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# Detection of *Ascosphaera apis*, Causing Chalkbrood Disease in the Colonies of European Honey Bee, *Apis mellifera* L. in West Bengal, India

Rakesh Das[1,2], Ritesh Kumar[3], Gautam Kunal[4], Suraj Goldar[5], Subrata Dutta[5], Shantanu Jha[2]

1 - Present Adress: School of Agriculture, Swami Vivekananda University, Barrackpore, West Bengal-700121, India
2 - Department of Agricultural Entomology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, West Bengal-741252, India
3 - Department of Plant Pathology, MSSSoA, Centurion University of Technology and Management, Odisha-761211, India
4 - Plant Protection (Entomology), KVK, Jale, Dr. Rajendra Prasad Central Agricultural University, Bihar-847302, India
5 - Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, West Bengal-741252, India

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**Corresponding author**
Ritesh Kumar
Department of Plant Pathology, MSSSoA, Centurion University of Technology and Management, Odisha-761211, India.
E-Mail: ritesh.kumar@cutm.ac.in

**Abstract**

The decline of honey bee (*Apis mellifera* L.) populations is of great concern worldwide. Among the several key drivers, disseminating pests and pathogens is a potential one. Chalkbrood is a widespread fungal disease of the honey bee caused by *Ascosphaera apis.* The present study surveyed the prevalence of diseases in *A. mellifera* beekeeping in the Gangetic plains of West Bengal, India. The results confirmed the occurrence of chalkbrood disease in different apiaries, where dead and mummified bee larvae with cotton-like chalky white or greyish-black covering were found as physical symptoms of the disease. From three surveyed apiaries, 16 hives out of 113 were infected with the disease, and 46 frames out of 132 of the infected 16 hives were affected by the pathogen. Microscopic examination reflected that nearly 87% of the samples collected from the infected frames were positive for the *Ascosphaera* spore. The fungus associated with the disease was isolated on Potato Dextrose Agar medium, pure cultured, and its genomic DNA was isolated to perform PCR based on 18s rDNA sequencing using a specific primer pair of ITS-1 and ITS-4. The fungus was identified as *Ascosphaera apis*.

## Introduction

Due to the eusocial lifestyle with an advanced level of social organization and close relatedness amongst nest mates, honey bees face significant parasite and pathogen pressure. Pathogen dissemination has been attributed as one of the leading causes of honey bee population loss (Neumann & Carreck, 2010). Chalkbrood is one of the important fungal diseases commonly occurring in managed honey bee colonies (*Apis mellifera* L.). In the previous several decades, the disease has been reported to have spread globally (Baker & Torchio, 1968; Hitchcock & Christensen, 1972; Yacobson et al., 1991; Anderson & Gibson, 1998), and in recent years the incidence has increased (Kluser & Peduzzi, 2007). Human activities associated with growing food demand have been proven to have direct and indirect impacts that could be at least partially responsible for this trend (Aizen et al., 2009). The fungus *Ascosphaera apis*, which causes chalkbrood disease in honey bees (Spiltoir, 1955; Spiltoir & Olive, 1955), was first discovered in *A. mellifera* from Europe in the early twentieth century and in India from Punjab in 2001 (Chhuneja et al., 2001).

The disease-causing *A. apis* is a heterothallic, spore-cyst fungus, while the ascospores are the predominant source of inoculum, and infection begins with consumption (Spiltoir, 1955; Hitchcock & Christensen, 1972; Rose et al., 1984) and

spores germinate in the larval gut when the colony temperature falls below the optimal temperature of 32-35 ºC, either before or after capping (Chorbinski & Rypula, 2003). Adults are not vulnerable to this infection and may only transmit the disease inside and between colonies via food sharing (Gilliam & Vandenberg, 1997), while young bee larvae of 3-4 days old are the most susceptible (Bailey, 1981). Spores can quickly accumulate and remain viable for at least 15 years on hive parts and in beehive products, serving as long-lasting sources of inoculum (Gilliam, 1986; Anderson et al., 1997; Flores et al., 2005a, b), and these contaminated materials also help in the transmission of the disease in between managed colonies (Gilliam & Vandenberg, 1997). Although individual bee larvae are more vulnerable, there have been no reports of entire bee colonies being destroyed (Aronstein & Murray, 2010).

