from *H. itama* larval food on larval survivorship and to characterise and identify the isolated beneficial fungus. This study could be the starting point to elucidate and establish their contributions in the Malaysian stingless bee queen of *in-vitro* rearing technique.

MATERIALS AND METHODS

Samples collection

The larval food of *H. itama* used in this study was collected from two Meliponiculture farms, Big Bee Honey Farm (4°57'08.0''N 103°20'25.0''E) and Taman Penyelidikan Alam Universiti Malaysia Terengganu (5°21' 5578''N 103°16'21.62''E), where both sites are located in Marang, Terengganu, Malaysia. All materials used during sample collection were sterilised to minimise the potential contamination from fields. The samples of young brood combs were collected aseptically using a knife and transferred into a container with minimal exposure to the environment. The young brood combs can be easily recognised, appearing darker in colour than the pale colour of old brood combs. All samples were then transferred into a sterile Falcon tube and stored on ice during transportation to the laboratory. In the laboratory, the eggs were gently removed using a needle from the brood combs. The brood combs were then squeezed into a 50 mL centrifuge tube for extracting the larval food.

Evaluation of bee fungus on *in-vitro* stingless bee queen rearing

Three treatments were tested to evaluate the interaction of symbiotic-bee fungus to the growth of the larvae under *in-vitro* queen rearing procedures. The three treatments were: (1) microbes-free larval food that acts as a control, (2) fresh larval food and (3) microbes-free larval food with fungus supplement. To remove the presence of microbes in Treatment 1, the larval food was sterilised using an autoclave at 121 °C for 15 min at 15 psi. In Treatment 3, the sterile larval food was supplemented with the symbiotic-bee fungus isolated from natural brood cells cultivated in the laboratory.

Fungus inoculation was cultivated in a 30 g broth (pH 6.0) that was obtained from 10-15 days old of fungus culture in 30 g agar medium (pH 6.0) at 29 °C, which then followed by serial dilution with 0.02% of Tween 80 in dH_2O. Six folds of serial dilutions were prepared and

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tested. A total of 1 µL of each inoculum was tested on a well containing ten larvae. The cultures were monitored daily until the completion of larvae development, which required about 40-45 days. The inoculum with the highest survivability was supplemented for Treatment 3.

The *in-vitro* queen rearing of the stingless bee procedure was carried out based on Razali *et al.* (2021), with some modifications. Each Elisa plate (F-bottom type) with 96 cell cavities was volumised with 150 µL of larval food. The eggs were grafted into Elisa plate vertically as they were deposited in natural brood cells to prevent them from drowning from asphyxiation due to the airflow blockage (dos Santos *et al.*, 2016). All three treatments were placed in an incubator with humidity 100% for the first seven days and 75% for the rest of larval development at room temperature (25 ± 2.5 °C). Each treatment were replicated five times with seven samples per treatment. Overall samples used in the study was 105 (n=105). The percentage of larval survival was recorded as below:

Percentage of larval survival (%) = (Number of survived individuals/Total individual grafted) × 100

Isolation of fungus

Fungus isolation was conducted using methods described by Paludo *et al.* (2019). High osmolarity of selective media, namely International Streptomyces Project media 2 (ISP-2) was prepared to successively isolate the growing fungus from larval food of Treatment 2. The ISP-2 comprised of 30 g of glucose, 3 g of malt extract, 3 g of yeast extract and 2% of basis agar diluted with 100 mL of deionised water with pH adjusted to pH 4.5 or pH 6.0 using hydrochloric acid (HCl) 1 N (Paludo *et al.*, 2018). The larval food of *H. itama* was streaked onto ISP-2 media and incubated at 30 °C until the growth. The colony formed on ISP-2 media was then streaked onto Potato Dextrose Agar (PDA) (Oxoid, UK) to obtain the colony of pure culture. Pure fungus isolates were grown on PDA and incubated at 28 °C for 4 to 6 days.

