MORPHOLOGICAL AND MOLECULAR CONFIRMATION OF THE CHALKBROOD (*Ascosphaera apis*) INFECTION IN HONEY BEE COLONIES

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ABSTRACT

Infection by the fungal pathogen *Ascosphaera apis* causes chalkbrood, a major larval disease that threatens colony health and productivity of honey bee (*Apis mellifera* L.) globally. This study aimed to determine the prevalence and molecular identity of the pathogen and to access its phylogenetic relationship with internationally reported isolates. In this study, *A. apis* was identified using morphological and molecular approaches, and the prevalence of chalkbrood infection was assessed in 140 bee colonies. Analyses of chalkbrood mummies revealed that 40 colonies (28.57%) were infected with *Ascosphaera* spores. Pure isolates cultured on Potato Dextrose Agar (PDA), and PCR-based amplification using ITS gene-specific primers produced a specific 485 bp band, confirming *A. apis* in the samples. Sequence analysis confirmed the pathogen's identity, and the sequences of *A. apis* (PQ199405 and PQ282636) were deposited in GenBank. BLAST and phylogenetic analyses of the ITS region sequences showed close similarity (99.12%) with *A. apis* isolates reported worldwide. This study provides the first molecular confirmation of *A. apis* in Pakistan and establishes its phylogenetic relationship with global chalkbrood isolates. A baseline dataset generated in this study is essential for future epidemiological studies and supports the development of effective management strategies against chalkbrood disease in regional apiculture.

Keywords: Chalkbrood disease, disease prevalence, honey bee, microscopic analyses, phylogenetic analysis

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INTRODUCTION

Honey bee (*Apis mellifera* L.) is widely recognized for its intricate social structure and remarkable pollination abilities. It also produces valuable products such as honey, propolis, beeswax, bee pollen and royal jelly (Hung *et al.*, 2018; Abuagla *et al.*, 2025). These ecosystem services and economic contributions make honey bees essential to both agriculture and biodiversity conservation (Iqbal, 2009; Alam *et al.*, 2025). Honey bees play a critical role in the pollination of numerous fruits, vegetables, nuts, oilseed crops and melons in Pakistan (Ahmad and Aziz, 2017). Over 10,000 beekeepers manage nearly 1.1 million bee colonies, producing about 15,750 metric tons of honey annually in Pakistan (Usman *et al.*, 2022). The economic value of pollination-dependent crops has been estimated at approximately $ 1.59 billion. Furthermore, beekeeping in Pakistan contributes substantially to the national economy, with an annual honey production exceeding 15000 tons and export valued of worth $ 9.8 million to international markets (Irshad and Stephen, 2013; Irshad and Stephen, 2014). Despite their ecological and economic importance, honey bees are facing multiple threats, including diseases, parasites, pesticides and environmental stressors, all of which significantly contribute to population decline (Iqbal and Mueller, 2007; Parveen *et al.*, 2022; Brunet and Fragoso Fabiana, 2024; Alam *et al.*, 2025; Iqbal *et al.*, 2025).

Chalkbrood is a globally prevalent fungal disease affecting honey bee brood and is caused by *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir (Castagnino *et al.*, 2020; Das *et al.*, 2023; Mráz *et al.*, 2023). This fungal pathogen poses a significant threat to colony health, particularly under intensive beekeeping systems. In addition to *Apis* species, chalkbrood-like diseases have also been reported in several non-*Apis* bees, including bumblebees, carpenter bee (*Xylocopa* spp.), leafcutter bees, mason bees, and sweat bees, often caused by *A. apis* or closely related *Ascosphaera* species bees

(Gilliam *et al.*, 1994; Maxfield-Taylor *et al.*, 2015; Rutkowski *et al.*, 2023). This indicates that *Ascosphaera* can infect a broader range of bee hosts beyond honey bees.

Larval infection by *A. apis* weakens brood development, disrupts colony productivity and reduces honey production (Aronstein and Murray, 2010; Evison, 2015). Infected larvae become mummified, with color changing from healthy pearly white to a conspicuous white or grayish hues and eventually turning black (Castagnino *et al.*, 2020). The presence of white or grayish masses within the infected larvae also serve as a diagnostic feature of the disease (Borum and Ulgen, 2008; Tejerina *et al.*, 2019). *A. apis* is a spore-forming fungus with specialized structures called asci, which contain fruiting bodies known as ascomata. The spores are typically oval or elliptical in shape (Deneke *et al.*, 2023). Spores enter the environment from mummified infected larvae, and can be spread during hive inspections, through beekeeping activities, or via contaminated equipment, honey and other bee products (Simone-Finstrom *et al.*, 2018). Furthermore, adult bees act as vectors, transmitting spores within the colony through food-sharing behaviors known as trophallaxis (Aronstein and Murray, 2010). Foraging bees can inadvertently transport fungal spores back to the hive, where nurse bees subsequently transfer contaminated food to larvae during feeding (Dosselli *et al*., 2016).

