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MORPHOLOGICAL AND GENOTYPIC CHARACTERIZATION OF *ASCOSPHAERA APIS,* THE CAUSAL ORGANISM OF CHALKBROOD DISEASE, ISOLATED FROM EUROPEAN HONEY BEES (*APIS MELLIFERA L.*) COLLECTED IN SEVERAL APIARIES IN THE PHILIPPINES[1]

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ABSTRACT

Chalkbrood is a contagious brood disease of European honeybees (*Apis mellifera* L.) which lead to significant reduction of bee population and colony productivity. It is caused by the fungus *Ascosphaera apis,* and its diagnosis in the country involves inspection of colonies for mummified larvae but does not include cultivation and identification of the pathogen. The study, therefore, aimed to isolate, characterize, and identify the organism causing chalkbrood in several apiaries in the country. It also determined possible genetic variation which may affect their virulence in honeybee larvae. Mummified brood from eight apiaries in Luzon and Mindanao, and 21 commercially available local honey were used to isolate *A. apis*. A total of 12 mold isolates exhibited phenotypes typical of the fungus. Molecular identification using the internal transcribed sequence (ITS)-PCR revealed that all isolates had 100% ITS sequence similarity with *A. apis*. Genotypic characterization with repetitive sequence-based PCR using enterobacterial repetitive intergenic consensus (ERIC) sequences-specific primers revealed that isolates CALGS, CCLGS, LBLWM, LBLGM, BTCWM, MLBGS, CCLWM and LCBGS belonged to one cluster while LUDWM, SMRWM, BTCGS, and MLBWM were each placed in separate groups. Isolates BTCWM and BTCGS coming from the same apiary were placed moreover, into different groups while CCLGS and LCBGS from different apiaries clustered in the same group. These results indicate that genetic variation is observed among the *A. apis* isolates, and that such variation is not associated with the location of the chalkbrood-infected apiaries where the isolates were obtained.

Keywords: *Ascosphaera apis,* Chalkbrood, European honey bee, genotypic variation, honey bee disease

INTRODUCTION

European honey bees (*Apis mellifera* L.) just like other living organisms, are susceptible to a variety of diseases (Food and Agriculture Organization [FAO], 2006). Most of these diseases are specific to the brood or adult bees, but the most virulent diseases are those which affect the brood. Honey bee diseases are caused by bacteria, fungi, viruses and protozoa (Yadav & Kaushik, 2017).

Migratory beekeeping is a widely extended practice of providing crop pollination services and increasing yield of honey bee products (Jara et al., 2020). This, however, can be detrimental to honey bees for it can cause stress which will make them more susceptible to diseases (Martinez-Lopez et al., 2022). Honey bee colonies subjected to migratory practice were reported to have higher infestation rate and abundance of bee pathogens which include *Nosema ceranae* (Zhu et al., 2014), and some RNA viruses (Welch et al., 2009). It also allows the spread of pathogens and parasites between managed colonies and wild pollinators (Martinez-Lopez et al., 2022). This is demonstrated by the inter-species transmission of the honey bee RNA virus Israeli Acute Paralysis virus (IAPV) from infected honey bees to bumble bees and vice versa (Singh et al., 2010). Migratory practice and transport of colonies over great distances have caused rapid local, regional and global spread of honey bee pathogens and parasites such as *Vairimorpha ceranae, Varroa destructor* and *Ascosphaera apis* (Owen, 2017; FAO, 2006).

Chalkbrood is one of the many honey bee diseases observable in many apiaries worldwide. It is a contagious disease caused by the filamentous fungus, *A. apis* which affects the honey bee larvae of queens, workers and drones (Aronstein & Murray, 2010). It is an invasive mycosis that exclusively affects bee brood. Chalkbrood is found throughout the world and has been detected in most beekeeping areas in Chile, Central America, and Japan (Reynaldi et al., 2003). It was first reported in the Philippines in the 1980s (Nixon, 1982; Heath, 1985) but the observed cases of chalkbrood infection were not significant to devastate the colonies (Cervancia, 1997). This disease, however, can cause significant reduction in bee population and colony productivity (Aronstein & Murray, 2010). Cervancia (2018) reported that almost all *A. mellifera* colonies in the country are infected with the chalkbrood disease.

Conventional methods are commonly used to detect the fungal pathogen among honey bees which usually involves cultivation on rich culture media. Cultural, morphological and physiological characterizations are then being done to determine the identity of the isolates. Molecular methods involving an amplification of the internal transcribed spacer (ITS) regions of the ribosomal DNA of the fungal pathogen is also used to rapidly identify it (James & Skinner, 2005).

