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Prevalence and phylogenetic analysis of *Ascosphaera apis* (Maassen ex Claussen) LS Olive & Spiltoir (1955) isolates from honeybee colonies in Turkey

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Abstract

Ascosphaera apis (Maassen ex Claussen) Olive et Spiltoir is a causal agent of chalkbrood disease and is widely found in mainly honeybee colonies worldwide. Chalkbrood disease rarely causes colony death, but severe infections can lead to a decrease in honeybee populations and as a result, a decrease in honey production or beekeeping byproducts. Therefore, it is important to determine the distribution and prevalence of this disease agent in a certain region in terms of controlling of it. In this study, it was aimed to identify chalkbrood disease agents affecting honeybee colonies especially in beekeeping places in Turkey, to isolate and identify them using morphological and molecular methods, especially ITS gene sequencing. Morphologically, pure cultures of clinically suspected samples in terms of chalkbrood disease were created and microscopically evaluated. After that, genomic DNA isolation was performed from pure cultures to amplify ITS gene region and the resulting gene sequences were used in molecular identification and phylogenetic analysis. For this purpose, a total of 1.193 hives were randomly selected from 400 apiaries in 40 provinces throughout Turkey between 2018 and 2019. As a result, the presence of *A. apis* was detected in 7 hives in 2018 and 19 hives in 2019, as a result a total of 26 samples were found to be positive. Accordingly, the infection rate was determined as 2.18%. The obtained results are thought to be important in terms of determining the spread rate of *A. apis* and the rate of chalkbrood disease in Turkey.

Keywords Honeybee · *Ascosphaera apis* · Diagnosis · ITS · Turkey

Abbreviations

ITS	Internal transcribed spacer
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
USA	United States of America
MY20	Malt yeast agar
PDA	Potato dextrose agar
dNTP	Deoxynucleotide triphosphates
NCBI	The National Center for Biotechnology Information
MEGA X	Molecular Evolutionary Genetic Analysis X
BOX PCR	BOX-A1R-based repetitive extragenic palindromic-PCR
REP PCR	Repetitive element polymerase chain reaction
ERIC PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction

Introduction

The fungus *Ascosphaera* (Ascomycota: Eurotiomycetes: Ascosphaerales) is a pathogen associated with mainly honeybee, honeybee products and byproducts. Up to now, it was determined that the genus of *Ascosphaera* contains defined 28 species worldwide. Many of these species have been identified as saprophytes in pollen stores, honey, larval excrement, and nesting materials (Wynns et al. 2012). However, there are

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some pathogenic species in the genus, and these were defined as *A. aggregata* Skou, *A. apis* (Maassen ex Claussen) Olive et Spiltoir (1955), *A. atra* Skou et Hackett, *A. major* (Prokschl et Zobl) Skou and *A. proliperda* Skou (Maxfield-Taylor et al. 2015). Pathogenic species in the *Ascosphaera* genus can infect many hosts such as European honeybee (*Apis mellifera* L.), leaf-cutting bees (*Megachile* spp.), masonic bees (*Osmia* spp.), and sweat bees (*Nomia* spp.) (Bissett 1988; Anderson et al. 1998; Stephen and Vandenberg 1981).

In general, chalkbrood disease affects European honeybee larvae. Although chalkbrood disease is seen in almost every region of the world, it is mainly seen more widely in the Northern Hemisphere (Bailey and Ball 1991). Chalkbrood disease is mainly caused by *Ascosphaera apis*, and this fungus was first identified by Maassen as P*ericystis apis* in 1913 (Maassen 1913). Later, it was reclassified as *Ascosphaera apis* in 1955 (Spiltoir and Olive 1955). The fungus is often described as an opportunistic pathogen, causing larvae to die during the development of pupae, weakening the colony and decreasing production capacity (Evison 2015). *A. apis* rarely causes the mass death of a colony, but the loss of larvae leads to a decrease in the adult bee population, thereby reducing the efficiency and production of honey and pollen. Sometimes, serious cases of this disease have been reported in terms of colony deaths, but this is unusual (Campano et al. 1999).

