Indian Journal of Entomology Online published Ref. No. e24198 DoI. No.: 10.55446/IJE.2024.2198

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION AND MATING TYPE DETECTION OF CHALKBROOD FUNGAL PATHOGEN *ASCOSPHAERA APIS* IN *APIS MELLIFERA* L. IN SOUTHERN INDIA

V Karthik[1], M R Srinivasan[1]*, V R Saminathan[1], S Karthikeyan[2] and V Balasubramani[1]

[1]Department of Agricultural Entomology; [2]Centre for Post Harvest Technology,
Tamil Nadu Agricultural University, Coimbatore 641003, Tamil Nadu, India
*Email: mrsrini@tnau.ac.in (corresponding author): ORCID ID 0000-0002-5922-8208

ABSTRACT

Chalkbrood *Ascosphaera apis* is a fungal brood pathogen that exerts considerable biotic stress on honey bees worldwide. Chalkbrood was noticed at Coimbatore in *Apis mellifera* colonies for the first time. Precise identification of the fungal pathogen and their mating types is crucial to develop effective disease management strategies. Hence, the present study aimed to isolate and identify fungal pathogen morphologically and genomically. Scanning Electron Microscopic (SEM) analysis was done and confirmed the essential morphological characteristics of the isolated fungus such as spore cysts (51-86 µm in diameter), spore balls (8-15 µm in diameter), and ascospores (1.7-2.6 µm in length). Molecular characterization using internal transcribed sequence (ITS)-PCR of the fungal isolate indicated 99-100% sequence similarity to *A. apis*. In addition, the multiplex PCR assay was performed and the mating types MAT1-1 and MAT1-2 were successfully detected and named TNAU CBD MAT1 and TNAU CBD MAT2, respectively.

Key words: *Apis mellifera*, fungal pathogen, *Ascosphaera apis*, identification, morphology, SEM analysis, ITS, multiplex PCR, mating types, spore cysts, spore balls, ascospores

The honey bee fungal brood pathogen *Ascosphaera apis* Olive and Spiltoir, heterothallic fungus is a global threat to the beekeeping industry, causing individual larval mortality, and greatly affects the colony productivity (Spiltoir, 1955; Spiltoir and Olive, 1955; Baily, 1963; Wood, 1998). The genus *Ascosphaera* comprises four species known to occur in honey bee colonies: *Ascosphaera apis* (Chalkbrood), *A. major*, *A. atra*, and *A. duoformis* (Anderson and Gibson, 1998). The defining character of the *Ascosphaera* genus is the formation of spherical ascomata or sporocysts containing multiple spore balls composed of numerous ascospores. These species exhibit differences in spore cyst size and variations in the shape and size of ascospores (Bissett et al., 1997). Chalkbrood opportunistically infects bees combined with other forms of stress (Yoder et al., 2014), while recent research indicates that honey bee viruses may have the capability to infect and multiply within the fungal pathogen *A. apis* (Li et al., 2014; Cheng et al., 2022). The development of chalkbrood fungus takes place when bee colonies experience cool, damp environments with inadequate airflow, particularly during the spring season (Mehr et al., 1976; Gilliam et al., 1978; Puerta et al., 1994; Flores et al., 1996; Borum and Ulgen, 2008).

Chalkbrood disease was first reported in Europe in the early 1900s (Maassen, 1913), and the incidence was inconspicuous outside Europe until the early 20th century. Currently, the disease has spread globally with an increasing incidence in many countries (Kluser and Peduzzi, 2007; Sevim et al., 2022: Das et al., 2023). The honey bee larvae aged 1-4 days old are most susceptible to the chalkbrood fungal infection, mainly transmitted through contaminated food fed by the nurse bees (Bailey 1963; Gilliam et al., 1978; Holloway et al., 2012). Initially, the ingested spores get activated in an anaerobic environment of the larval gut under the CO_2 condition followed by hyphal ramification, resulting in cessation of feeding by larvae (Nelson and Gochnauer, 1982; Koenig et al., 1987; Bamford and Heath, 1989). The spores of *A. apis* remain persistent and viable for 15 years in the hive environment, serving as the source of inoculum for further infection (Bailey and Ball, 1991). All castes of honey bee broods (worker, drone, queen) can be infected by a chalkbrood fungus (Wynns, 2012). *Apis cerana* was the major honey bee species in northern India till 1960s. *A. mellifera*, the Italian honey bee was introduced successfully into northern India during the 1960s and southern India during the 1990s (Atwal and Sharma, 1968; Chaudhary, 1997). Now *A. mellifera* has been successfully established throughout northern India while *A. cerana* is still an important honey bee species