Chalkbrood has been identified as one the major threat to *A. mellifera* in surveys conducted in selected areas of the Punjab and Khyber Pakhtunkhwa provinces of Pakistan (Khan *et al.*, 2024). Chalkbrood is more prevalent in spring when the weather is cold and humid (Flores *et al.*, 1996), and rapid brood growth makes it difficult for worker bees to maintain a constant brood nest temperature (Borum and Ulgen, 2008). The younger larvae (1-2 days old) are highly vulnerable to infection by this pathogen, while adult bees often not (Jensen *et al.*, 2009). The detection of chalkbrood disease in honey bees relies on various methods, including morphological, and molecular approaches. Morphological detection involves visually inspecting larvae and pupae for characteristic symptoms such as mummification, abnormal coloration, and distorted body shapes. Molecular methods that amplify targeted nucleotide sequences, allow precise identification of *A. apis* using the internal transcribed spacer (ITS) region (Nilsson *et al.*, 2008; Jensen *et al.*, 2013). Molecular detection has been crucial for identifying *A. apis* using specific primers such as 3-F1, and 3-R1 (James and Skinner, 2005), or AscosF3 and AapisR3 (Murray *et al.*, 2005), providing high sensitivity and specificity even in asymptomatic individuals (Jensen *et al.*, 2013; Aziz and Alam, 2024). These molecular tools enable early detection and can significantly aid in disease management strategies in apiaries.

Although the occurrence of chalkbrood is increasing globally, dedicated investigations in Pakistan remain limited. Existing literature is sparse and mainly descriptive primarily reporting chalkbrood as a disease of honey bees (Munir *et al.*, 2024) or identifying it as major threat in survey-based assessments (Khan *et al.*, 2024). The available work has largely addressed its management (Sarwar, 2016) rather than diagnostic confirmation or pathogen characterization. Consequently, there is a clear need for systematic diagnosis to verify the causative agent, clarify its phylogenetic relationship with global isolates, and quantity its incidence in bee colonies in Pakistan. The present research was designed to address these gaps through a detailed investigation, including the detection of *A. apis*, its morphological and molecular identification, and the determination of infection prevalence in selected colonies. The findings provide baseline data to support further research on geographical occurrence, temporal trends, and seasonal patterns of chalkbrood in Pakistan.

MATERIALS AND METHODS

Sample Collection and Chalkbrood Identification: Samples collected from 140 *A. mellifera* colonies, maintained at different locations (Table 1) were examined to access the occurrence of chalkbrood disease. The study employed proportional sampling based on the available colony numbers at each location, while maintaining a uniform sampling protocol across all sites. Study locations were selected based on the availability of honey bee colonies at different apiaries during the study period, and sampling periods were adopted from previously reported seasonal patterns of chalkbrood occurrence. Although sampling schedules varied among locations across months due to operational constraints, the same standardized sampling procedures were consistently applied at all sites to ensure methodological comparability. Consequently, sample sizes varied due to natural differences in colony availability across locations. Disease incidence in bee colonies was recorded on a weekly basis, from first week of March to the second week of May 2022. Visible mummies (5-20), complete or partially cannibalized, were observed inside the frames, on the bottom boards, or in dead bee traps. Chalkbrood mummies (hard, white or gray masses found within brood cells) were collected and transferred to Bee Research Unit Laboratory, PMAS-Arid Agriculture University, Rawalpindi, Pakistan. The collected samples were then stored at -20^{o}C for subsequent analysis and confirmation. During sample collection, frames and specimens were carefully examined, focusing on scattered lidless cells, small holes in sealed brood, presence of white mycelium in the combs, and larvae coated with mycelium. Chalkbrood infection was more prevalent in cool and humid conditions. These observations facilitated to identify the potential chalkbrood infections.

Fungal isolation and Purification

Hyphal-tip Isolation: Isolation and purification were carried out following the protocol of Jensen *et al.* (2013). Briefly, each mummy was surface-sterilized in 10% sodium hypochlorite (NaClO) for 10 min. Following sterilization, the mummy was washed twice with sterile distilled water for 2 min. The mummy was then cut into smaller pieces and placed on Potato Dextrose Agar (PDA) plates. The plates were kept in darkness at 30-34°C, and fungal growth generally appeared within 2–4 days after incubation.