There are many factors affecting the spread of *A. apis* in honeybee colonies. It is known that honeybee larvae show great susceptibility to the fungus when they are exposed to temperature drop (32–35 °C) (Castagnino et al. 2020). The spores of *A. apis* are more easily reproduced, especially in cold and humid areas. Therefore, the disease is more common in the cool spring and autumn months when the rain is abundant. Also, the disease may also occur during the rainy summer months when the night temperature decreases. In addition, many factors such as climatic conditions, stress factors, other diseases and pests, the use of old honeycombs, air and environmental pollution, excessive use of antibiotics and sensitive colonies are effective in spreading of the disease (Borum and Ülgen 2010).

Different methods are used to identify A. *apis*. The diagnosis in field is usually based on the presence of chalky mummies. After the field diagnosis, it is necessary to verify the presence of spore cysts in the samples using the microscope slide spreading technique in the laboratory (Jensen et al. 2013). In addition, molecular methods can be used for more precise and accurate identification. For this purpose, Polymerase Chain Reaction (PCR) has been increasingly used for the detection of microorganisms (Aziz and Lafta 2022; Dubey et al. 2016). ITS gene region is the most widely sequenced DNA marker for molecular identification of fungi and in molecular ecology of fungi. Today, it is considered as general fungal marker barcode and has typically been most useful tool for molecular systematic at the species to genus level (Nilsson et al. 2008; Schoch et al. 2012). There is almost no variation between *A. apis* strains in terms of ITS gene sequence and therefore, some specific primers which are specific to *A. apis* were designed. No matter which primers are used, the presence of a band from PCR amplification indicates the presence of *A. apis* (Jensen et al. 2013).

Chalkbrood disease was first seen in Germany in 1913 and later detected in Europe, Asia, and the USA (Maassen 1913; Aronstein and Murray 2010). In Turkey, this disease was first diagnosed in 1988, and 82.61% of beekeepers in Hatay province stated that honey yield decreased due to *A. apis* and 18% said that they could not get any honey due to this pathogen (Şahinler and Şahinler 1996; Balkaya et al. 2016). In addition, many studies were carried out on the spread and distribution of *A. apis* in Turkey, mainly in a more limited area. However, it is of great epidemiological importance to investigate the distribution and spread of disease factors in different time periods and in a wider region. Therefore, in this study, it was aimed to determine the infection rate of *A. apis* in 1.193 hives belonging to 400 apiaries from 40 provinces of Turkey. This is the first detailed study to investigate the distribution of *A. apis* throughout Turkey.

Materials and methods

Collection of larvae samples

First, larvae samples from different apiaries (400 apiaries and 1.193 hives) in several provinces throughout Turkey between 2018 and 2019 were brought to Samsun Veterinary Control Institute. The sample numbers, institutes, and provinces where samples were collected are given in Table 1. After the preliminary examination of samples, the suspicious samples were sent to Kırşehir Ahi Evran University and the detailed morphological and molecular analyses were performed in terms of the presence of *A. apis*. During the collection of samples, suspicious specimens were carefully examined by beekeepers and accompanying specialist teams. For this, a special attention was paid to irregular wax coverings on nest, the presence of scattered lidless cells on the nest frames, small holes in the cell cover, white mycelium in the combs, larvae covered with mycelium, and adult bees covered by fungal spores in the hive entrance (Jensen et al. 2013). Location, coordinate, altitude, and climate information were obtained from the relevant institution where samples detected positively in terms of *A. apis* and were given in Table 2.

Table 1 Number of the examined hives for the presence of *A. apis* between 2018 and 2019 and the provinces and institutes where they were taken