contributing to honey production in south India. At present, in south India beekeepers have started rearing the Italian honey bees, utilizing the major honey crops such as moringa and sunflower (Kishan et al., 2017). For the first time in the Coimbatore district of Tamil Nadu, we detected the occurrence of chalkbrood in *Apis mellifera* colonies during June 2022. The occurrence of the chalkbrood disease was so serious in *A. mellifera* that it caused infection in 80% of the colonies and caused a colony loss of up to 45% during Oct 2022. In some parts of the world, the chalkbrood fungus *A. apis* was found to infect the otherwise less susceptible *Apis cerana cerana* and even resulted in high bee mortality (Gilliam et al., 1993; Chen et al., 2018). Since most of the Tamil Nadu beekeepers depend on the Indian honey bee *Apis cerana indica,* for their honey production and livelihood, any possible cross-infection of chalkbrood *A. apis* might pose a serious threat to the Tamil Nadu beekeeping industry. Hence, as a prerequisite, the fungal pathogen's precise identification is highly essential for effective disease management.

Understanding the molecular basis of fungal development and reproduction could directly contribute to improving disease control strategies. Typically, the existence of chalkbrood mummies validates disease presence during field diagnosis. Consequently, confirming the presence of spore cysts using microscopy in the laboratory is crucial (Jensen et al., 2013). Polymerase Chain Reaction (PCR) and Internal Transcribed Spacer (ITS) gene region-based DNA markers are widely used to identify fungi at genus to species level. Furthermore, molecular techniques are used to obtain rapid, reliable, and accurate identification. The fungus *A. apis* exhibit heterothallism, possessing two mating types (Mat-1 and Mat-2) that cannot be differentiated based on morphology (Aronstein and Murray, 2010; Aronstein et al., 2007). Recognition of the mating types is crucial for gaining a deeper insight into fungal reproduction, biology, and pathogenic characteristics. To conduct the mating type assay, the reference strains namely ARSF 7405 and ARSF 7406 are required. Unfortunately, no reference strain is available in India. Hence, we initially separated the unknown mating type isolates as described by Jensen et al. (2013), and the mating type was determined by a multiplex PCR assay developed by Aronstein and Colby (2015). Probably, this could be the first report on the mating type detection of the fungus *A. apis* in India. Considering the above conditions, the current study intended to determine the mating type and identify the fungus *A. apis* phenotypically by Scanning Electron Microscopy (SEM) and genomically by using an ITS gene marker; phylogenetic analysis was also performed to study its close relatives.

MATERIALS AND METHODS

The *A. apis* infected *Apis mellifera* colonies were identified by the presence of mummified larvae in the brood comb, bottom board, and hive entrance, preferably the white mummies were collected to facilitate mate separation, and black mummies were collected for microscopic examination. All the samples were collected from the *Apis mellifera* colonies located at the Apiary ($11^0$0'59''N; $76^0$55'47''E), Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India in June 2022. All the samples were stored in a deep freezer at -20^0C until used for isolation. The fungal strain was isolated from the fresh white mummies that were collected from infected brood frames or bottom boards. Then, the collected mummies were surface sterilized with 10% sodium hypochlorite for 10 min followed by rinsing with sterile distilled water for 2 min under aseptic conditions. Sterilized mummies were incised into small pieces, placed on the Potato Dextrose Agar (PDA) plates amended with 50 ppm of streptomycin to prevent bacterial contamination, and incubated in dark condition at 30-34^{0}C for 3-6 days in an incubator. During incubation, the growth of mycelia and sporulation was observed. Once the sporulation point was observed, mating types were separated on PDA plates without antibiotics and purified by the hyphal tipping technique with the aid of a microscope. For further studies, the separated fungal strains were maintained at -20^0C with 15% glycerol (v/v).