Single-Spore Separation: The mummies were first surface-sterilized, chopped and placed into a tube filled with sterile water. The mixture was vortexed to obtain spore suspension. The suspension was diluted to concentrations from 10^{-2} to 10^{-4} and evenly spread onto PDA plates. The media plates were incubated in complete darkness at 30°C for 2-3 days. A fungal plug was then extracted and transferred to fresh growth agar (PDA), which were incubated at 30°C for 4-6 days to promote further growth (Cheng *et al.*, 2022).

Morphological Examination: The morphological confirmation of *A. apis* was conducted in the Laboratory of Fungal Plant Pathology of the University. After success culturing of the fungus at 30ºC for 2-3 days, a small piece of hyphae was taken from the culture medium and suspended in 5 ml of sterile double-distilled water (ddH_2O) in a 5 ml sterile tube, followed by gentle mixing. Two drops of homogenized suspension were placed on a new sterile slide and directly observed under a light microscope (Olympus Nea, Japan) (Jensen *et al.*, 2013; Cheng *et al.*, 2022).

Pathogenicity Bioassay: Ascospore cysts, produced from the mating of two opposing *A. apis* types, were carefully removed from the colony's surface to prepare a spore suspension. For this purpose, the spore cysts were added into tubes having sterile water along with glass beads. The tubes were vortexed for 30 seconds, and centrifuged for 1 min at 14000 rpm. The supernatant was discarded, and the spore pellet was re-suspended. These steps were repeated three time. The spore suspension was adjusted at spore concentrations of 5×10^6 spores/ml after counting with a hemocytometer; the spore concentration was adopted from the protocol described by Cheng *et al.* (2022). Three days old honey bee larvae were randomly collected from three different colonies, housed in 96-well plates, and fed 10 µl of the spore suspension, while sterile water served as the control. Three replicates, each containing four larvae (total 12 larvae) were cultured at 30°C and 90% relative humidity (RH) for 7-14 days, and larval morphology was observed daily (Cheng *et al.*, 2022).

Molecular Confirmation of *A. apis*

DNA Extraction: DNA of purified fungi was extracted following the protocol of Reynaldi *et al.* (2003). A 100 mg of mycelium was transferred in a 1.5 ml centrifuge tube, mixed with 400 µl of extraction buffer and 200 µl of 10% SDS, and incubated at 80°C for 5 min. The samples were allowed to cool at room temperature for 10 min, followed by the addition of 300 µl of protein neutralizing solution, and subsequently the sample were kept on ice for 5 min. The samples were gently vortexed and centrifuged at 12000 rpm for 30 min at 4ºC. Approximately 500 µl of the supernatant was transferred to a new 1.5 ml centrifuge tube, mixed with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and centrifuged at 14000 rpm for 15 min at 4ºC. The supernatant was carefully transferred to a new tube, then 600 µl of cold ethanol was added and mixed using a vortex mixer. The DNA was then precipitated by centrifugation at 10000 rpm for 20 min at 4°C. After carefully removing the ethanol, the DNA pellet was allowed to air dry for 5 min at room temperature. Finally, the DNA was resuspended in 30 µl of TE buffer and stored at -20ºC. DNA quality and integrity was verified on agarose gel (2%) with ethidium bromide (2 µl). The DNA samples were stored at -20ºC for subsequent PCR analysis.

PCR Amplification: PCR amplification was performed in the Center Laboratory, Faculty of Agriculture, PMAS-Arid Agriculture University Rawalpindi, following the described protocol of Cheng *et al.* (2022). The final 50 µl reaction mixture contained 2 µl genomic DNA, 25 µl 2x Green Master mix (Thermo Scientific), 1 µl of forward and reverse primers, and 21 µl PCR grade water. The species-specific forward (AscoF3) and reverse (AapisR3) primers were adopted from Murray *et al.* (2005), which amplify the target ITS region of rDNA of *A. apis*, and producing a PCR band of 485 bp (Table 2). PCR conditions were optimized based on established protocols for *A. apis*, with slight modifications to achieve optimal amplification. The amplification was carried out using a My-Gene-™ Series Peltier thermal cycler (Model MGG96G) with an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min (James and Skinner, 2005; Murray *et al.*, 2005). Amplified PCR products were separated on a 1% agarose gel stained with ethidium bromide and visualized under UV illumination (MS Major Science USA).