Furthermore, the disease infection can significantly reduce colony productivity, resulting in a 5-37 percent drop in honey production (Heath, 1982; Yacobson et al., 1991; Zaghloul et al., 2005). Members of the *Ascosphaera* genus live in colonies with social or solitary bees (Wynns et al., 2012; Klinger, 2013). Most are saprophytes, feeding on larval debris, feces, or pollen, while others are bee pathogens causing brood disease (Bissett, 1988). Several species of *Ascosphaera* have similar life cycles and pathogenesis to *A. apis*. However, they infect solitary rather than honey bees (Skou, 1972, 1988; Bissett, 1988; Anderson et al., 1998).

A deeper understanding of the competitive interactions between *A. apis* strains and their bee hosts will help disease management efforts. We must, however, be able to discriminate between various species of pathogens first. The internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit is the most widely used locus for molecular species identification and subgeneric phylogenetic inference within the fungal kingdom (Nilsson et al., 2008). The ITS region of rDNA has been found helpful in examining the genetic links of *Ascosphaera* species (Anderson et al., 1998; Chorbinski, 2004), and this also facilitates the creation of species-specific primers (James & Skinner, 2005; Murray et al., 2005). The objective of this study was to evidence the occurrence of the chalkbrood disease through fungal isolation and molecular study from West Bengal, India, as there is no documentation of this disease from the state, and also to record the infestation percentages.

## Materials and Methods

### *Survey and collection of diseased samples*

A survey was carried out in the Gangetic plains of West Bengal, India, during 2020-21 to collect information regarding the prevalence of major diseases associated with *A. mellifera* beekeeping, the frequency of their occurrence, and farmers' perception of them. The climate of Gangetic West Bengal is tropical monsoon, with oppressively hot summers, high humidity all year, and well-distributed rainfall during the southwest monsoon season. For the study, beekeepers were selected from three districts of West Bengal covering Gangetic plains, namely, Sahapur under North 24 Pargans district, Sikra under Purba Barddhaman, and Bangaria under Nadia district (Fig 1), as it includes the most potential beekeeping zone of the state, and also the majority of beekeepers belong from these areas. The selection was used to identify the people rearing bees commercially and having more than 30 bee boxes. Based on farmers' information in prepared questionnaires, three apiaries with 30, 45, and 38 hives from the mentioned three places were examined for this study. Each comb from all the 113 hives of three apiaries was examined thoroughly. Cells with white cretaceous larvae were collected from the comb and brought to the laboratory to confirm the disease type. Other field symptoms were also recorded for disease confirmation from the infected hives. Simultaneously, data were also recorded for the percent disease infestation.

### *Fungal isolation*

Isolation from different parts of the diseased samples showing characteristic symptoms was made on Potato Dextrose Agar (PDA) medium augmented with broad-spectrum antibiotics (50 ppm streptomycin and ampicillin). As soon as the mycelial growth was visible around the inoculated samples, kept at 28±2 ºC for 3-4 days, the growing fungal tips were transferred to the sterilized PDA medium previously poured into sterilized Petri dishes and grown. After 3-4 days, they were again transferred to a fresh PDA slant to obtain pure culture by hyphal tip isolation. Regular transfer of hyphal tip pure culture of the pathogen was done during the investigation to maintain the pathogen.

### *Microscopic examination*

Fungal tissue was slide mounted for microscopy in distilled water, and morphological identification of that fungal culture was performed using a Motic BA210 microscope.