Initially, the presence of sporocysts in the field-collected samples was verified under a stereo microscope (Leica M205A stereo microscope, Leica Microsystems) followed by fungal hyphae were slide-mounted with distilled water and seen under a phase contrast microscope. Furthermore, the ultrastructure of sporocyst, spore balls, and ascospores were observed under an SEM. For SEM analysis, the matured sporocysts harvested from PDA plates were used. The harvested sporocysts were mounted on pin stubs supported with double-sided tape. Then the mounted sporocysts were randomly incised with a sterile scalpel to ensure the release of spore balls. Mounted specimens were subjected to gold sputter coating and observed under the SEM (FEI Quanta 250, Netherlands) having an Everhart Thornley Detector with tungsten as an

electron source. The size of the sporocyst, spore balls, and ascospore were also determined.

Morphologically confirmed fungal strains were further subjected to molecular analysis by rDNA-ITS studies. A total of 50mg of fresh mycelium was taken in 2% CTAB (Cetyl trimethyl ammonium bromide) buffer and the genomic DNA was extracted as per the protocol given by Wu et al. (2001). The extracted DNA samples were stored at -20^{0}C for further studies. The primer pair of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as a forward and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') as a reverse was used for the PCR amplification of ITS1-5.8S-ITS2 and 18S regions of fungal isolates. The total volume of the reaction mixture was 40 µl, which comprised of 4 µl genomic DNA, 4 µl forward primer, 4 µl reverse primer, 8 µl of nuclease-free water, and 20µl master mix (Smart prime). PCR was performed in a thermal cycler (Applied Biosystems, Thermo Fisher Scientific, USA) with the following conditions: 5 min initial denaturation at 95^{0}C; 40 cycles of denaturation (1 min at 95^{0}C), annealing (1 min at 60°C) and extension (1min at 72°C); final extension at 72°C for 10 min. The resulting PCR product (5 µl each) was electrophoresed at 90 V for 45 min using 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. The remaining PCR products were Sanger sequenced by Syngenome (Coimbatore, India). The obtained DNA sequences were edited and aligned in BioEdit 7.2. Sequences were compared to NCBI Gen Bank database using BlastN. The ITS gene sequence of the fungal isolate was successfully deposited in the NCBI GenBank database under the accession number OQ641236. For constructing a phylogenetic tree, the ITS nucleotide sequences of *Ascosphaera apis* from various regions of the world were retrieved from NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and matched with our fungal isolates to compare similarity or diversity. MEGA11 (Version 11.0.13) software was employed to construct a phylogenetic tree using the Neighbor-Joining method along with 1000 bootstrap replicates.

Once the single mate type was designated as described earlier in the isolation part, the dual test was performed to verify the opposite mating type. To conduct a dual test, 6 mm dia of the fresh mycelial discs were taken, and opposite strains were placed on PDA at a distance of 4 cm. The plates were incubated at 30-34^{0}C for 3-6 days. After incubation, the production of the reproductive structure between the meeting point of the two mycelia confirms the presence of opposite mate types. After the confirmation, each mating type was maintained separately on PDA plates for further analysis. The mating types were identified through a multiplex PCR assay created by Aronstein and Colby (2015). Initially, the genomic DNA (gDNA) of the undetermined fungal mating types and mixed idiomorphs were extracted as described in the molecular identification part. The extracted gDNA samples were stored at -20°C for later use. The multiplex PCR test was performed using two sets of primers specified as given here's:

Primer- Sequence 5′ to 3′- Gene- Product size (bp)- References:

Mat1F-83; AGCAGACGCTAAAGAACTTG; Mat1-1; 478; (Aronstein and Colby, 2016)

Mat1R-562; ATTGGGTGGAACAATGCCTA; Mat1-1; 478; Aronstein and Colby (2016)

Mat2scaf74F; AAA ATA CCA AGG CCA CCG A; Mat1-2; 212; Aronstein et al. (2007)

Mat2scaf74R; GGAGCATATTGGTAATTTGG; Mat1-2; 212; Aronstein et al. (2007).