DNA Sequencing: Purified PCR products were submitted for sequencing through Sanger dideoxy sequencing performed by Microgen Pvt. (https://hardydiagnostics.com/microgen). The obtained sequences were cleaned, edited and submitted to BLAST analysis in the GenBank database for identification. The sequences of *A. apis* isolates were added in GenBank with accession codes PQ199405 and PQ282636. Phylogenetic analysis was conducted using MEGA 11 (Molecular

Evolutionary Genetics Analysis) by the Neighbor-Joining method (NJM) with 1,000 bootstrap values. This analysis aimed to access the evolutionary relationships between the isolates from this study and previously reported global isolates (Das *et al.*, 2023),

Statistical analysis: The prevalence of chalkbrood disease from different apiaries was calculated using descriptive statistics with a 95% Confidence interval (Statistix 8.1 Software).

RESULTS

Prevalence of chalkbrood: Chalkbrood disease was assessed in bee colonies across different locations through sampling conducted from March to May (Table 3). A total of 40 colonies (28.57%) were found to be infected with chalkbrood across the sampled apiaries. Chalkbrood prevalence varied across the sampling period and among the surveyed apiaries. The disease prevalence was higher during the month of March compared to April and May. Variation in the number of positive colonies across sampling months reflects temporal variation in infection frequency. Temporal interpretations are based on available field data and should therefore be considered with appropriate caution due to variability in sample availability. The bee colonies from Mandra showed highest infection (48.39%, 15/31 colonies) during March, followed by University Research Farm Koont (40.91%, 9/22 colonies) and Panjgran (40.54%, 15/37 colonies). The lowest prevalence was found in Rawat (8.33%, 2/24 colonies) during the month of May (Table 3).

Symptoms of chalkbrood: The characteristic symptoms of chalkbrood disease were observed in the sealed brood areas during colony inspection. A scattered brood pattern was evident with the presence of mummified bee larvae (chalk mummies) in the cells (Fig 1A). The white and black mummified bee larvae were also noted at the bottom boards of the hive (Fig 1B).

Morphological and pathogenic characteristics

Pure culture and microscopic examination: The hyphal-tip isolation method revealed that chalkbrood produced small, white to gray, spherical colonies on PDA medium seven days after inoculation (Fig. 2A). Black spores of *A. apis* were observed at the junction points of colonies in cultured medium after 30 days of incubation at 30ºC (Fig 2B). Microscopic examination revealed septate and branched hyphae with distinct conidiophores under 100x magnification. The hyphae were septate, 2.5–8 µm in diameter, with distinct dichotomous branching (Fig 2C). Spherical conidia were visible, enclosed in nearly hyaline, spherical spore cysts at 400x magnification (Fig 2D). Mature ascomata were brick-shaped, containing spherical hyaline asci, with the release of spores from ruptured spore cysts (Fig 2E).

Pathogenic assay: The pathogenicity assay showed larval morphological changes and mortality following in vivo inoculation of three days old larvae with *A. apis* spores (Fig. 3). The inoculation revealed that all larvae (12 per replicate) died within 24 h post-inoculation. The dead infected larvae displayed white mycelia and ascospores on the surface of the dead bee larvae seven days post-inoculation (Fig. 3B). Fourteen days after inoculation, the larvae body shrank and transformed into black mummies (Fig. 3C). The control larvae (fed sterile water) showed no fungal growth and retained their normal white appearance throughout the observation period.

Molecular confirmation of *A. apis* in bee colonies: Molecular identification of *A. apis* was performed on bee samples collected from various locations. PCR-based analysis amplified specific bands of 485 bp in five positive samples, confirming the presence of *A. apis* (Fig. 4) using specific primers (AscoF3 and Aapis R3) (Table 2). The amplified PCR products were sequenced, and sequences of *A. apis* isolates were submitted to GenBank (PQ199405 and PQ282636).

Phylogenic Tree analysis: *Ascosphaera apis* sequences (PQ199405 and PQ282636) were identified using BLAST analysis in the GenBank nucleotide database. Phylogenetic analysis of the ITS region revealed 99.12% identity with *A. apis* isolates reported from different countries. These closely related isolates ITS region sequences included NR_178140, MH859367, and KJ158165 (USA); KT373974 (Argentina); (PV056025 and PV056026 (Italy); KM242591 (Russia); and (MK910077 (China) (Fig. 5).