Observation of fungal isolates morphology

The morphological characteristics of the fungal isolate were observed under a light microscope [Carl Zeiss (V12)] based on the standard identification technique by using Lacto Phenol Cotton Blue (LPCB) staining. LPCB was dropped onto the microscopic slide. The pure culture was distributed onto the microscopic slide using a sterilised needle. The coverslip was then placed on the microscopic slide. The slide was examined under 100× magnification light microscope to observe the fungal characteristics.

The microstructure of spore shape and conidiophore were observed under a scanning electron microscope (SEM). The fungus samples were cut into small pieces using a sterile blade into a 1.5 mL microcentrifuge tube. The samples were fixed using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 to 4 h. The samples then were rinsed with 0.1 M sodium cacodylate buffer (pH 7.2) for 30 min with three times repetition. The samples were post-fixed using 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 to 4 h. The samples were then rinsed with 0.1 M sodium cacodylate buffer (pH 7.2) for 30 min for three times. The samples were dehydrated with ethanol ranging from 35% to 100% for 15 min each concentration with three times repetition at 100% of concentration. The sample was air-dried and mounted with carbon. Lastly, the samples were coated with gold using Auto Fine Coater and ready to be analysed by Analytical Scanning Electron Microscope (Model: JEOL6360LA).

DNA extraction and 23S rRNA gene sequencing method for fungal DNA barcoding

Molecular analysis was conducted using methods described by Romanelli *et al.* (2014) and Paludo *et al.* (2019) with some modifications. The fungal isolate was identified by sequence analysis of the 23S rRNA gene. The fungal Internal Transcribed Spacer (ITS) gene was amplified using universal primers, ITS1 and ITS4 (Romanelli *et al.*, 2014), that was subsequently used to amplify a 500 bp gene fragment from genomic DNA of three different isolates of fungus (SP 3, SP 4, SP 5) which taken from the larval food liquid prior to the appearance of whitish fungus selective structure based on previous study described by Paludo *et al.* (2018). The total reaction volume of 25 µL contained gDNA purified using in-house extraction method, 0.5 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 200 uM each), 0.5 U DNA polymerase, supplied PCR buffer and water. Positive control was sequenced with DNA fragments carrying 1 µL priming site of the primers, whereas for the negative control, 1 µL of sterile distilled water was mixed into the PCR mix. The DNA purified samples were performed as follows: 1 cycle (98 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 15 sec; 60 °C for 30 sec; 72 °C for 30 sec) for annealing and extension of the amplified DNA, and 1 cycle for a final extension of the amplified DNA (72 °C for 10 min). The PCR products were purified by standard method and directly sequenced with universal sequencing primers M13F (-20) and M13R-pUC (-26) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing service was conducted by Apical Scientific Sdn. Bhd., Selangor, Malaysia.

Data analysis

Data were log (x + 1) transformed to improve normality and homogeneity of variance. One-way ANOVA with Tukey's post hoc test was used to evaluate differences in the treatments between microbes-free larval food, fresh larval food and microbes-free larval food with fungus supplement. Statistical analyses were performed using SPSS (version 21.0).

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RESULTS AND DISCUSSION

In this study, there were significant differences in the survival of *in-vitro* stingless bee queen larvae in response to the larval food treatments (F = 17.215, P<0.001) (45.68 ± 21.17%) (Table 1). The larvae treated with the fresh larval food (Treatment 2) showed the highest larval survival (85.71%), followed by the larvae provided with microbes-free larval food plus bee fungal supplement (Treatment 3; 57.14%). The beneficial bee fungus was observed appeared at the border of the Elisa plates of Treatments 2 and 3, and was found eaten by the larvae (Figure 1). However, the larvae in microbes-free larval food (Treatment 1) resulted in the least larval survival (37.14%).