Institute name	Province	Number of the examined hives	
		2018	2019
Adana VKEM*	Adana	15	15
	Hatay	15	15
	Kahramanmaraş	15	14
	Mersin	15	15
	Osmaniye	15	15
Etlik VKMAEM	Ankara	15	15
	Bolu	15	14
	Çankırı	15	15
	Çorum	15	15
	Kastamonu	15	15
Bornova VKEM	Aydın	15	12
	Denizli	14	15
	İzmir	15	15
	Manisa	15	15
	Muğla	15	15
Konya VKEM	Aksaray	15	15
	Antalya	15	15
	Burdur	15	15
	Karaman	15	15
	Konya	15	15
Elazığ VKEM	Bingöl	15	15
	Diyarbakır	15	15
	Elazığ	15	15
	Hakkari	15	14
	Van	15	15
Pendik VKEM	Balıkesir	15	15
	Çanakkale	15	15
	Edirne	15	15
	İstanbul	15	15
	Kırklareli	15	15
Erzurum VKEM	Ağrı	15	15
	Ardahan	15	15
	Artvin	15	15
	Erzincan	15	15
	Erzurum	15	15
Samsun VKEM	Giresun	15	15
	Ordu	15	15
	Samsun	15	15
	Sivas	15	15
	Trabzon	15	15
Total		599	594

*Veterinary Control Institute Directorate

Morphological identification

After the field diagnosis, microscopic examination was performed to confirm the presence of spore cysts in the samples using a binocular microscope. For this purpose, the spores and mycelia were placed between glass slide and cover slip in a drop of distilled water and examined at 100 to 400 x magnification. During the examination, hypha showing significant double branching and having reproductive structures (spore balls) were considered as *A. apis* (Bissett 1988).

Culturing of *Ascosphaera apis* samples

After morphological examination, *A. apis* samples were isolated from fresh or dried mummified larvae. The samples were first subjected to surface sterilization by dipping into 10% sodium hypochlorite solution for 10 min and then were washed with sterile distilled water twice. After washing, they were left to dry and cut into small pieces with sterile surgical blade. Finally, the larval pieces were placed on MY20 agar (20% dextrose agar) containing 50 µg/mL tetracycline and 75 µg/mL ampicillin to prevent bacterial growth and the petri dishes were incubated at 32 °C (Ruffinengo et al. 2000). A different agar plate was used for each sample. During incubation, petri dishes were monitored daily, and growing fungal colonies were transferred to another MY20 agar that did not contain antibiotics (Jensen et al. 2013). All samples were subjected to hyphal tip isolation. The isolated fungi were cryopreserved at -20 °C with 15% (v/v) glycerol for further studies. To prove the accuracy of the surface sterilization in larval samples, 100 µL from the last water sample used in washing was spread on PDA (potato dextrose agar) and incubated at 28 °C for a week. PDAs plates with no growth were considered successful in terms of surface sterilization (Gurulingappa et al. 2010).

Molecular identification

Morphological identification of the fungal isolates was molecularly verified using ITS gene sequencing. Genomic DNA isolation for each fungus was performed with the E.Z.N.A. Soil DNA kit (OMEGA-BIO-TEK) according to the manufacturer's recommendations. The isolated DNAs were stored at -20°C until use. After that, the primer pairs of ITS5: 5'- GGAAGTAAAAGTCGTAACAAGG-3' as forward and ITS4: 5'TCCTCCGCTTATTGATATCG- 3' as reverse were used for PCR amplification of ITS1-5.8 S-ITS2 gene region between the 18 and 23 S rRNA subunits (White et al. 1990). The PCR reaction mixture was prepared to include 200 µM from each dNTP, 50 pmol from each opposing primer, 2.5 U *Taq*-DNA-polymerase, 5 µL 10× *Taq* DNA polymerase reaction buffer and 50 ng genomic DNA. The final volume was completed to 50 µL with ddH$_2$O. The reaction mixtures were incubated in a thermal cycler (Bio-Rad, CA, USA) as follows: 5 min initial denaturation at 95 °C; 35 cycles of denaturation (60 s at 94 °C), annealing (50 s at 55 °C), and extension (1 min s at 72 °C); a final extension at 72 °C for 10 min (De Muro et al. 2005; Pellegrino and Bellusci 2009; Sevim et al. 2010). 5 µL of each PCR product was electrophoresed for 45 min at 90 V on 1% agarose gel with 0.5 µg/mL ethidium bromide. The remaining

Table 2 Location, coordinate, altitude, and climate information of samples which are positive for *Ascosphaera apis*