Genotyping of *A. apis* isolates from several countries by Jensen et al. (2012) revealed seven genetic strains of the bee fungal pathogen. Similarly, multiple genetic strains of the fungus from Australia were identified (Gerdts et al., 2021). Strain diversity was reported to be significantly higher in colonies with higher levels of chalkbrood infection and that some strains were observed as being dominant in infecting colonies (Roberts & Armstrong, 2021).

Since studies on cases of chalkbrood in the country only reported incidence of the disease in local apiaries through observation of field symptoms and presence of mummified larvae, this study was conducted to phenotypically characterize the causal organism isolated from chalkbrood-infected broods of *A. mellifera* which were collected from several apiaries in the country and screen for genetic variation.

MATERIALS AND METHODS

Sample collection

Honey bee brood samples that are fluffy white, swollen and sponge-like or look like white or grey mummies were collected from apiaries of eight cooperator-beekeepers of the UPLB Bee Program who reported case of chalkbrood infection during the time of sampling. Most of the samples came from *A. mellifera* apiaries in Luzon with three from Laguna, two from Batangas and one each from the Rizal Province and Metro Manila. One apiary from Lupon, Davao Oriental in Mindanao also provided mummified honey bee broods. The samples were collected from the bottom board as well as in brood cells with perforated cappings and in unsealed cells. Mummies obtained were placed in sterile containers which were brought to the laboratory for processing.

Commercially available local honey samples were also used as possible sources of the pathogen. Honey samples were obtained from 15 provinces; 10 from Luzon and five from Mindanao. There were no honey samples obtained from the Visayas. Most of the samples were produced by *A. mellifera* and the rest by *A. breviligula* (**Table 1**).

The causal organism of chalkbrood disease *A. apis*, can be isolated from either fresh or dried mummified larvae coming from the brood frames or the bottom board (**Figure 1**). Both white and dark colored mummies can be used for isolation of the pathogen (Jensen et al., 2012). Chalkbrood mummies appear white when they are infected by only one haploid mating type idiomorph (either + or -) but they turn grey or black when infected by both idiomorphs resulting to the formation of fruiting bodies or ascomata (Hornitzky, 2001; Aronstein & Holloway, 2013). Honey, on the other hand, can also be used in the detection of honey bee pathogens (Ribani et al., 2020). It is one of the bee products that does

not undergo transformation prior to sale, and is considered as a disease disperser due to the possible presence of infectious agents (Teixeira et al., 2018).

Table 1. Sources of honey samples screened for *Ascosphaera apis* and their producing bee species.

PROVINCE	BARANGAY/MUNICIPALITY/ CITY	HONEYBEE SPECIES
Ilocos Norte	Batac	*Apis breviligula*
Ilocos Sur	Bantay	*A. mellifera*
La Union	Supiden	*A. mellifera*
Benguet	Atok	*A. breviligula*
	Cabiten	*A. mellifera*
	Sapid	*A. mellifera*
	Tuba	*A. mellifera*
Mt. Province	Catengan,	*A. breviligula*
	Tadian	*A. breviligula*
	Tubo	*A. mellifera*
Bataan	Mariveles	*A. breviligula*
Laguna	Bay	*A. breviligula*
Batangas	Pulo, Lipa	*A. mellifera*
Quezon	Dolores	*A. mellifera*
Oriental Mindoro	Victoria	*A. breviligula*
Misamis Oriental	Cagayan de Oro	*A. mellifera*
Davao Oriental	Hamiguitan	*A. mellifera*
Davao del Sur	Bansalan	*A. breviligula*
Cotabato	Mlang	*A. mellifera*
South Cotabato	Koronadal	*A. breviligula*
	Lake Sebu	*A. breviligula*

Figure 1. Mummified larvae of *Apis mellifera* L. collected from an apiary in Maraoay, Lipa City, Batangas.

Isolation and purification

In isolating *A. apis* from mummies of brood with symptoms of chalkbrood, the procedure described by Jensen et al. (2012) with minor modification was followed. Fungal spores which may be present in honey were isolated following the methods of Anderson et al. (1997) and Reynaldi et al. (2003). Each collected mummified brood was surface-sterilized with 10 % sodium hypochlorite for 10 min, rinsed thrice with sterile distilled water for 2 min and sliced into three pieces with a sterile scalpel. These sections were then placed on Potato Dextrose Agar (PDA) plates supplemented with ampicillin and streptomycin at 6 µg/ml each and incubated in the dark at 35 ^{o}C under microaerophilic conditions. After a 48-hour incubation, the plates were then incubated aerobically at 35 ^{o}C. Fungal colonies which were visible after five to seven days were isolated, purified by repeated point inoculation on fresh PDA plates and maintained using the same agar medium.