These results are consistent with those from other studies that reported the stingless bee's larvae need to consume beneficial fungus to survive during the larval stage (Menezes *et al*., 2018; Paludo *et al*., 2018). Thus, it indicated that the larval foods contained microorganism that is very important in contributing to larval survival. Though the larvae in Treatment 3 were provided with 1 × 10^6 CFU/mL fungal concentration of *Panus lecomtei*, the individual survival has deviated from the larvae in Treatment 2. We observed that the critical stage of *H. itama* larvae was before the pupation stage that required continuous inspection until the pupation stage. In certain cases, the larval food could contribute to the high survival rate as a consequence of the excessive number of microorganisms in the larval food (Baptistella *et al*., 2012). To the best of our knowledge, the fresh larval food might contain many other beneficial microbes which are not yet discovered. However, we selectively isolate the fungus depending on the stingless bee eating mechanism that feed on the fungus appeared around the Elisa plate and in natural brood cell of stingless bee hive.

The composition of ISP-2 contained nutrients that can support bacteria and fungi because no additional antibiotics were added to inhibit the bacteria. During isolation, only whitish fungi appearance was observed and it is very unlikely that bacteria is present in the Treatment 2. The identification of isolates was carried out using 23S rRNA confirmed the presence of *P. lecomei* as the source of food by larvae. This discovery has shown the roles of *Panus* in the larvae food ecosystem. This finding suggests that symbionts interaction has significantly enhanced the probability of larval survival against a broad range of pathogenic microbes by protecting the larval food contents. Besides, it is also suggested that the fungus naturally existed in larval food could be responsible for producing secondary metabolites for the larvae's longevity as reported by Paludo *et al.* (2018).

Based on microscopic examination and morphological characteristics, the fungus isolated from *H. itama* larval food was identified as *Panus lecomtei* belonging to the family of Polyporaceae (Figure 2). Under the light microscope, the fungus resembled a pseudomycelium (Figure 2a) in characteristics and ascospore (Figure 2b) formation. The formation of ascospore proposes a

Table 1: Percentage of larval survival in response to three treatments (%) (n = 105).

Treatment		Percentage (%)
Treatment 1	Microbes-free larval food	37.14
Treatment 2	Fresh larval food	85.71
Treatment 3	Microbes-free with fungus supplement	57.14

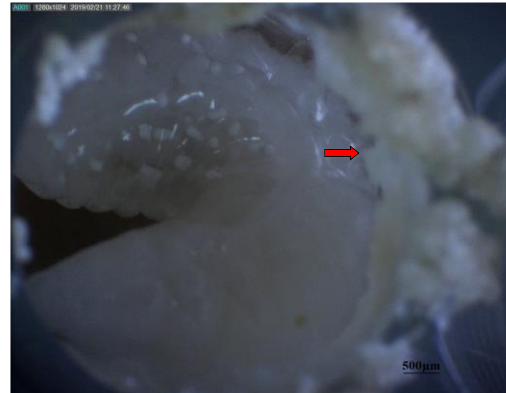

Figure 1: Red arrow as an indicator showing the whitish fungus feed by the larvae of stingless bee, *H. itama* under *in-vitro* state.

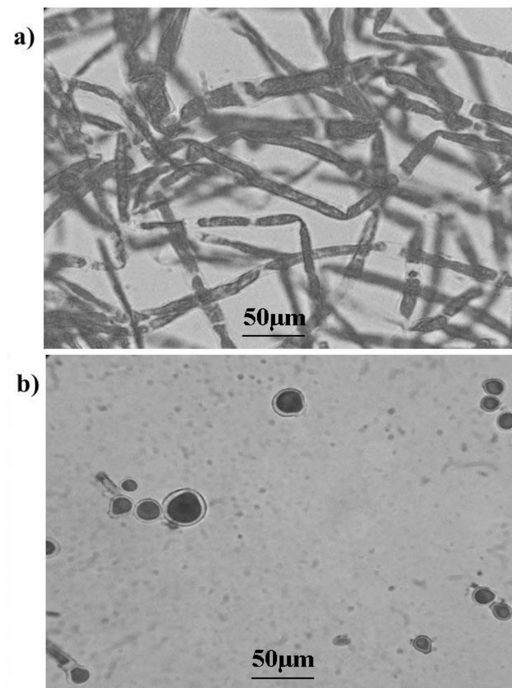

Figure 2: The microscopic characteristics of symbiotic bee fungus show the arrangement structures. (a) Pseudomycelium of fungus; (b) Ascospore of fungus.