Table 1. Details of sampling locations of apiaries for *Ascosphaera apis* collection

Sampling location		City, Province	Latitude	Longitude	Altitude (meter)
S1	University Research Farm Koont	Rawalpindi, Punjab	33.114789	73.012807	513
S2	Mandra	Rawalpindi, Punjab	33.371635	73.234334	501
S3	PMAS- AAUR (Arid Agriculture University)	Rawalpindi, Punjab	33.650454	73.080688	515
S4	Rawat	Rawalpindi, Punjab	33.496426	73.194274	570
S5	Panjgran	Rawalpindi, Punjab	33.149551	73.02945	528
S6	Salgran	Rawalpindi, Punjab	33.822453	73.275163	821

Table 2. Sequence of primer used for the *Ascosphaera apis* identification

Name	Sequence	PCR Product	Reference
AscoF3 (Forward Primer)	GCACTCCCACCCTTGTCTA	485bp	(Murray *et al.*, 2005)
AapisR3 (Reverse Primer)	CCCACTAGAAGTAAATGATGGTTA		

Table 3. Prevalence and molecular detection of *Ascosphaera apis* in different *Apis mellifera* colonies

S. No	Sampling location	Months	Humidity %	Positive / Total colonies	% Infected apiaries
S1	University Research Farm Koont	March	43.66	9/22	40.91
S2	Mandra	March	44.02	15/31	48.39
S3	PMAS- AAUR	April	27.88	7/18	38.89
S4	Rawat	May	18.62	2/24	8.33
S5	Panjgran	March	42.52	15/37	40.54
S6	Salgran	March	29.8	2/8	25.00
Total				**40/140**	**28.57**

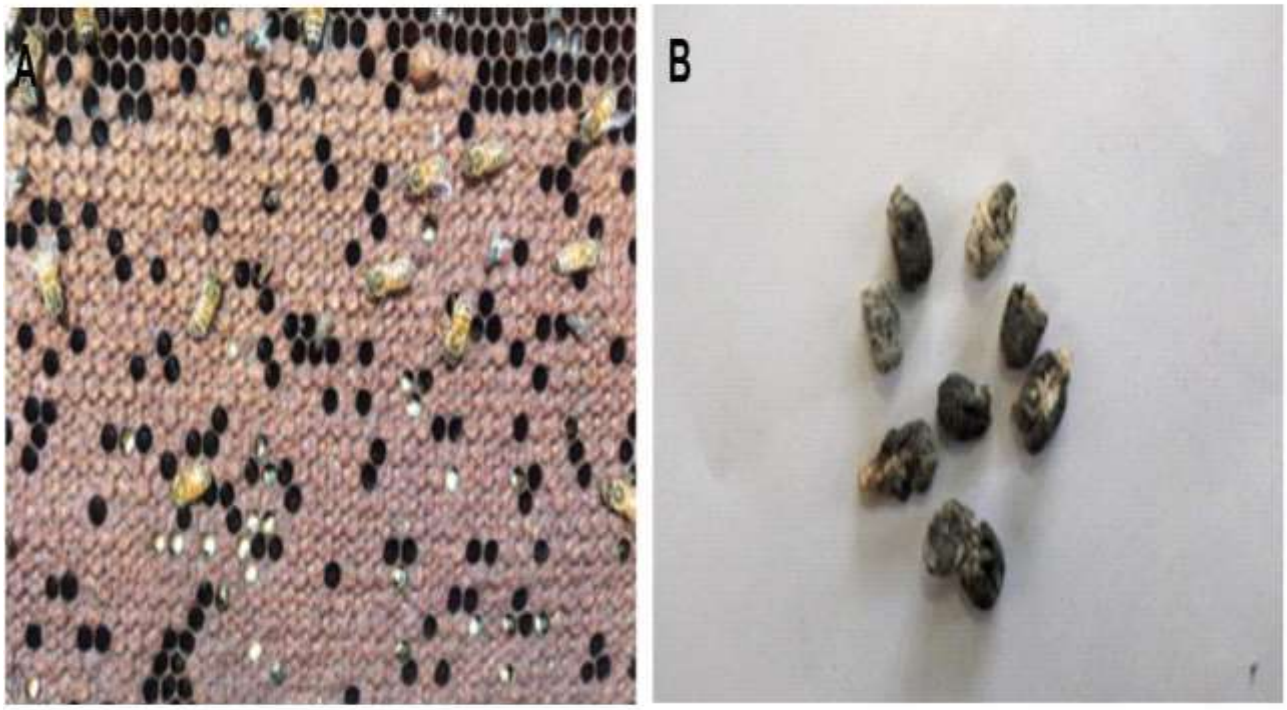

Figure 1. A) Chalk-like material covering infected bee larvae; B). Dead, mummified bee larvae from infected colonies.