For the honey, 50 grams sample was homogenized in a water bath kept at 40 ^{o}C. Ten milliliters of the sample were then obtained and mixed with equal volume of 0.01M sodium phosphate buffered saline, pH=7.2 (PBS). The mixture was then centrifuged at 3,500 x g for 45 min at 5 ^{o}C. The resulting supernatant was discarded. The pellet was resuspended in 3 ml of PBS and mixed vigorously prior to spread-plating on PDA plates and incubated under the same conditions.

Morphological characterization

The fungal isolates obtained were observed for the visibility of aerial mycelia, ascospore formation, growth diameters in cm, and surface texture of colonies produced. Microscopic examination of the isolates was done by using the slide culture technique. This examination allowed observation of fungal growth in its natural state without the risk of disrupting the mycelia during slide preparation. Agar blocks of 1 cm^2 area were prepared from sterile PDA and placed aseptically on glass slides with coverslips positioned on top of the block. Four sides of each agar block were then inoculated with the isolate before the tissue paper lining at the bottom of the plate was moistened with sterile water. All slide culture plates were then incubated at 30 ^{o}C for three to five days. The presence of septate hyphae with dichotomous branching and spherical fruiting bodies as well as sizes of the spore cysts and spore balls were determined.

Molecular identification

The method developed by Cenis (1992) was used in extracting genomic DNA from the putative *A. apis* isolates. About 500 µl of Potato Dextrose Broth (PDB) contained in a 1.5 ml microcentrifuge tube was inoculated with several hyphal threads and incubated for three days at 25 ^{o}C. The mycelial mat produced was pelleted by centrifugation at 13,000 rpm for 5 min and washed twice with 500 µl of Tris-EDTA (TE) buffer. The pellet was then treated with 300 µl of

extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and subsequently crushed manually with a conical grinder. Following this, 150 µl of 3M sodium acetate (pH=5.2) was added and the tube was placed at -80 °C for 10 min. The mixture was centrifuged again at 13,000 rpm for 5 min and the resulting supernatant was transferred to a clean tube and diluted with equal volume of cold isopropanol. The mixture was incubated for 15 min at -20 °C. After incubation, the mixture was centrifuged at 14,000 rpm for 10 min before the supernatant was carefully removed with the white pellet washed with 1 ml of 70 % ethanol. Centrifugation at 14,000 rpm for 3 min was done to remove the ethanol followed by drying under the laminar hood. Around 50 µl of distilled water was used to solubilize the extracted pellet.

Detection of extracted DNA from the fungal isolates was done by electrophoresing 4 µl of the sample mixed with 2 µl loading buffer on 1% agarose gel immersed in 0.5X Tris-Acetate EDTA (TAE) buffer using a Mupid® submarine electrophoresis system (Advance Corporation, Chuoku, Tokyo, Japan) set at 100V. Viewing of the agarose gel with the separated DNA fragments was done under ultraviolet light using a UVP Doc It® gel documentation system (UVP, LLC, Upland, California, USA) with GoodView™ serving as nucleic acid stain.

A PCR was performed to amplify the internal transcribed spacer (ITS) region of the ribosomal DNA of the fungal isolates. Two ITS primers, ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (5'- TCCTCCGCTT ATTGATA TGC-3') (White et al., 1990) were used to amplify the ITS regions including the 5.8S rRNA gene. The DNA fragments were amplified using Veriti™ Thermal Cycler (Applied Biosystems, Massachusetts, USA). The 50 µl reaction mixture contained 25 µl Vivantis Mastermix (2X) PCR buffer, 1.5 µl of 50mM $MgCl_2$, 3 µM of each primer, 5 µl of template DNA and 12.5 µl molecular biology grade water. The thermal cycling program consisted of 3 min initial denaturation at 95 °C, followed by 35 cycles of 40 sec denaturation at 94 °C, 50 sec primer annealing at 52 °C, 1 min extension at 72 °C, and a final 10 min extension at 72 °C (Lacap-Bugler et al., 2003). Four µl of amplicon with 2 µl loading buffer were electrophoresed in a 1.0 % (w/v) agarose gel in 0.5X TAE buffer at 100V and visualized under UV light using the same nucleic acid stain.