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Table 2: The fungus strains isolated from *H. itama* larval food and the identity of gene sequences generated from the strains. The similarities of the closest relative are shown as percentage. Taxonomic relationship was identified by comparing the sequences in the National Centre for Biotechnology Information (NCBI).

Strain	Sequence length (bp)	Closely related genus/species from Genbank database	Similarity percentage (%) per identity
SP 3	693	*Panus lecomtei* (MK603978.1)	99.70%
	779	*Panus neostrigosus* (KM411451.1)	99.55%
	687	*Panus lecomtei* (KP135329.1)	100.00%
	688	*Panus lecomtei* (KP135328.1)	99.85%
	667	*Panus lecomtei* (KP135327.1)	99.85%
	655	*Panus rudis* (KU863048.1)	99.85%
	676	*Panus lecomtei* (MK168585.1)	99.85%
	648	*Panus lecomtei* (MK851549.1)	99.85%
	647	*Panus rudis* (KU863051.1)	99.69%
	641	*Panus rudis* (KU863049.1)	99.84%
SP 4	693	*Panus lecomtei* (MK603978.1)	99.70%
	779	*Panus neostrigosus* (KM411451.1)	99.55%
	687	*Panus lecomtei* (KP135329.1)	100.00%
	688	*Panus lecomtei* (KP135328.1)	99.85%
	667	*Panus lecomtei*(KP135327.1)	99.85%
	655	*Panus rudis* (KU863048.1)	99.85%
	676	*Panus lecomtei* (MK168585.1)	99.85%
	648	*Panus lecomtei* (MK851549.1)	99.85%
	647	*Panus rudis* (KU863051.1)	99.69%
	641	*Panus rudis* (KU863049.1)	99.84%
SP 5	693	*Panus lecomtei* (MK603978.1)	99.70%
	779	*Panus neostrigosus* (KM411451.1)	99.55%
	687	*Panus lecomtei* (KP135329.1)	100.00%
	688	*Panus lecomtei* (KP135328.1)	99.85%
	667	*Panus lecomtei*(KP135327.1)	99.85%
	655	*Panus rudis* (KU863048.1)	99.85%
	676	*Panus lecomtei* (MK168585.1)	99.85%
	648	*Panus lecomtei* (MK851549.1)	99.85%
	647	*Panus rudis* (KU863051.1)	99.69%
	641	*Panus rudis* (KU863049.1)	99.84%

possible spread inside the colonies and between colonies during swarming. The role of fungus on bees has been extensively studied years back. This finding was supported by Paludo *et al.* (2019), who suggested that the fungal propagation inside *S. depilis* colonies and between colonies was due to the formation of the ascospore. In terms of fungal microstructure, we observed the dense of hyphal elements under SEM as shown in Figure 3. These fungal mycelia were found eaten by the larvae of *S. depilis* which were started to grow from the brood cells cerumen at the day-1 old and produced a complex filamentous view (Paludo *et al*., 2018). In SEM analysis, the *P. lecomtei* after seven days incubation period manifold of blastoconidia, hyphae and pseudohyphae in appearance (Figure 3). This should facilitate the fungal specimen recognition to the different phenotypic and microscopy indicators of *P. lecomtei*. To be of value for the fungal isolation and identification, these indicators are likely to encounter in species identification. The outcomes revealed that the fungus isolated from the larval food of stingless bees, *H. itama* was identified as *P. lecomtei*. Interestingly, this study is the first report on the existence of *Panus* sp. in the stingless bee larval food. This study strongly indicates that *Panus* sp. is coexisted with other *Trigona* species due to similar geographical localities.