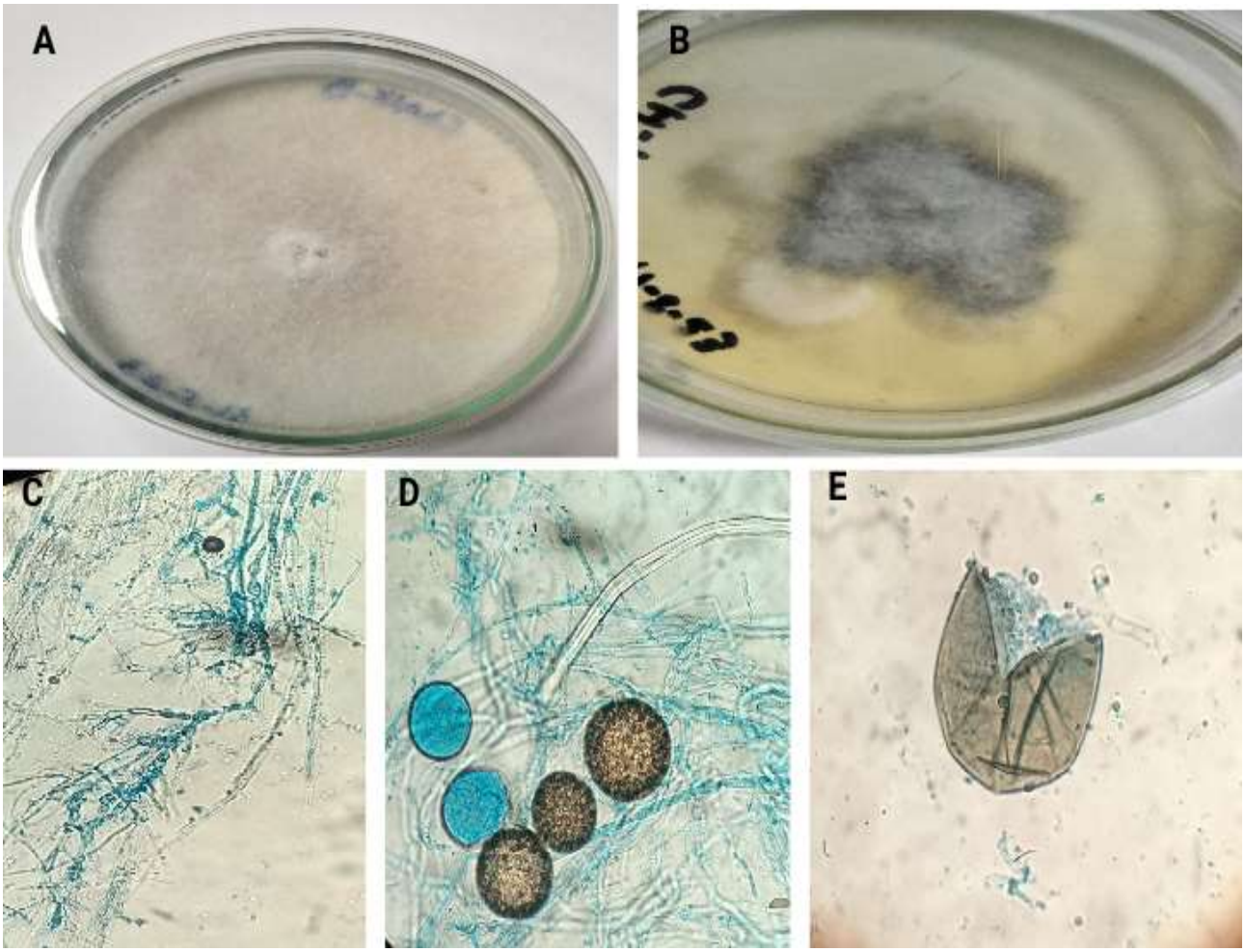

Figure 2. (A) Morphology of *Ascosphaera apis* cultured on PDA; (B) Opposing mating types of *A. apis* producing spores at the junction point; (C). Septate mycelium and conidiophores of *A. apis*; (D) Spore balls enclosed in spherical, nearly hyaline spore cyst; (E). Release of spores from ruptured spore cyst.

Figure 3. In vivo bioassay: morphological changes in 3 days old bee larvae inoculated with *A. apis* spores: A) bee larvae one day after inoculation; (B) White mycelia and ascospores on the surface of infected larvae after seven days; (C) Black mummies formed 14 days after inoculation.