### *Molecular identification and phylogenetic analysis*

The fungal DNA was isolated using the Wizard SV Genomic DNA Purification System (Promega Biotech India Pvt. Ltd) following manufacturer protocols. Molecular identification of fungal cultures was performed by sequencing and analysis of the Internal Transcribed Spacer (ITS) region of the nuclear rDNA. PCR amplification of ITS region was performed using universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') as forward primer and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') as a reverse primer (White et al., 1990) in Thermal cycler. The PCR Amplification was carried out in 25 µl reaction mixture containing 2.5 µl of 10x *Taq* buffer, 1.0 µl of each, forward as well as reverse primer (5 pM/µl), 0.4 µl of 2.5 mM dNTP mix, 0.2 U of *Taq* polymerase (5 U/µl) and 16.9 µl of sterile nuclease-free water along with 3 µl (10-200 ng/µl) of DNA template.

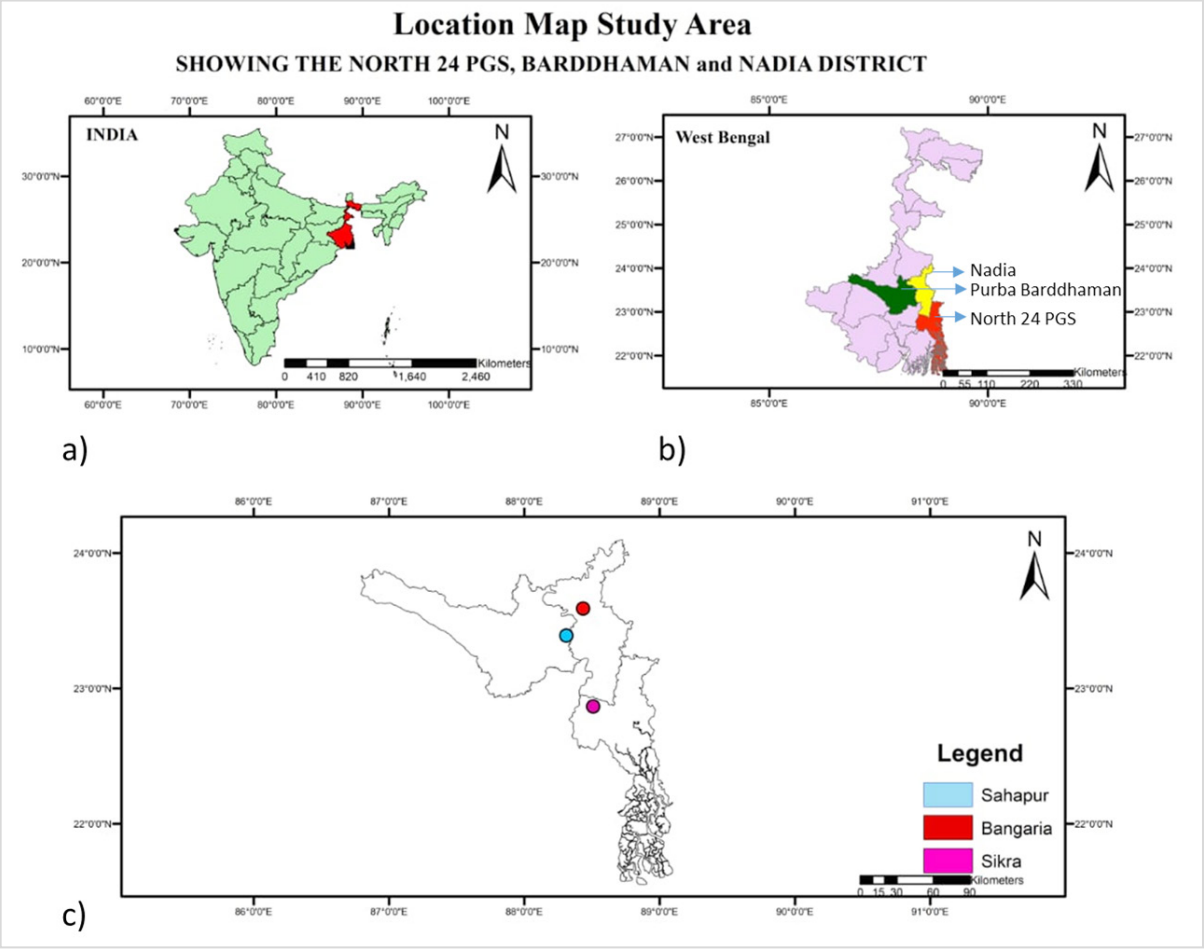


**Fig 1.** Map showing West Bengal under India a), Surveyed three districts under West Bengal b) and Area of survey under three districts c).