Isolate	City/County	Collection date		Latitude	Longitude	Altitude (m)	Temperature (°C)	Humidity (%)	Daily Rainfall (mm)
24	Adana/Yüreğir	04.04.2018		36.991421	35.330830	27	19.2	28.2	0
57	Osmaniye/Düziçi	25.04.2018		37.367367	36.556738	150	22.1	37	0
60	Osmaniye/ Düziçi	25.04.2018		37.33777	36.558711	150	22.1	37	0
104	Aydın/İncirliova	8.05.2018		37.870000	27.730000	46	19.3	76.5	3.3
105	Aydın/Center	9.05.2018		37.853695	28.052134	75	21.3	71.5	0
176	Ağrı/Taşlıçay	28.05.2018		39.635437	43.433407	1.827	15.3	70.4	0
192	Bingöl/ Center	28.06.2018		38.900000	40.340000	1.500	24.7	39.5	0
3	Adana/Yüreğir	12.03.2019		36.986550	35.337011	27	15.7	78.8	0
LS12	Hatay/ Defne	9.04.2019		36.120000	36.110000	85	17.4	76.3	4.1
LS16	Osmaniye/Düziçi	12.04.2019		37.243448	36.462250	440	15.1	74	0.7
LS17	Osmaniye/Kadirli	12.04.2019		37.374021	36.097416	68	16.4	75.4	0
LS56	Çankırı/Kızılırmak	24.04.2019		40.340000	34.000000	555	9.4	67.5	2.1
LS102	Ardahan/Center	11.06.2019		41.110000	42.690000	1.900	16.7	58.4	0
LS105	Ardahan/Center	11.06.2019		41.040000	42.930000	1.900	16.7	58.4	0
LS107	Elazığ/Center	12.06.2019		38.680969	39.226398	1.067	23.8	32.5	0
LS113	Diyarbakır/Çermik	11.06.2019		38.130000	39.450000	694	27.4	36.5	0.6
LS115	Diyarbakır/Çermik	11.06.2019		38.150000	39.360000	694	27.4	36.5	0.6
LS125	Bingöl/Genç	13.06.2019		38.748401	40.553931	1.087	22.1	48.5	0
LS129	Van/Edremit	18.06.2019		38.419980	43.250000	1.730	22	32.4	0
LS139	Ağrı/Center	20.06.2019		39.721668	43.056667	1.632	18.5	57.4	1.4
LS171	Mersin/Tarsus	3.07.2019		37.139919	34.572276	23	26.1	75.6	0
LS173	Mersin/Tarsus	3.07.2019	36.916469		34.895246	23	26.1	75.6	0
LS174	Mersin/Tarsus	3.07.2019	36.916668		34.900002	23	26.1	75.6	0
LS175	Mersin/Tarsus	3.07.2019	36.976227		34.925537	23	26.1	75.6	0
LS188	Bolu/Dörtdivan	18.07.2019	40.694371		32.095356	1.340	15.7	89.1	0
LS189	Bolu/Center	18.07.2019	40.728798		31.603046	726	17.4	90.8	21.7

PCR products were sequenced by Macrogen company (The Netherlands). The obtained DNA sequences were compared with the most related fungal species in NCBI GenBank and used in phylogenetic analysis to confirm species identification (Benson et al. 2012). Finally, the DNA sequences were