Three preserved strains, 1, 2 and 3 formerly isolated from three samples of fresh larval food were used to identify the fungus species. The evaluating parameter was that the only growing fungus on the selective media preferred by *Panus* sp. These strains that were temporarily named for fungal isolative comparative analysis against GenBank database disclosed that the strains exhibited high nucleotide similarity to the known fungus taxa, *P. lecomtei* (99.70%) (Figures 3, 4 and 5; Table 2). Although limited information of *P. lecomtei* with stingless bee, this species was identified to play an important role as a source of novel secondary metabolites with many expressions of biological activities (Erkel *et al*., 1996). Interestingly, the capability of this species to exhibit antimicrobial activity might be the consequences from high survival rate of larva in Treatment 1 when compared to Treatment 3 (Wang *et al*., 2020). Antimicrobial properties produced by *Panus* sp. essentially served to prevent the larva of *H. itama* from being attacked by pathogenic microorganisms. Severe

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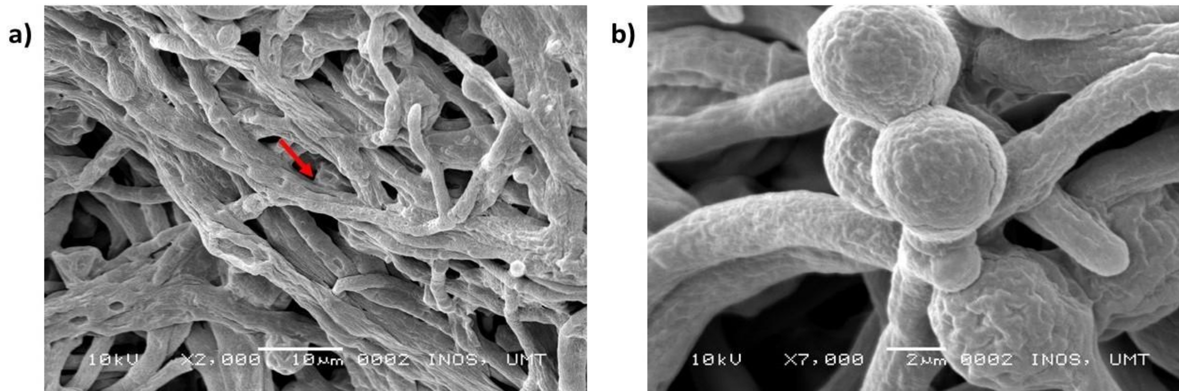

Figure 3: Scanning electron microscopy (SEM) analysis of *Panus lecomtei* at seven days of incubation period. (a) Arrow showed the dense of mature fibrous hyphal elements connecting between cells; (b) Budding formation of blastoconidia.

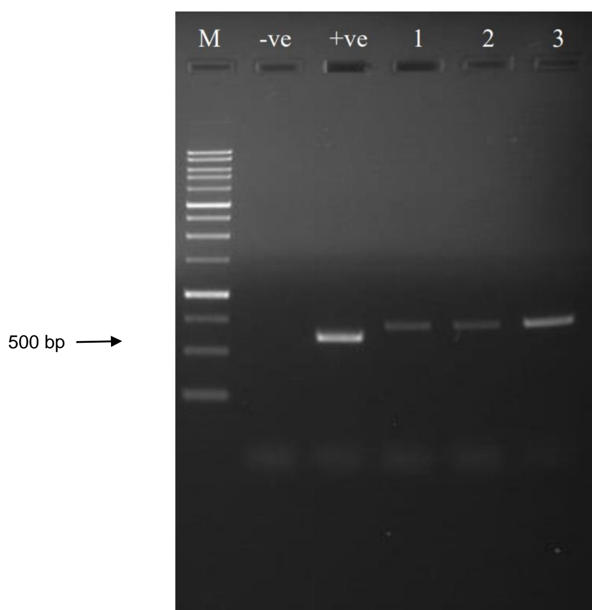

Figure 4: Gel visualization on UV transilluminator after performing PCR amplification. Lanes 1, 2 and 3 were the temporary name for the fungal isolates from *H. itama* larval food that has been identified by the PCR amplification technique. M: Marker (1 Kb DNA ladder); -ve: Negative control; +ve: Positive control; 1: Fungus strain SP 3; 2: Fungus strain SP 4; 3: Fungus strain SP5.

invasion of pathogenic microbes in *H. itama* larval food could cause a high mortality rate of the individual organism. This study suggests that biological aspects in *Panus* sp. could improve the quality of nutritional value in *H. itama* larval food.