The PCR amplification was carried out by initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 sec., annealing at 57.6 °C for 1 min, extension at 72 °C for 1 min 30 sec., and final extension at 72 °C for 10 min after cycling. Amplified PCR products were observed in 1.2% agarose gel with 0.5µl of Ethidium bromide staining (10 mg/ml) in 1X TAE buffer and visualized under the Gel documentation unit.

To compare the similarity or diversity, the ITS nucleotide sequences of different *Ascosphaera apis* were retrieved from NCBI GenBank (http://www.ncbi.nlm.nih.gov/) of different geographical regions of the world and were matched with the three native fungal isolates after end-trimmed with the ClustalW (Thompson et al., 1994) software. Using the Mega X program (Kumar et al., 2018), a phylogenetic relationship was built by the Neighbor-joining (NJ) technique (Saitou & Nei, 1987) with 1000 bootstrap to elucidate the evolutionary relationships of isolates used in the current study with the isolates from worldwide.

*Statistical analysis*

Statistical data analyses were conducted to obtain simple descriptive statistics like mean and standard error using Microsoft Excel, 2010.

**Fig 2.** Cotton like chalky white material covering bee larvae inside comb.

## Results and Discussion

*Disease occurrence and percent infestation*

During the inspection, the initial field symptoms recorded were the presence of chalky-white dead bee larvae in the comb cells (Fig 2), mummified dead bee larvae inside the hive (Fig 3) as well as in front of the hive entrance (Fig 4) and the mummified larvae were of white or black (Fig 5). All these field symptoms revealed the presence of chalkbrood disease in the inspected bee hives. White, black, or grey mummies near the hive entrance, on the bottom board, or in sealed and unsealed brood cells are used to diagnose this disease condition in a bee colony (Gilliam et al., 1978; Heath, 1982; Shimanuki & Knox, 1990). We evaluated 113 (30, 45, and 38) hives from three different beekeepers.

**Fig 3.** Dead larvae inside the hive.

**Fig 4.** Dead larvae at hive entrance.

As shown in Table 1, four hives were found to be infected in the first apiary, which reflected 13.33% of infection in the hives (n=30). Seven of the second apiary's hives were infected, indicating a 15.56% infection in the hives (n=45). The third apiary had five infected hives, showing 13.16% infection in the hives (n=38). Thus, from a total of 113 hives in three apiaries, 16 were infected, showing 14.15% overall infection. In the first apiary, an average of 8.75 frames per hive was measured, and an average of 3.00 frames per hive were infected, showing 34.29% infection. In the second apiary, 8.00 frames per hive were found, and an average of 2.43 were infected, indicating 30.38% of infection. From the third apiary, an average of 8.2 frames per hive were found; among them, 3.4 were infected; 41.46% of infection was recorded. So, in a total of 132 frames from 16 infected hives of three apiaries, 46 were found to be infected with the disease, reflecting 34.85% of overall frame/comb infection (Table 1).

**Fig 5.** Chalky white colouration changes into black.

**Table 1.** The frequency of presence of the chalkbrood disease affected cells in three apiaries in West Bengal, India.

| Apiary | No. of hives | No. of infected hives | Infected hives (%) | Frames/hive (Average ± SEM) | Infected frames/hive (Average ± SEM) | Infected frames (%) |
|---|---|---|---|---|---|---|
| 1 | 30 | 4 | 13.33 | 8.75 (±**0.18**) | 3.00 (±**0.41**) | 34.29 |
| 2 | 45 | 7 | 15.56 | 8.00 (±**0.20**) | 2.43 (±**0.20**) | 30.38 |
| 3 | 38 | 5 | 13.16 | 8.20 (±**0.22**) | 3.40 (±**0.51**) | 41.46 |

SEM: Standard Error of the Mean.