The amplified PCR products were sent to 1stBase-Asia (Malaysia) for sequencing. The resulting DNA sequences were then cleaned by excluding the PCR primer binding sites as well as the overlapping and low signals produced by nucleotides with Mega6.06 (http://www.megasoftware.net/). In identifying the closest match, sequences were compared against reference sequences for *A. apis* available in the International Nucleotide Sequence Databases using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Genotypic characterization

Following the rep-PCR protocol of Redondo et al. (2009) the genotypes of *A. apis* isolates were determined. The DNA primers with sequences: 5'-ATGTAAGCTCCTGGGGATTCAC-3'(ERIC1R), and 5'- AAGTAAGTGACTGGGG TGAGCG-3'(ERIC2) (Hulton et al., 1991) were used. PCR analyses were carried out in a final volume of 50 µl consisting of 1X of Taq Master Mix (Vivantis) and a final concentration of 2 mM $MgCl_2$ and 1.2 µM primers. The reaction conditions involved an initial activation step (5 min, 94°C), 40 cycles of denaturation at 94 °C for 1 min, annealing at 52°C for 1 min and elongation at 72°C for 8 min. This was followed by a final elongation step at 72°C for 10 min. Ten µl of the amplified samples were analyzed on a 1.6% agarose gel. The DNA bands stained with GoodView™ nucleic acid dye were visualized under UV light. Manual scoring of the resulting fingerprints was done and the data were analyzed using NTSYSpc2.1 program. Isolates which exhibited the same banding pattern were considered as one genetic variant of the fungal pathogen and clustered as one group.

RESULTS AND DISCUSSION

Colony characteristics of the isolates

Twelve mold isolates were obtained from the chalkbrood mummies which came from the eight sampled apiaries**.** All isolates produced dense white mycelia having aerial, surface and subsurface hyphae with average colony diameters ranging from 6.7 cm to 8.2 cm after seven days of incubation (**Table 2**). A ring of grey to black mycelia resulting from spore formation was observed beyond the center after four days of incubation for five of the isolates namely BTCGS, CCLGS, CALGS, LCBGS and MLBGS (**Figure 2**). This was confirmed when black zones of dark ascomata were visible at regions where hyphae of two possible mating types met (**Figure 3**). The rest of the isolates only formed thick white mycelia even after incubation for seven days which indicates that each of the isolate was probably composed of a single idiomorph. There were no fungal colonies with characteristics typical of the fungal pathogen obtained from the honey samples processed although several mold colonies were detected.

Morphological characteristics of the isolates

An observation of fungal morphology specifically, the fruiting bodies as well as septation and branching of the hyphal filaments are commonly used in the identification of *A. apis*. It is reported to produce distinct spherical ascomata or spore cysts containing the spore balls. Hyphal filaments of the said fungal pathogen have septations and show pronounced dichotomous branching (Hornitzky, 2010).

All of the isolates were observed to produce septate hyphae with dichotomous branching (**Figure 4**). In the case of sporulating isolates, average diameters of the ascomata and spore balls were observed to fall within the range of 56.4 to 78.3 μm and 13.2 to 18.8 μm, respectively (**Table 3**).

Table 2. Cultural characteristics and colony diameter of the twelve mold isolates on Potato Dextrose Agar (PDA) and incubated for 7 days at 30 ^{o}C.

LOCALITY	ISOLATE	CULTURAL CHARACTERISTICS	COLONY DIAMETER (cm)
Bicutan, Taguig City	BTCGS	Thick, white, aerial mycelia with a ring of gray, sporulating mycelia	8.2
	BTCWM	Thick, white, aerial mycelia	7.9
San Mateo, Rizal	SMRWM	Thick, white, aerial mycelia	7.3
Calamba City, Laguna	CCLGS	Thick, white aerial mycelia with a ring of gray, sporulating mycelia	7.2
	CCLWM	Thick, white, aerial mycelia	6.7
Calauan, Laguna	CALGS	Thick, white, aerial mycelia with a ring of gray, sporulating mycelia	7.9
Los Baños, Laguna	LBLGM	Thick, white, aerial mycelia	7.3
	LBLWM	Thick, white, aerial mycelia	7.0
Lipa City, Batangas	LCBGS	Thick, white, aerial mycelia with a ring of gray, sporulating mycelia	7.3
Maraoay, Lipa City, Batangas	MLBGS	Thick, white, aerial mycelia with a ring of gray sporulating mycelia	7.6
	MLBWM	Thick, white, aerial mycelia	7.2
Lupon, Davao Oriental	LUDWM	Thick, white, aerial mycelia	7.1

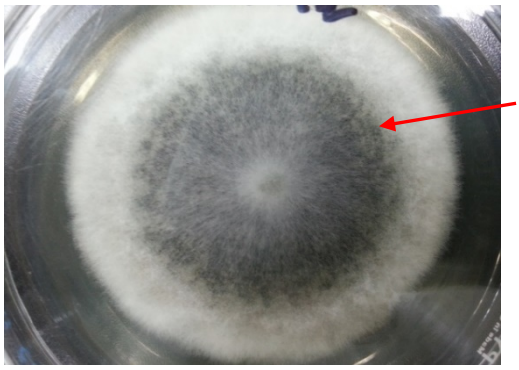

Figure 2. Colony of fungal isolate BTCGS grown on Potato Dextrose Agar showing the ring of grey to black mycelia after 4 days of incubation at 30 ^{0}C. Arrow indicating the area of spore formation.