Significantly differ from the previous study reported by Menezes *et al.* (2018) which identified *Monascus* sp. was not detected in the microbial mass eaten by *S. depilis* larvae. The research was then thoroughly studied by Paludo *et al.* (2018) who found *Zygosaccharomyces* sp. in *S. depilis* colony. To confirm the chemical interaction between *Monascus* sp. and *Zygosaccharomyces* sp., another essential study was conducted by Paludo *et al.* (2019), which has revealed the presence of fungi in *S. depilis* colony. The study identified the isolated fungi from different brood cells of *S. depilis* colonies were *Monascus ruber*, *Candida* sp. and *Zygosaccharomyces* sp. (Paludo *et al.*, 2019). Paludo *et al.* (2019) affirmed that volatile organic compounds (VOCs) were responsible for the growth stimulation. Molecular reaction revealed that ethanol and isoamyl alcohol produced by *Candida* sp. can significantly promote the growth of *Zygosaccharomyces* sp. while the growth of *Candida* sp. was supported by monascin produced by *M. ruber*. In our study, we observed *P. lecomtei* in *H. itama* colony, instead. Following the biologically active compounds derived from *P. lecomtei*, ethyl acetate compound from acetic acid and ethanol formation is suspected to be responsible to initiate a precursor of ecdysteroid biosynthesis to stimulate the insect moulting hormone (Kinnear *et al*., 1978), thus, speeding the metamorphosis process in stingless bee. Biosynthesis of ecdysteroids have been reported by Paludo *et al.* (2018), who proposed that chemical synthesis of these compound have resulted to a high average of larval pupation after a high amount of ergosterol consumption.

This finding suggests that *in-vitro* queen mass production is regulated from biological activities of *Panus* sp. Symbiotic bee fungi contribute to the survival of stingless bee larvae by providing specific nutrients and producing secondary metabolites, which help to protect larval food free from microbial contamination (Menezes *et al*., 2015; Menezes *et al*., 2018). Thus, this study may be the first to report about microorganisms isolated from the larval food of stingless bee especially for the Malaysian stingless bee, *H. itama*. Findings of this study are helpful to determine the presence of the microbes in *H. itama* larval food. Although it is accepted that the effectiveness of the BLAST result that is accurate and precise to identify microorganisms at genus level, further taxonomic studies using specific molecular tools such as species-