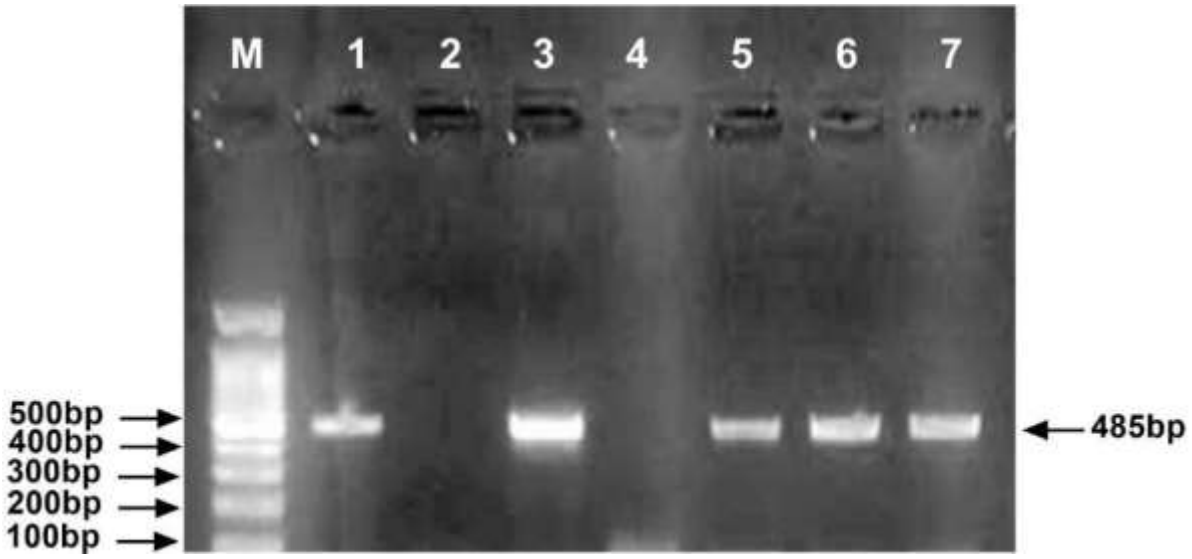

Figure 4. Image of agarose gel electrophoresis showing PCR-amplified products with specific 485 bp bands in positive samples of *Ascosphaera apis*. M = 100-bp DNA ladder; Lane 1 = Postive control; Lane 2, 4 = negative control & negative sample (no band indicating absence of *A. apis*); Lane 3, 5-7 = postive samples showing presence of *A. apis*.

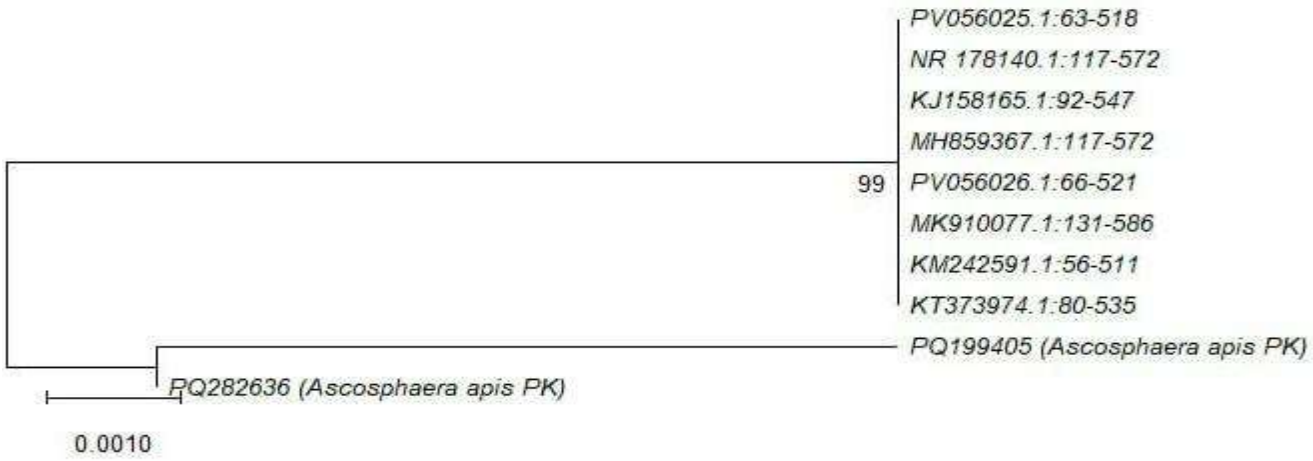

Figure 5. Phylogenetic analysis of *Ascophaera apis* isolates (PQ199405 and PQ282636) based on ITS region sequences using the Neighbor-Joining method.

DISCUSSION

The fungus *Ascosphaera apis* infects honey bee larvae, causing chalkbrood disease and leading to substantial colony losses under favorable environmental conditions (Deneke *et al.*, 2023; Aziz and Alam, 2024). The rapid spread of this disease has been reported across many countries. Its occurrence and severity are closely influenced by environmental factors such as humidity and geographical location (Castagnino *et al.*, 2020). This study provides the first molecular confirmation of the pathogen causing chalkbrood disease in Pakistani honey bee colonies, using DNA sequencing of samples collected from multiple locations of Punjab, and validated through laboratory pathogenicity assays. The epidemiological information on chalkbrood based on diagnostic studies offers baseline data for surveillance in the province and for devising effective chalkbrood management strategies.