Interestingly, no adult bees were found dead, having such typical symptoms; even beekeepers agreed with it. As expected, adults are not vulnerable to this infection but can transmit the disease (Gilliam & Vandenberg, 1997). In the infected combs, the dead larvae were found sparsely distributed in both worker and drone cells, reflecting the disease pathogenesis of the fungus, which can infect the brood of workers, drones, or queens (Wynns, 2012). Here, the absence of dead queen larvae in cells may be due to a prolific queen in each hive. Beekeepers claimed that the disease severity was observed during February-March in local conditions. It is the spring, and the cool and humid condition enhanced the fungal growth, resulting in the most prevalent disease in hives (Mehr et al., 1976; Puerta et al., 1994; Flores et al., 1996; Borum & Ulgen, 2008). According to beekeepers, they did not pay serious attention to this infection severity, and the disease disappeared with the onset of time. The disease disappearance may be due to the presence of prolific new queen bees, as the beekeepers replaced their old queens with new ones in every colony before the honey season (before winter) every year to boost their honey collection. Replacement of the queen in the colony has become one of the most common practices for dealing with chalkbrood (Gilliam et al., 1983; Palacio et al., 2000).

*Microscopic examination*

In Table 2, we present the results obtained from the microscope examination of the samples taken from the affected frames. From the first apiary, 11 samples were observed; among them, nine were found with the spore of *Ascosphaera*, which reflected 81.81% of affirmative samples for the existence of the pathogen. Six of seven samples of the second apiary had *Ascosphaera* spores, representing an 85.71% positive rate for pathogen presence. In the third apiary, 13 samples were collected, 12 containing *Ascosphaera* spores, producing a 92.30% positive rate for pathogen presence. Overall, from the three apiaries, 31 samples were taken, and out of that, 27 were found with the spores of *Ascosphaera*, showing 87.09% of the positive samples of the pathogen under microscopic examination. The presence of ascospores in samples can be considered a positive diagnostic for the disease (Chorbinski & Rypula, 2003). The pathogen, *A. apis,* was reflected with dense white mycelium on a solid medium containing aerial, surface, and subsurface septate hyphae of nearly 2-8 µm in diameter (Skou, 1988). Beekeepers addressed that the disease does not occur yearly and claimed the severity of occurrence in recent years. It is confirmed that the fungal spores can remain viable for a long period and germinate with the onset of favorable conditions to serve as a source of inoculum (Heath & Gaze, 1987; Bailey & Ball, 1991; Gilliam & Taber, 1991; Anderson et al., 1997). Simultaneously, disease spreading in the colonies is attributed to faulty management practices. For instance, beekeepers contribute to transmission by transferring the contaminated combs between colonies (Gilliam & Vandenberg, 1997). Another situation is the migration of bee colonies, leading to a higher incidence of *A. apis* fungus than stationary ones (Jara et al., 2018) due to the stress upon honey bees translocation (Simone-Finstrom et al., 2016). Sometimes, drifting or robbing bees and/or drones may also make a minor contribution to the spread of infection. However, pollen can transfer pathogens across bee species during rearing and foraging (Singh et al., 2010). Finally, recent studies have found that pathogen cross-infection between species is more common than previously thought (Graystock et al., 2013; Furst et al., 2014).

**Table 2.** The results obtained at the microscope examination of the slide mounted with sample material from affected cells from apiaries in West Bengal, India.

| Apiary | Infected frames/ hive (Average) | No. of Samples Examined | No. of samples with *Ascosphaera* spore | Positive samples (%) |
|---|---|---|---|---|
| 1 | 3.00 | 11 | 9 | 81.81 |
| 2 | 2.43 | 7 | 6 | 85.71 |
| 3 | 3.40 | 13 | 12 | 92.30 |

*Identification of the pathogen*

For the validation of isolates, genomic DNA from three isolated fungi was extracted, ITS sections were amplified using specific primers ITS-1 and ITS-4, and the amplified product was sequenced using Sanger sequencing (AgriGenome Labs Pvt. Ltd., Kerala, India). The NCBI's BLASTn program was used to confirm the sequences retrieved. According to BLAST analyses, isolates of West Bengal had 99 to 100% similarity to *Ascosphaera apis* sequences in the GenBank database. The sequences of isolated fungus were submitted to the NCBI database and assigned Accession Numbers (Table 3). Taxonomic resolution utilizing ITS sequence analysis has been proposed as the principal fungal barcode identifier and is thus widely used to identify fungi (Schoch et al., 2012). Previous research has also found 100% sequence identity of this region between numerous strains of *A. apis* isolated from European honey bee larvae collected from several continents (Anderson et al., 1998).