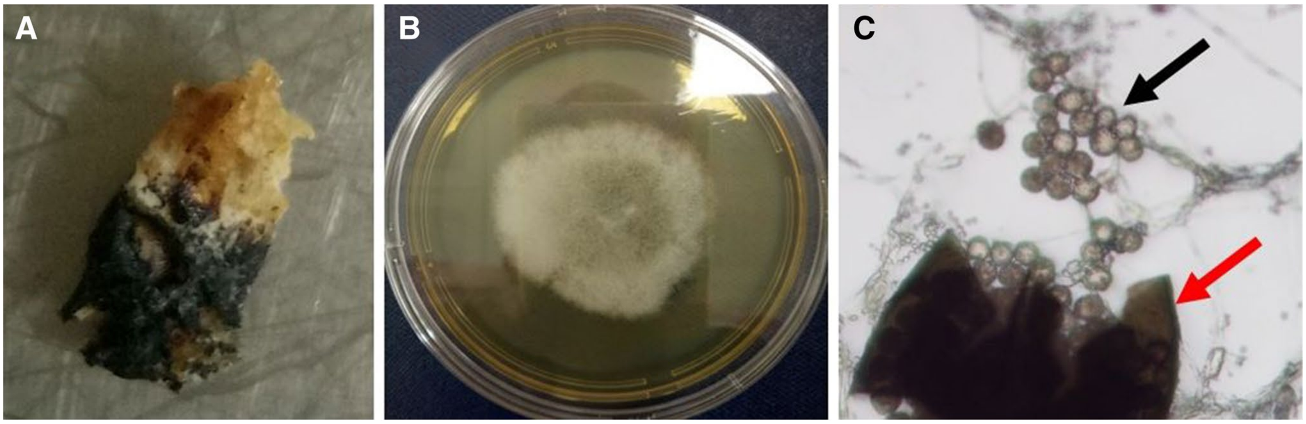


Fig. 1 Morphological images of *Ascosphaera apis*. **A**; infected larva, **B**; *A. apis* colony on MY20 agar, **C**; spore cyst (red arrow) and spore balls (black arrows)

Table 3 Percentage (%) similarities of the fungal isolates with the most related fungal species or isolates in GenBank according to Blast search using ITS gene sequences (ITS1-5.8 S-ITS2)

Isolate	Species	GenBank ID number	Query coverage (%)	Similarity (%)
24	*Ascosphaera apis* SX	MK910078	100	99.67
	Ascosphaera apis GZ	MK910077	100	99.67
	Ascosphaera apis CBS 402.96	MH862580	99	99.67
	Ascosphaera apis 7405	GQ867785	99	99.67
57	*Ascosphaera apis* SX	MK910078	100	99.50
	Ascosphaera apis GZ	MK910077	100	99.50
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
60	*Ascosphaera apis* SX	MK910078	100	99.50
	Ascosphaera apis GZ	MK910077	100	99.50
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
104	*Ascosphaera apis* SX	MK910078	100	99.51
	Ascosphaera apis GZ	MK910077	100	99.51
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
105	*Ascosphaera apis* SX	MK910078	100	99.50
	Ascosphaera apis GZ	MK910077	100	99.50
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
176	*Ascosphaera apis* SX	MK910078	100	99.67
	Ascosphaera apis GZ	MK910077	100	99.67
	Ascosphaera apis CBS 402.96	MH862580	99	99.67
	Ascosphaera apis 7405	GQ867785	99	99.67
192	*Ascosphaera apis* SX	MK910078	100	99.50
	Ascosphaera apis GZ	MK910077	100	99.50
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
3	*Ascosphaera apis* SX	MK910078	100	100
	Ascosphaera apis GZ	MK910077	100	100
	Ascosphaera apis CBS 402.96	MH862580	100	100
	Ascosphaera apis CBS 534.69	MH859367	100	100
LS12	*Ascosphaera apis* SX	MK910078	100	99.51
	Ascosphaera apis GZ	MK910077	100	99.51
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
LS16	*Ascosphaera apis* SX	MK910078	100	99.50
	Ascosphaera apis GZ	MK910077	100	99.50
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
LS17	*Ascosphaera apis* SX	MK910078	100	99.34
	Ascosphaera apis GZ	MK910077	100	99.34
	Ascosphaera apis CBS 402.96	MH862580	99	99.34
	Ascosphaera apis 7405	GQ867785	99	99.34
LS56	*Ascosphaera apis* SX	MK910078	100	99.34
	Ascosphaera apis GZ	MK910077	100	99.34
	Ascosphaera apis CBS 402.96	MH862580	99	99.34
	Ascosphaera apis 7405	GQ867785	99	99.34

Table 3 (continued)