PCR amplification was performed in 30 µl of the final reaction volume, containing 15 µl of Master mix (Smart prime), 1 µl of each of four primers, 1 µl of gDNA, and 10 µl of nuclease-free water. The PCR reactions were carried out in a thermal cycler (Applied Biosystems, Thermo Fisher Scientific, USA) with the following conditions: initial denaturation at 95 for 3 min; 35 cycles of denaturation at 95°C (30 s), annealing at 56°C (30 s), and extension at 72°C (30 s); final extension at 72°C (2 min). The PCR bands were verified with 1.8% agarose gel and documented using the Gel Doc imaging system (Mediccare, Gelstan, India).

RESULTS AND DISCUSSION

Chalkrood mummies were noticed on the brood frame (Fig. 1A), and bottom board during field diagnostics. Examining the black mummies collected from the field under a stereo-zoom microscope confirms the presence of sporocysts (Fig. 1E), which is a characteristic feature commonly found in the *Ascosphaera* genus (Bissett et al., 1996; Chorbinski and Rypula, 2003; Aronstein and Murray, 2010). Also, isolates produced dense mycelia (Fig. 1D) with pronounced dichotomous branching (Fig. 1F) as previously reported by Skou (1988) and Hornitzky and Anderson (2010). Furthermore, the isolated fungi produced black-colored ascomata after 6 days of post-inoculation (Fig. 1B). SEM analysis

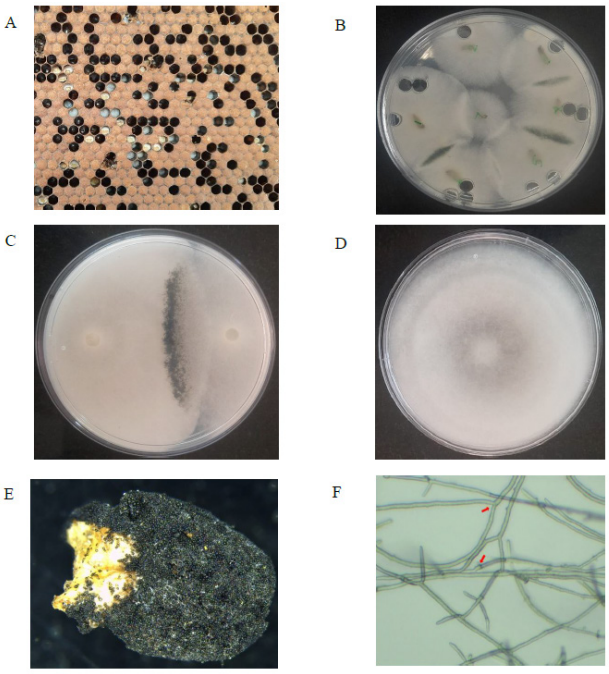

Fig. 1. Symptom and identification of *Ascosphaera apis*. A. Chalkbrood-infected brood frame. B. Mating type separation from field-collected mummies. C. Verification of fungal MAT type by dual test. D. Dense mycelial growth of *Ascosphera apis*. E. Chalkbrood mummy with numerous sporocysts (60x magnification). F. Hyphae of isolate fungi - Pronounced dichotomous branching (upper arrow) with septation (lower arrow) (400X)

indicated that the wall of a spherical ascoma or spore cyst was double-layered (Fig. 4A), with a smooth outer surface (Fig. 4B) and highly verrucate inner wall (Fig. 4C) (Bissett, 1988; Skou, 1988; Anderson and Gibson, 1998; Chorbinski and Rypula, 2003). The size of the fully mature ascoma ranges from 51 to 86 µm in diameter, consistent with previous studies (Christensen and Gilliam, 1983; Chorbinski and Rypula, 2003). The spore balls were oval with a diameter of 8-15 µm and had no apparent outer membrane (Fig. 4D). Ascospores are typically ellipsoidal or fusiform in shape, bend often, and are 1.7-2.6 µm long with rounded ends (Chorbinski and Rypula, 2003). Conclusively, SEM examination findings validate the diagnostic approaches used. The outcomes confirmed that the examined strains are classified within the *Ascosphaera* genus and specifically belong to the *Ascosphaera apis* species. This was the first time, chalkbrood appeared in Tamil Nadu, India, probably due to prolonged damp weather along with intermittent rain and scarcity of the floral resource as well. Eventually, the population of the affected colony dwindled and the colony perished.