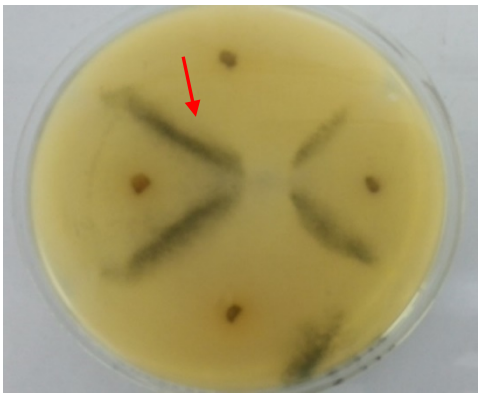

Figure 3. Characteristic black regions of ascomata at the interface of two possible mating types of the fungal isolate BTCGS. Arrow indicating the region of ascomata formation.

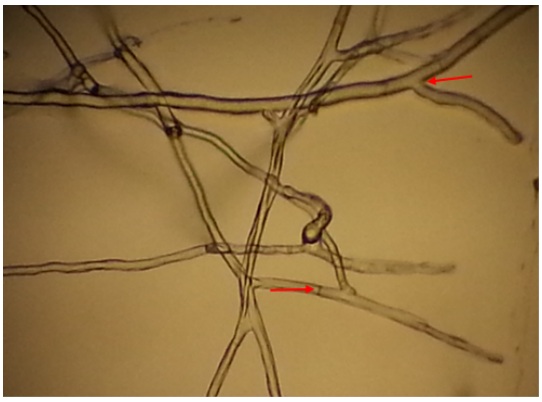

Figure 4. Hyphae of isolate LBLGM showing septation and dichotomous branching (400X). Arrow indicating septation in the hyphal filament.

Table 3. Average diameters of the ascomata and spore balls of the sporulating fungal isolates.

ISOLATE	ASCOMATA DIAMETER (µm)	SPORE BALL DIAMETER (µm)
BTCGS	56.4	13.2
CCLGS	78.3	18.6
CALGS	63.8	13.5
LCBGS	62.3	13.5
MLBGS	67.5	13.5

The ascomata produced by *Ascosphaera* are considered as its most distinctive characteristic. Their spherical ascomata are about 47 to 140 µm in diameter and contain numerous spore balls having hyaline spores (Aronstein & Holloway, 2013). Spore balls are 7 to 20 µm in diameter while the spores are less than 2 µm wide (Bisset, 1988). Based on the data obtained for the five spore forming isolates, measurements of the ascomata and spore balls were all in the range described for the fungal pathogen with isolate CCLGS with the biggest ascomata and spore ball measuring of 78.3 and 18.6 µm diameters, respectively **(Figure 5**).

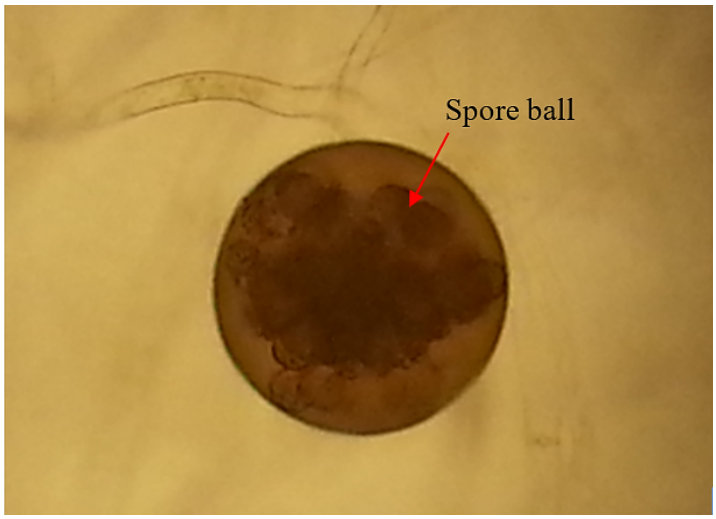

Figure 5. Ascoma produced by isolate CCLGS containing several spore balls (400X).

Polymerase chain reaction-based identification

Since *Ascosphaera* lacks distinguishing characteristics, the genus is considered as a difficult group to identify. Most identification methods developed were based on observed characteristics of the ascomata, spore ball and spores. Overlaps in such characteristics however, were observed among eight species of *Ascosphaera* (James & Skinner, 2005).