**Table 3.** Identification of different fungus isolates from various areas of West Bengal, India.

| Apiary | Isolate | Close related to | Percent Identity | Gene bank Accession No. | Submitted Gene bank Accession No. |
|---|---|---|---|---|---|
| 1 | Aap-N24P | *Ascosphaera apis* | 99.23 | MK910078.1 | ON819759 |
| 2 | Aap-PB | *Ascosphaera apis* | 98.97 | KX622168.1 | ON819761 |
| 3 | Aap-N | *Ascosphaera apis* | 100.00 | MN900311.1 | ON819763 |

*Phylogenetic analysis*

For preliminary alignment and phylogenetic tree construction, ITS sequences of different types of species of *Ascosphaera apis* were downloaded from the NCBI database. The ITS sequences of three isolated fungi (Aap-N24P, AaP-PB, and AaP-N) were aligned with all the downloaded sequences, end-trimmed for missing nucleotides, and a phylogenetic tree was built. Evolutionary analyses were performed in MEGA X using the Neighbour Joining method with 1000 bootstrap values and the Maximum Composite Likelihood model (Fig 6). A total of 31 nucleotide sequences were used for the analysis.

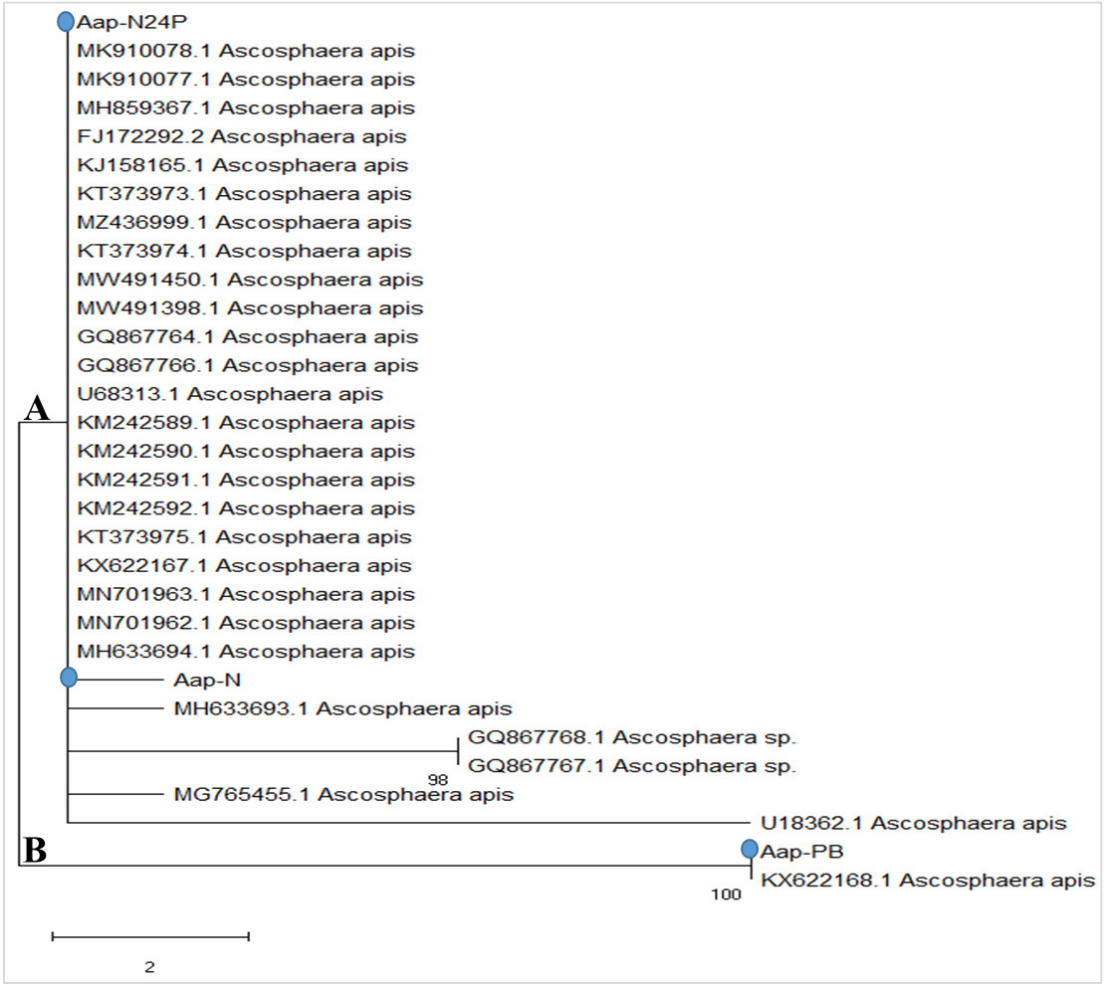


**Fig 6.** Phylogenetic tree constructed by using neighbor joining analysis between ITS sequences of the isolates collected and sequences from selected *Ascosphaera apis* reference isolates obtained from NCBI.

The branch lengths are in the same units as the evolutionary distances used to estimate the phylogenetic tree, and the tree was drawn to scale with the sum of branch length 22. The evolutionary distances were calculated using the number of differences method (Nei & Kumar, 2000) and are in base difference per sequence units. Gaps and missing data were removed from all positions, and the total number of positions in the final dataset was 208. All the isolates have been grouped into two major clusters, A and B. Cluster A, which has various sub-clusters, contained the isolate Aap-N24P, which is more taxonomically related to other *Ascosphaera apis*. The isolate Aap-N also falls under cluster A's sub-divisions, showing its relatedness to *Ascosphaera apis*. Cluster B contains AaP-PB, which shows the distance relationship of the isolates with the sequences of other isolates. The recent advancements in sequencing technology, coupled with genus and species resolution of specific DNA region-based phylogeny, are proving comparably good enough to avoid the complex DNA-DNA hybridization and are considered an excellent standard in phylogeny, and therefore used for identification and taxonomy worldwide. (Janda & Abbott, 2007).