Regularly, the honey bee colonies contaminated with *A. apis* have not shown any visible symptoms. Hence, the early detection of disease is vital for disease management. This can be achieved by molecular diagnostic methods, which allow us to detect the disease even at the subclinical level. Initially, the genomic DNA of the isolated fungi was extracted successfully. Using the primer pair ITS1 and ITS4, the fungal DNA was amplified with a band size of 600-700bp (Fig. 2). The ITS (internal transcribed spacer) sequence analysis has been widely used in fungal barcoding and identification (Schoch et al., 2012). The amplified products were Sanger sequenced and the obtained sequences were confirmed using NCBI's database and BLASTn programs. Based on BLASTn analysis, the isolate of Coimbatore had 99 to 100% similarity with *Ascosphaera apis* sequences available in the GenBank database. The fungal sequences were submitted successfully to the NCBI database under the accession number OQ641236. Previous studies have demonstrated 100% sequence similarity among *A. apis* strains recovered from European honey bee larvae collected across continents (Anderson et al., 1998). However, recent investigations found no substantial variations in *A. apis* isolates based on ITS gene region (Jensen et al. 2013). These results are consistent with our findings. Reynaldi et al. (2003) recommend using PCR-based DNA fingerprinting methods such as BOX, REP, and ERIC PCR to identify genetic variation among isolates. In this way, epidemiological investigations may help in establishing the clonal identity or relatedness of *A. apis* isolates.

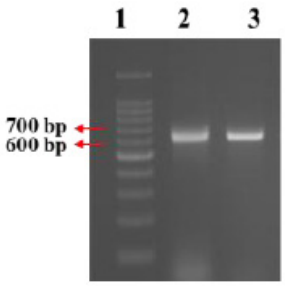

Fig. 2. PCR amplification of isolated fungi.
Lane 1: 100 bp ladder; Lane 2,3: isolated fungi

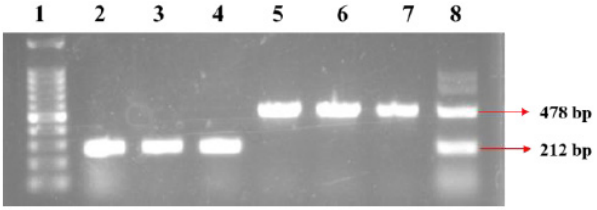

Fig. 3. Multiplex PCR amplification of Mating types. Lane 1: 100bp ladder; Lane 2,3,4: Mat1-2 (TNAU CBD MAT 2); Lane 5,6,7: Mat1-1(TNAU CBD MAT 1); Lane 8: Mixed idiomorphs

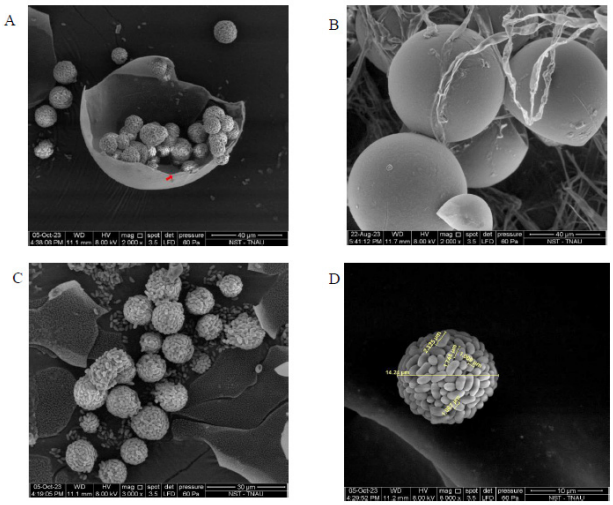

Fig. 4. SEM images analysis of *Ascosphera apis* fruiting body. A. Double-layered wall of the spore cyst. B. Spore cysts with smooth outer surface C. Verrucate inner wall of spore cyst. D. Single spore ball with no apparent outer membrane.