Polymerase chain reaction (PCR) using primers specific to the internal transcribed spacer (ITS) regions were also used to identify the species of *Ascosphaera*. The ITS regions are sections of the rDNA with non-functional sequences situated between structural ribosomal RNAs. These are excised as non-functional by-products and are rapidly degraded during rRNA maturation. Murray et al. (2005) made use of differences in sequences of the ITS to develop primers which differentiated four species of *Ascosphaera* associated with honey bees.

Using the primer pair ITS 1 (forward) and ITS 4 (reverse), the internal transcribed spacer (ITS) regions including the 5.8S and small portions of 18S and 28S rDNA sequences of the fungal isolates were amplified via PCR. Each of the 12 fungal isolates was observed to form a single band with size falling within the range of 600 to 800 bp (**Figure 6**). Possible identities of the isolates were then determined based on comparison of ITS sequences with those listed in the DNA database. All of these isolates were observed to have 100% identity match with several strains of *A. apis* namely Strain 7405, ST -OR11-A1, m05, m10 and ATCC MYA-4450.

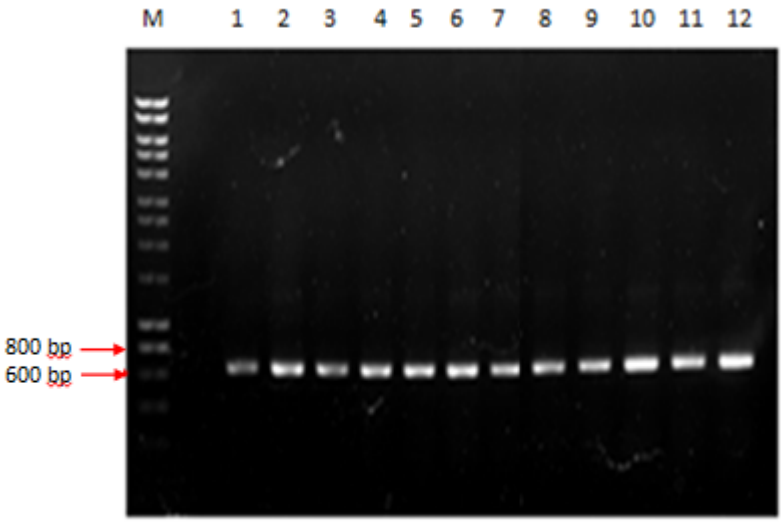

Figure 6. PCR products using ITS 1 and ITS 4 primers. Lane M: Hyperladder™ kb, (1) CALGS, (2) CCLGS, (3) LBLWM, (4) LBLGM, (5) BTCWM, (6) MLBGS, (7) CCLWM, (8) BTCGS, (9) LUDWM, (10) SMRWM, (11) MLBWM, (12) LCBGS.

Genotypic characterization of *Ascosphaera apis* isolates

Along with bacteria and archaea, eukaryotic microorganisms possess repetitive sequences with unknown functions. These sequences however, are being utilized in PCR to generate unique DNA profiles or fingerprints of individual microbial strains (Ishii & Sadowsky, 2009). The use of repetitive sequence-based PCR (rep PCR) technique with either BOX, REP or ERIC sequences-specific primers can help distinguish different genotypes of *A. apis* (Reynaldi et al., 2003).

Twelve to 14 amplicons were generated with sizes ranging from slightly above 2,500 bp to values about 200 bp although one isolate MLBWM, produced an amplicon having a size below 200 bp (**Figure 7**). Majority of the isolates produced 14 amplicons except for isolates LUDWM and SMRWM having 13 and BIMGS with only 12 amplicons.