## Conclusion

Based on microscopic and molecular analyses, the study documented the occurrence of chalkbrood disease caused by the fungus *Ascosphaera apis* in the colonies of *Apis mellifera* from West Bengal, India, for the first time. The disease symptoms are attributed to the presence of the dead, mummified bee larvae, covered in a cotton-like chalky white or greyish-black covering, in comb cells, on the bottom board, and in front of the hive opening. Approximately 87% of the samples collected from the contaminated frame were positive for *Ascosphaera* spore on microscopic analysis. The fungus was also identified as *Ascosphaera apis* based on ITS sequence data. Understanding that chalkbrood disease exists and that a colony can become infected over time, leading to considerable losses in bee numbers and colony production, is crucial. The fungal spores remain viable in bee hives for long periods, and even migratory beekeeping enhances the dispersion of the fungus. Beekeepers should consider this possibility, as disease and parasite dispersal are some drivers of pollinator decrease on a global scale. Therefore, management activities should be carried out properly to reduce the spread of fungus spores and the potential for disease development.

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## Authors' Contributions

RD: conceptualization, methodology, investigation, formal analysis, writing-original draft, writing-review and editing.
RK: conceptualization, methodology, investigation, formal analysis, writing-review and editing.
GK: conceptualization, methodology, investigation, formal analysis, writing-review and editing.
SG: conceptualization, methodology, investigation, formal analysis, writing-review and editing.
SD: conceptualization, methodology, formal analysis, supervision, writing-review and editing.
SJ: conceptualization, methodology, formal analysis, supervision, writing-review and editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

The data will be made available on request.

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