To construct a phylogenetic tree, ITS sequences from different *A. apis* species were acquired from the NCBI database. The ITS sequence from isolated *A. apis* (Coimbatore isolate) was aligned with downloaded sequences, trimmed at the ends for any missing nucleotides, and used to construct a phylogenetic tree (Fig. 5). The analysis included a total of 11 nucleotide sequences of *A.apis* and *A. atra* was set as an outgroup. The isolated fungus was more closely related to other *A. apis* isolates than *A. atra*. The *A. apis* isolate Aap-N24P from West Bengal (Das et al., 2023) and *A. apis* isolate Coimbatore were the most closely related isolates. This may be due to the introduction of Italian honey bee colonies from the northern part of India to the southern part of India (Chaudhary, 1997). In addition, beekeepers in the southern part of India are migrating and purchasing the *Apis mellifera* colonies from time to time from the northern part of India. In the heterothallic Ascomycota group, *A. apis* engage in sexual reproduction with morphologically identical haploid partners, which are differentiated solely by their mating type locus (Bissett, 1988; Anderson and Gibson, 1998; Poggeler, 2001).

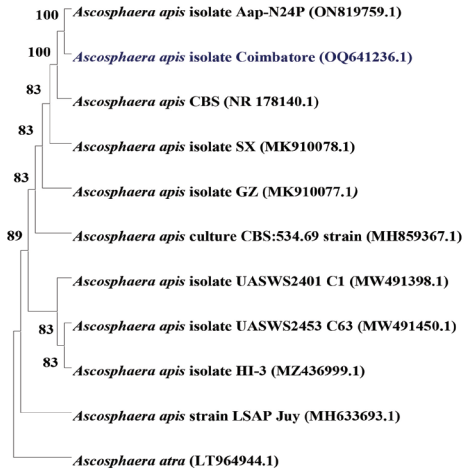

Fig. 5. Phylogenetic tree of the isolated fungi

Recognition of mating type in fungi is crucial for better understanding their lifecycle, reproduction, and pathogenicity. To detect the mating types requires any one of the reference strains (ARSF 7405 and ARSF 7406). Unfortunately, in India, there is no reference strain available. Hence, for the first time, we attempted to detect the mating type by multiplex PCR assay developed by Aronstein and Colby (2015). The multiplex PCR assay replaces the indispensable need for reference to detect the mating types. Initially, the dual test (Fig. 1C) revealed the presence of an opposite mating type from the isolated fungus *A. apis*. This result verified the heterothallic nature of isolated fungi *A. apis* as described in several studies (Aronstein et al., 2007; Aronstein and Murray, 2010). (Fig. 3) shows the result of amplified gDNA from multiplex PCR assay of the isolated Mating types. MAT1-1 and MAT 1-2 specific primers were amplified to a fragment length of 478 bp (lanes 5-7) and 212 bp (lanes 2-4), respectively. Lane 8 shows two bands in respective sizes, indicating the presence of both mating types that served as an internal amplification control (IAC). All the obtained results of the multiplex PCR assay were consistent with the results of Aronstein and Colby (2015), indicating the successful detection of mating types. Finally, the detected mating types MAT1-1 and MAT 1-2 were named TNAU CBD MAT1 and TNAU CBD MAT2, respectively, and stored in 15% glycerol (v/v) at -20^{0}C.

ACKNOWLEDGEMENTS

Authors thank the Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore for providing research facilities and their technical support.

FINANCIAL SUPPORT

No funding sources were involved in the research.

CONFLICT OF INTEREST

No conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

VK: Sample collection, isolation and identification; drafting the manuscript. MRS: Advisor for the research work, conceptualization and for drafting the manuscript and reviewing the manuscript. VRS, SK and VB corrected the drafted manuscript.

REFERENCES

Anderson D L, Gibbs A J, Gibson N L. 1998. Identification and phylogeny of spore-cyst fungi (*Ascosphaera* spp.) using ribosomal DNA sequences. Mycological Research 102(5): 541-547.

Anderson D L, Gibson N L. 1998. New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. Australian Systematic Botany 11(1): 53-72.