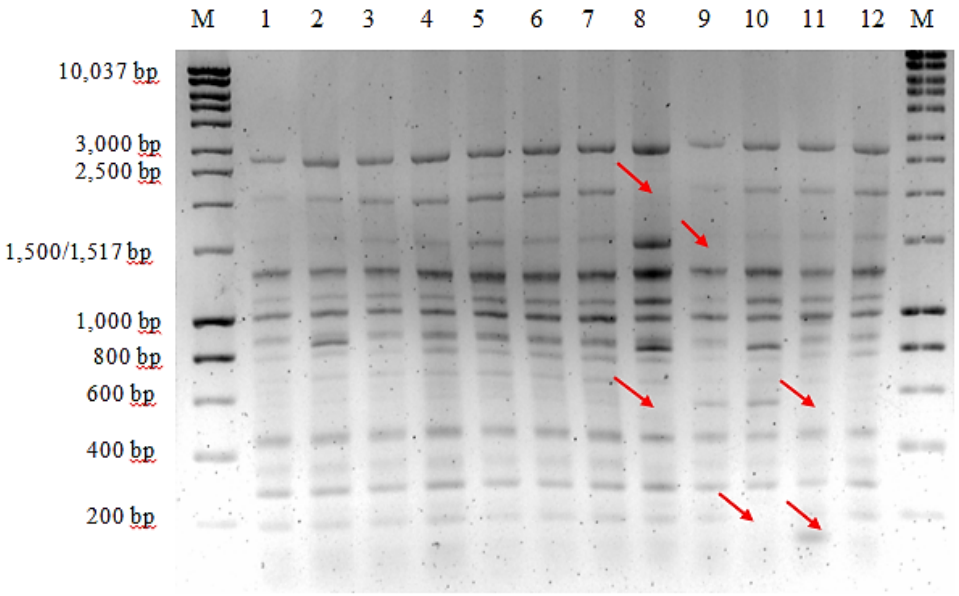

Figure 7. Characteristic rep-PCR fingerprint patterns of the fungal isolates obtained from chalkbrood mummies using ERIC sequences-specific primers. Lane M: Hyperladder ™ 1: kb; (1) CALGS, (2) CCLGS, (3) LBLWM, (4) LBLGM, (5) BTCWM, (6) MLBGS, (7) CCLWM, (8) BTCGS, (9) LUDWM, (10) SMRWM, (11) MLBWM, (12) LCBGS. Arrows indicating absence or presence of bands.

Majority of the 12 isolates exhibited the same DNA fingerprint or band pattern except for four isolates where there were missing or additional amplicons. Amplicons with sizes of about 2,000 bp and 600 bp were not observed in isolate BTCGS while isolate LUDWM lacked an amplicon having a size of about 1,500 bp. A 200 bp-sized amplicon was also not observed in Isolate

SMRWM. Meanwhile, Isolate MLBWM lacked a 600 bp amplicon but had an additional one with a size below 200 bp. Based on the DNA fingerprint generated, grouping of the isolates was determined using the NTSYS software (**Figure 8**).

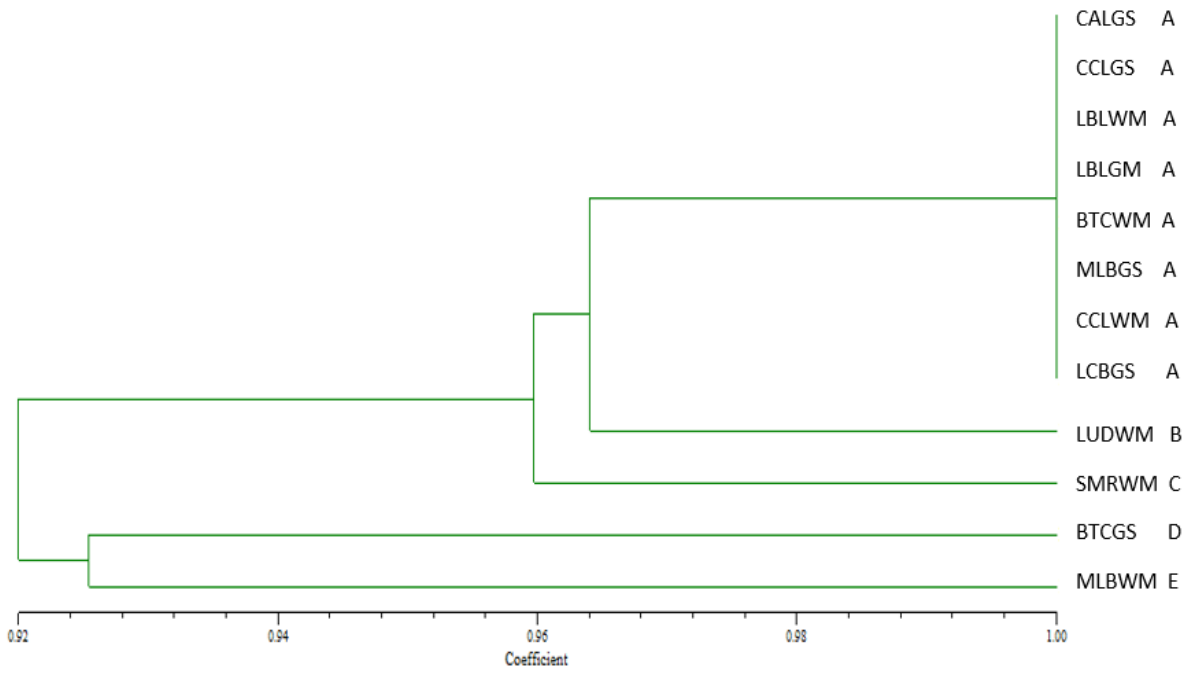

Figure 8. Dendrogram showing the similarity of the twelve isolates of *Ascosphaera apis* obtained from chalkbrood mummies based on the band pattern generated using ERIC sequences-specific primers in rep-PCR. Clustering is based on the UPGMA algorithm and DICE coefficient.