Isolate	Species	GenBank ID number	Query coverage (%)	Similarity (%)
LS102	*Ascosphaera apis* SX	MK910078	100	99.50
	Ascosphaera apis GZ	MK910077	100	99.50
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
LS105	*Ascosphaera apis* SX	MK910078	100	99.67
	Ascosphaera apis GZ	MK910077	100	99.67
	Ascosphaera apis CBS 402.96	MH862580	99	99.67
	Ascosphaera apis 7405	GQ867785	99	99.67
LS107	*Ascosphaera apis* SX	MK910078	100	99.51
	Ascosphaera apis GZ	MK910077	100	99.51
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
LS113	*Ascosphaera apis* SX	MK910078	100	99.67
	Ascosphaera apis GZ	MK910077	100	99.67
	Ascosphaera apis CBS 402.96	MH862580	100	99.50
	Ascosphaera apis 7405	GQ867785	100	99.50
LS115	*Ascosphaera apis* SX	MK910078	100	99.34
	Ascosphaera apis GZ	MK910077	100	99.34
	Ascosphaera apis CBS 402.96	MH862580	99	99.34
	Ascosphaera apis 7405	GQ867785	99	99.34
LS125	*Ascosphaera apis* SX	MK910078	100	94.76
	Ascosphaera apis GZ	MK910077	100	94.76
	Ascosphaera apis CBS 402.96	MH862580	99	94.59
	Ascosphaera apis 7405	GQ867785	99	94.59
LS129	*Ascosphaera apis* SX	MK910078	100	99.83
	Ascosphaera apis GZ	MK910077	100	99.83
	Ascosphaera apis CBS 402.96	MH862580	100	99.66
	Ascosphaera apis 7405	GQ867785	100	99.66
LS139	*Ascosphaera apis* SX	MK910078	100	98.84
	Ascosphaera apis GZ	MK910077	100	98.84
	Ascosphaera apis CBS 402.96	MH862580	99	98.84
	Ascosphaera apis 7405	GQ867785	99	98.84
LS171	*Ascosphaera apis* SX	MK910078	100	99.50
	Ascosphaera apis GZ	MK910077	100	99.50
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
LS173	*Ascosphaera apis* SX	MK910078	100	99.51
	Ascosphaera apis GZ	MK910077	100	99.51
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50
LS174	*Ascosphaera apis* CBS 402.96	MH862580	100	99.67
	Ascosphaera apis 7405	GQ867785	100	99.67
	Ascosphaera apis SX	MK910078	100	99.67
	Ascosphaera apis GZ	MK910077	100	99.67
LS175	*Ascosphaera apis* SX	MK910078	100	99.51
	Ascosphaera apis GZ	MK910077	100	99.51
	Ascosphaera apis CBS 402.96	MH862580	99	99.50
	Ascosphaera apis 7405	GQ867785	99	99.50

Table 3 (continued)

Isolate	Species	GenBank ID number	Query coverage (%)	Similarity (%)
LS188	*Ascosphaera apis* SX	MK910078	100	99.34
	Ascosphaera apis GZ	MK910077	100	99.34
	Ascosphaera apis CBS 402.96	MH862580	99	99.34
	Ascosphaera apis 7405	GQ867785	99	99.34
LS189	*Ascosphaera apis* CBS 402.96	MH862580	100	99.34
	Ascosphaera apis 7405	GQ867785	100	99.34
	Ascosphaera apis SX	MK910078	100	99.17
	Ascosphaera apis GZ	MK910077	100	99.17

compared with reference species included in the study of Klinger et al. (2013) using phylogenetic analysis.

GenBank accession numbers

ITS gene sequence of each fungal isolate was deposited in the GenBank database under the accession numbers of OM754488-OM754514.

Data analysis

All DNA sequences were edited with BioEdit 7.09 software and were subjected to NCBI Blast search to determine their percent similarities with the most closely related fungal species (Hall 1999; Benson et al. 2012). The obtained data were used to confirm the morphological identifications of the isolates. Cluster analysis of DNA sequences was done using BioEdit software with Clustal W and used in neighbor-joining (NJ) analysis with MEGA X phylogenetic software (Kumar et al. 2018). Alignment gaps were evaluated as missing data. The reliability of the generated phylogram was tested based on 1.000 replicates using the MEGA X with bootstrap analysis (Kumar et al. 2018).