Aronstein K A, Murray K D, De Leon J H, Qin X, Weinstock G M. 2007. High mobility group (HMG-box) genes in the honeybee fungal pathogen *Ascosphaera apis*. Mycologia 99(4): 553-561.

Aronstein K A, Murray K D. 2010. Chalkbrood disease in honey bees. Journal of Invertebrate Pathology 103: S20-S29.

Aronstein K, Colby D. 2015. A multiplex PCR assay for determination of mating type in isolates of the honey bee fungal pathogen, *Ascosphaera apis*. Journal of Apicultural Research 54(2): 105-107.

Atwal A S, Sharma O P. 1968. The introduction of *Apis mellifera* queens into *Apis indica* colonies and the associated behaviour of the two species. Indian Bee Journal 30 (2): 41-56.

Bailey L, Ball B V. 1991. Honeybee Pathology. Academic, London.

Bailey L. 1963. Infectious Diseases of the Honeybee. Land Books Limited, London. 176 pp.

Bamford S, Heath L A F. 1989. The infection of Apis mellifera larvae by *Ascosphaera apis*. Journal of apicultural research 28(1): 30-35.

Bissett J, Duke G, Goettel M. 1996. *Ascosphaera acerosa* sp. nov. Isolated from the alfaalfa leafcutting bee, with a key to the species of *Ascosphaera*. Mycologia 88: 797-803.

Bissett J. 1988. Contribution toward a monograph of the genus *Ascosphaera*. Canadian Journal of Botany 66(12): 2541-2560.

Borum A E, Ulgen M. 2008. Chalkbrood (*Ascosphaera apis*) infection and fungal agents of honey bees in north-west Turkey. Journal of Apicultural Research 47: 170-171.

Chaudhary O P. 1997. Successful introduction of Italian honey bee, *Apis mellifera* L. in South India. Journal of Insect Science (India). 23-29.

Chen D, Guo R, Xiong C, Zheng Y, Hou C, Fu Z. 2018. Morphological and molecular identification of chalkbrood disease pathogen *Ascosphaera apis* in *Apis cerana cerana*. Journal of Apicultural Research 57(4): 516-21.

Cheng X, Zhang L, Luo J, Yang S, Deng Y, Li J, Hou C. 2022. Two pathogenic fungi isolated from chalkbrood samples and honey bee viruses they carried. Frontiers in Microbiology 13: 843842.

Chorbinski P, Rypula K. 2003. Studies on the morphology of strains *Ascosphaera apis* isolated from chalkbrood disease of the honey bees. Electronic Journal of Polish Agricultural University 6:1-2.

Christensen M, Gilliam M. 1983. Notes on the *Ascosphaera* species inciting chalkbrood in honey bees. Apidologie 14(4): 291-7.

Das R, Kumar R, Kunal G, Goldar S, Dutta S, Jha S. 2023. Detection of *Ascosphaera apis*, causing chalkbrood disease in the colonies of European honey bee, *Apis mellifera* in West Bengal, India. Sociobiology 70(4): 9192-9192.

Flores J M, Ruiz J A, Ruz J M, Puerta F, Bustos M, Padilla F, Campano F. 1996. Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. Apidologie 27: 185-192.

Gilliam M, Lorenz B J, Prest D B, Camazine S. 1993. *Ascosphaera apis* from *Apis cerana* from South Korea. Journal of Invertebrate Pathology 61: 111-112.

Gilliam M, Taber S, Rose J B. 1978. Chalkbrood of honeybees, *Apis mellifera* L., a progress report. Apidologie 9: 75-89.

Holloway B, Sylvester H A, Bourgeois L, Rinderer T E. 2012. Association of single nucleotide polymorphisms to resistance to chalkbrood in *Apis mellifera*. Journal of Apicultural Research 51(2): 154-163.

Hornitzky M A, Anderson D L. 2010. Honeybee diseases, Australia and New Zealand Standard Diagnostic Procedures, 2003. Home page address: http://www. scahls. org. au.

Jensen A B, Aronstein K, Flores J M, Vojvodic S, Palacio M A, Spivak M. 2013. Standard methods for fungal brood disease research. Journal of Apicultural Research 52(1): 1-20.