Results showed that the twelve *A. apis* isolates clustered into five different groups at coefficient values above 0.97. Eight of the 12 isolates namely CALGS, CCLGS, LBLWM, LBLGM, BTCWM, MLBGS, CCLWM and LCBGS exhibited 100% similarity in their DNA fingerprints and were clustered into one group while isolates LUDWM, SMRWM, BTCGS and MLBWM were placed each into four separate groups. The observed clustering into different groups indicates genetic variation among the *A. apis* isolates.

Genetic variation among the 84 *A. apis* isolates obtained from infected brood and/or honey samples which were collected from Argentina and Chile were also demonstrated by Reynaldi et al. (2003) using rep PCR. They reported that the isolates clustered into six different groups based on the DNA fingerprints generated. Given the prevalence of one band pattern among the isolates, they suggested that a limited diversity is observed in the populations of *A. apis* from the said countries.

Twenty isolates of *A. apis* from Denmark were also reported to exhibit genetic variation. Using primers for the eukaryotic translation elongation factor 1 α (EF1 α) as well as scaffolds 300 and 1635, Vojvodic et al. (2011) showed that the Danish *A. apis* strains clustered into four different groups. Similarly, *A. apis* isolates from Australian apiaries with chalkbrood were also reported to exhibit genetic variation using the same set of primers. Six distinct groups were identified with two being unique in Australia while the rest were reported in other countries (Gerdts et al., 2021).

The location of the apiaries from where the isolates were obtained possibly had no effect on the clustering made based on the DNA fingerprint patterns. Organisms of similar origin were observed to be placed into different groups such as the case of BTCGS and BTCWM coming from the same apiary in Bicutan, Taguig City. Those coming from different locations were placed in the same group just like isolates CCLGS and LCBGS isolated from Calamba City, Laguna and Lipa City, Batangas, respectively. A similar case was also observed by Reynaldi et al. (2003) when they did DNA fingerprinting of several *A. apis* isolates coming from different locations in Argentina and Chile. They mentioned however, that comprehensive studies involving a lot more isolates should be made to prove that strain variation observed in *A. apis* is not affected by geographic origin.

SUMMARY AND CONCLUSION

Mummified larvae from eight *Apis mellifera* apiaries in the country with reported incidence of chalkbrood disease and 21 commercially available local honey were collected and used in the isolation of *Ascosphaera apis*. Sample processing and subsequent plating on appropriate culture medium led to the isolation of 12 mold isolates with five having colonies which sporulated and produced a ring of grey mycelia. This happened four days after inoculation while the rest had only dense white mycelia. Morphological characterization revealed that all of the isolates had septate hyphae with pronounced dichotomous branching. The five sporulating isolates were observed to produce ascomata with spore balls with sizes ranging from 56.4 to 78.3 µm and 13.2 to 18.8µm, respectively. Such phenotypic characteristics are typical with the fungal pathogen, *A. apis*. Molecular identification using the internal transcribed spacer (ITS) region of the ribosomal DNA revealed that all of the fungal isolates had a 100% identity match with several strains of the said bee fungal pathogen.

Genotypic characterization of the *A. apis* isolates through rep-PCR using ERIC sequences-specific primers revealed that the 12 isolates belonged to five different clusters. Isolates CALGS, CCLGS, LBLWM, LBLGM, BIMWM, MLBGS, CCLWM and LCBGS were grouped into one cluster while LUDWM, SMRWM, BIMGS, and MLBWM were each placed in a group of its own. The result also showed that such variation among the isolates is not associated with the location of the eight apiaries where the infected brood samples were obtained. Fungal

isolates BTCGS and BTCWM which originate from the same apiary, were observed to be placed into different groups. Isolates CCLGS and LCBGS on the other hand, clustered in the same group but were isolated from different apiaries.

Genetic variation as observed in the local isolates of the bee fungal pathogen, further investigation should be conducted to determine its possible effect on their virulence not only to *A. mellifera* but also to other honey bee species found in the country. The use of more primers should also be done to confirm genetic diversity among local isolates of *A. apis* and to obtain isolates from more apiaries in the country. This will then determine whether association exists between the fungal pathogen and the location of the apiary infected with chalkbrood.

This study conducted has proven to be the first reported molecular identification and demonstration of genetic variation in *A. apis* isolated from populations of the European honey bee, *Apis mellifera* L. in the country.

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