The current study recorded that 28.57% of the apiaries were infected with chalkbrood during the sampling period from March to May, likely due to increased humidity and temperature fluctuations favoring fungus growth and disease development. This disease prevalence during spring is particularly concerning, as this period coincides when bee population need to increase for maximizing honey production in early summer flows. Likewise, chalkbrood infection has

been reported from other countries, at infection rate of 17.4% in Ethiopia (Deneke *et al*., 2023), 24.1% in Japan (Yoshiyama and Kimura, 2011), 24.6% in France (Chauzat *et al*., 2010), 25% to 50% in Turkey (Aydin *et al*., 2006; Borum and Ulgen, 2008). The chalkbrood prevalence in Pakistan is comparable to that reported in many regions worldwide. However, the slightly higher prevalence compared to some countries may be attributed to local climatic conditions, genetic susceptibility of bee populations, or beekeeping management practices. It is important to note that temporal comparisons in the present study are based on field-collected data with variable sample sizes across months; therefore, observed differences in monthly prevalence should be interpreted with caution.

In the current study, microscopic examination of larval mummies from honey bee colonies revealed characteristics consistent with morphology of *A. apis* (Jensen *et al*., 2013). The culture medium was densely covered with white septate hyphae exhibiting dichotomous branching. Fungal isolates from both *A. mellifera* and *A. apis*, maintained on a common medium, produced black, globose spore cysts seven days after inoculation, with clearly visible spherical fruiting bodies. *A. apis* produces spherical spore cysts containing multiple spore balls composed of hyaline spores, and these morphological features closely match previous descriptions (Chen *et al*., 2018; von Knoblauch *et al*., 2024).

Pathogenicity bioassay showed that three days old larvae inoculated by feeding with spores in sugar syrup died, and the larvae transformed into black mummies 14 days after inoculation. These observations are consistent with previous reports, where larvae fed a spore-containing diet developed fungal mycelium beneath the cuticle within 72 hours, showed clinical signs of chalkbrood by 78 hours, and the larval corpses were eventually fully covered by fungal mycelium (Aronstein and Murray, 2010).

In this study, ITS gene was targeted for the molecular identification and genetic sequencing of *A. apis* isolated from *A. mellifera* larval samples. ITS gene is widely authentic to identify fungal species within the genus *Ascosphaera* at molecular level (Anderson *et al*., 1998). PCR confirmed the identification by producing amplified bands of 485bp using specific primers. Similarly, Jensen *et al*. (2013) also utilized PCR for molecular identification of *A. apis*. The combination of ITS-based identification and morphological confirmation of chalkbrood in the current study strengthens the reliability of the findings and reduces the risk of misidentification with closely related species of honey bee pathogens.

The DNA sequences of *A. apis* were deposited in NCBI GenBank (PQ199405 and (PQ282636). Phylogenetic analysis revealed 99.12% similarity of these isolates with sequences NR_178140, and MH859367 from USA (Vu *et al*., 2019), and (KJ158165) (Maxfield-Taylor *et al*., 2015), Argentina (KT373974), Italy (PV056025 and PV056026), Russia (KM242591) and China (MK910077) (Disayathanoowat *et al*., 2020). The high genetic similarity suggests a conserved genetic structure of *A. apis*, indicating its potential global dissemination through bee trade or colony migration. This highlights the importance of early detection and effective management, which are critical for preventing losses of both bees and honey production. However, as the present study was conducted in selected regions, it does not represent the nationwide disease prevalence; therefore, year-round investigations are required to better understand the epidemiology of chalkbrood in the country.

Conclusion: This study provides baseline data on the prevalence and molecular diversity of *Ascosphaera apis* in honey bee colonies in Pakistan. The study offers the first microscopic and molecular confirmation of the fungus in the region. Chalkbrood disease is characterized by dead, mummified bee larvae in comb cells and on the hive floor, covered with a chalky white or greyish-black coating resembling cotton. Microscopic examination revealed *Ascosphaera* spores in 28.57% of sampled apiaries, and molecular gene sequence analysis of the ITS region confirmed the isolates as *A. apis.* These findings highlight the need for effective management strategies to control the spread of fungal spores and minimize the risk of chalkbrood outbreaks in bee colonies. Moreover, continuous surveillance and preventive measures are also essential to protect honey bees' health and maintain colony productivity.

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