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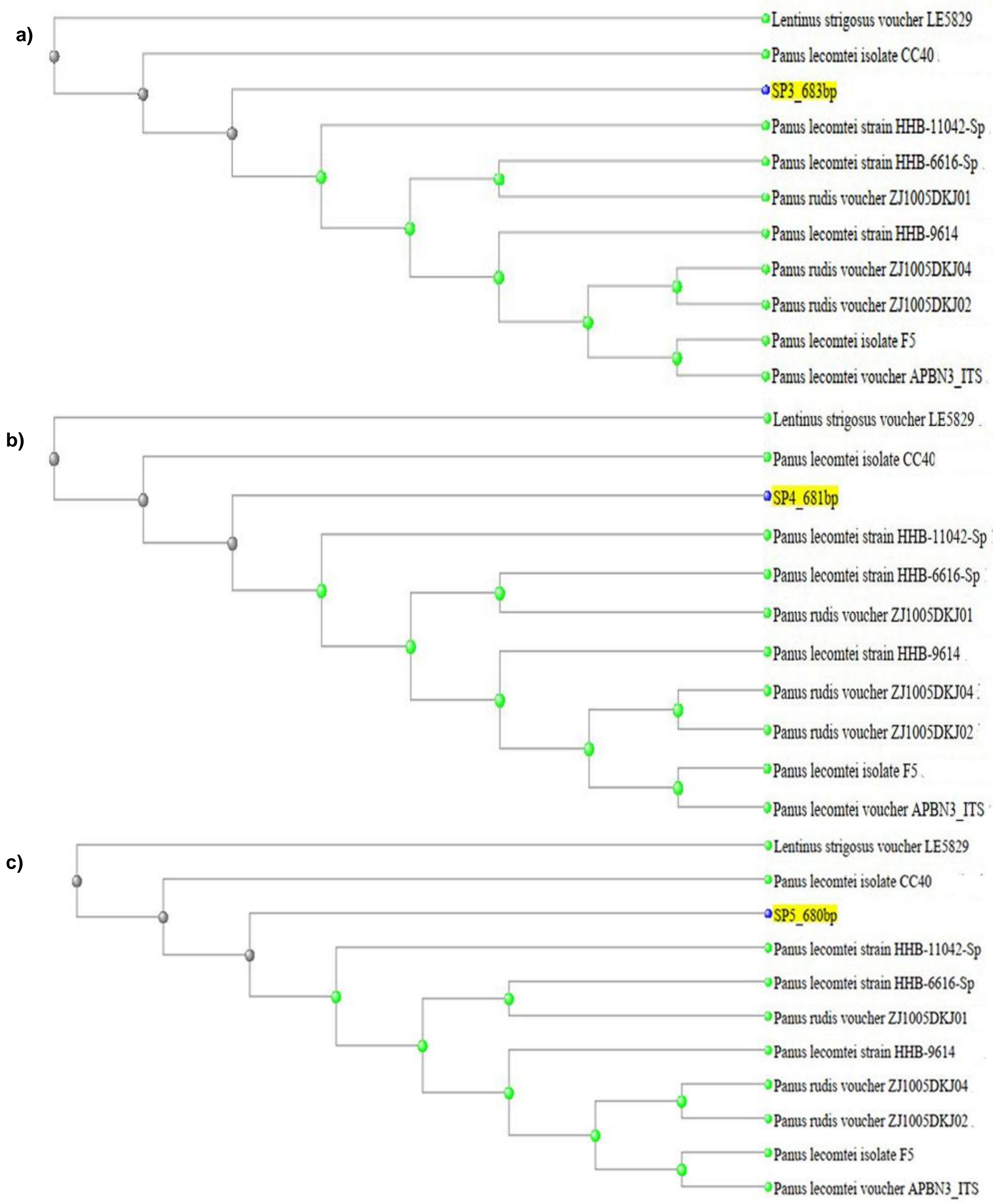

Figure 5: The phylogenetic trees comparing the ITS sequences of *Panus lecomtei*, fungus strains that were isolated from *H. itama* larval food (shown in font highlighted in yellow) with other species of *Panus* spp. and *Lentinus strigosus* (outgroup). The identity of gene sequences were generated from the strains (Note: a = SP3, b = SP4, c = SP5). Taxonomic relationship was identified by comparing the sequences in the National Centre for Biotechnology Information (NCBI).

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specific PCR was required to verify the taxonomic position of fungal species. Also, elucidation on other biological activities of fungi to serve as symbiotic relationship and their interaction with stingless bees would be interesting to be further studied.

CONCLUSION

Understanding the relationship between stingless bees and microbes has become one of the approaches to guarantee the sustainability of meliponiculture. This study proves that the larvae of *H. itama* depend on the presence of the beneficial fungus in order to pupate and survive. Somehow, a higher percentage of the larvae survival was observed from Treatment 2 (larvae provided with fresh larval food), which suggests that the stingless bee's larval food might contain other potentially beneficial microbes during the larval stage. Our results confirmed that the stingless bee larval food of *H. itama* contains symbiotic bee-fungus, namely *Panus lecomtei* (Basidiomycetes; Poriales; Polyporaceae) that are crucial for the pupation. This was verified again from the molecular approach, 23S rRNA gene sequencing. In conclusion, the study on microbial activity is crucial to gain an understanding for future research to explain the potential fungal isolates for mass queen production under *in-vitro* conditions. Nonetheless, more research should be explored in the future about the larval provision of the stingless bee, *H. itama* from the aspects of the identification of the other microorganisms for the stingless bee's larval development following the protocols in accordance and their biological activities and impacts on the quality of honey.

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