Kishan T M, Aruna R, Mishra G, Srinivasan M R. 2017. Beekeeping in India. Industrial Entomology 35-66.

Kluser S, Peduzzi P. 2007. Global pollinator decline: a literature review. Geneva: UNEP/GRID.

Koenig J P, Boush G M, Erickson E H. 1987. Effects of spore introduction and ratio of adult bees to brood on chalkbrood disease in honeybee colonies. Journal of Apicultural Research 26(3): 191-195.

Li Z, Su S, Hamilton M, Yan L, Chen Y. 2014. The ability to cause infection in a pathogenic fungus uncovers a new biological feature of honey bee viruses. Journal of Invertebrate Pathology 120: 18-22.

Maassen A. 1913. Weitere Mitteilungen uber der seuchenhaften Brutkrankheiten der Bienen (Further communication on the epidemic brood disease of bees). Mitteilungen aus der Kaiserlichen Biologischen Anstalt fur Land-und Forstwirtscshaft 14: 48-58.

Mehr Z, Menapace D M, Wilson W T, Sacket R R. 1976. Studies on the initiation and spread of chalkbrood within an apiary. American Bee Journal 116: 266-268.

Nelson D L, Gochnauer T A. 1982. Field and laboratory studies on chalkbrood disease of honey bees. American Bee Journal 122: 29-34.

Poggeler S. 2001. Mating-type genes for classical strain improvements of ascomycetes. Applied Microbiology and Biotechnology 56: 589-601.

Puerta F, Flores J M, Bustos M, Padilla F, Campano F. 1994. Chalkbrood development in honeybee brood under controlled conditions. Apidologie 25(6): 540-546.

Reynaldi F J, Lopez A C, Albo G N, Alippi A M. 2003. Differentiation of *Ascosphaera apis* isolates by rep-PCR fingerprinting and determination of chalkbrood incidence in Argentinean honey samples. Journal of Apicultural Research 42(4): 68-76.

Schoch C L, Seifert K A, Huhndorf S, Robert V, Spouge J L, Levesque C A, Chen W, Consortium F B, Bolchacova E, Voigt K, Crous PW. 2012. From the cover: nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109(16): 6241.

Sevim A, Akpınar R, Karaoglu S A, Bozdeveci A, Sevim E. 2022. Prevalence and phylogenetic analysis of *Ascosphaera apis* (Maassen ex Claussen) LS Olive & Spiltoir (1955) isolates from honeybee colonies in Turkey. Biologia 77(9): 2689-2699.

Skou J P. 1972. Ascosphaerales. Friesia 10: 1-24.

Skou J P. 1988. More details in support of the class Ascosphaeromycetes. Mycotaxon 31: 191-198.

Spiltoir C F, Olive L S. 1955. A reclassification of the genus *Pericystis* Betts. Mycologia 47(2): 238-44.

Spiltoir C F. 1995. Life cycle of *Ascosphaera apis* (*Pericystis apis*). American Journal of Botany 1: 501-8.

Wood M. 1998. Microbes help bees battle chalkbrood. Agricultural Research 1: 16-16.

Wu Z H, Wang T H, Huang W, Qu Y B. 2001. A simplified method for chromosome DNA preparation from filamentous fungi. Mycosystema 20(4): 575-577.

Wynns A A. 2012. The bee specialist fungus family Ascosphaeraceae and its allies: systematics, ecology and co-evolution with solitary bees (Doctoral dissertation, University of Copenhagen, Faculty of Life Sciences, Department of Agriculture and Ecology, Center for Social Evolution).

Yoder J A, Jajack A J, Cornacchione W S, Dunn A L, Cunningham E G, Matchett C L, Rosselot A E. 2014. In vitro evaluation of sugar syrups, antibiotics, and miticides on growth of honey bee pathogen, *Ascosphaera apis*: Emphasis for chalkbrood prevention is on keeping bees healthy. Apidologie 45: 568-78.

(Manuscript Received: April, 2024; Revised: May, 2024;
Accepted: June, 2024; Online Published: July, 2024)
Online First in www.entosocindia.org and indianentomology.org Ref. No. e24198