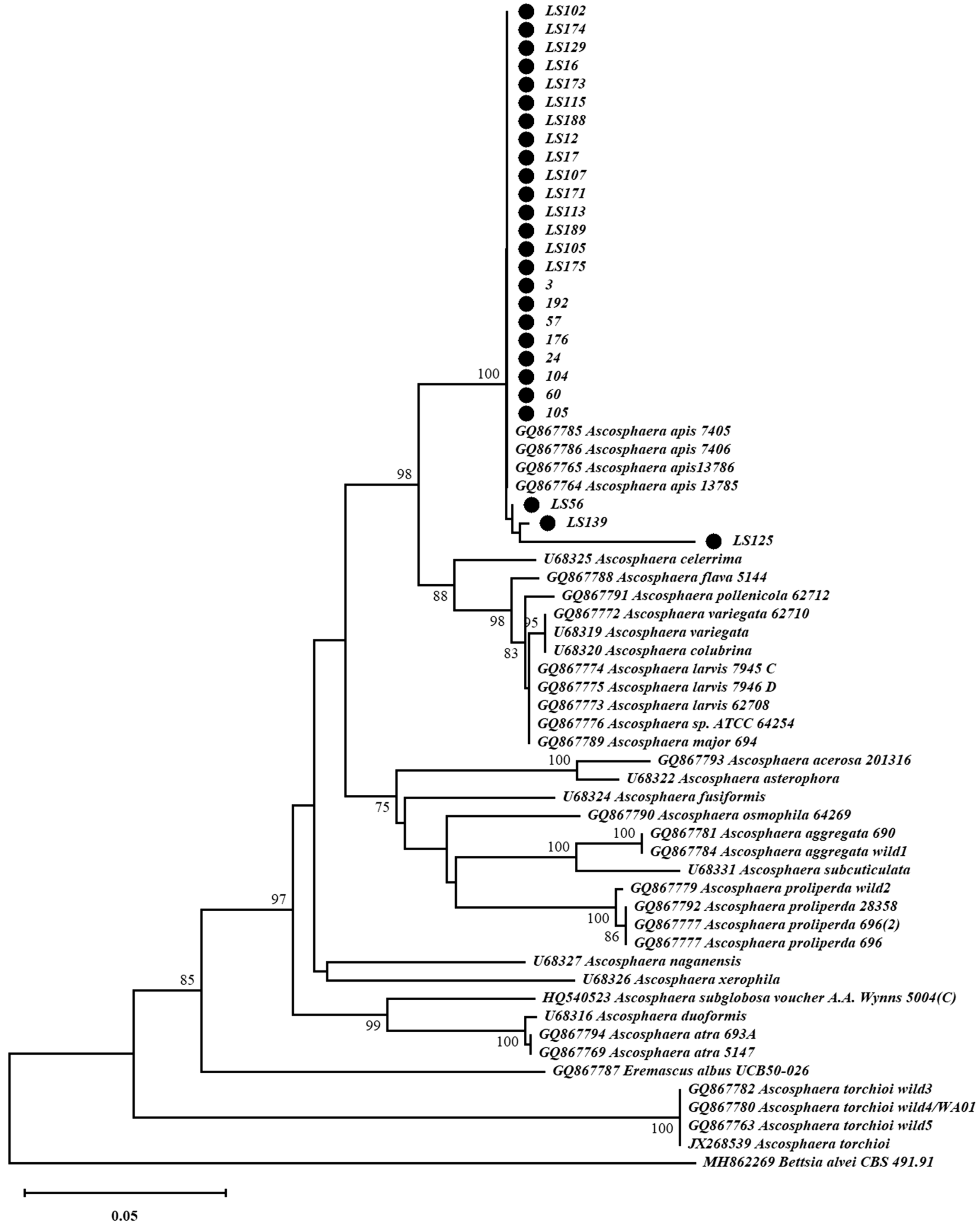
Results

A total of 400 apiaries and 1.193 hives in these apiaries throughout Turkey were examined with respect to the presence of *A. apis*. The fungus isolation was done from the suspected samples and their pure cultures were created (Fig. 1). Firstly, they were morphologically and microscopically characterized and a total of 26 isolates were found to be positive in terms of the presence of *A. apis*. The morphological characterization of the isolates was also confirmed by NCBI Blast search and phylogenetic analysis using ITS gene sequences. Based on the Blast search, all isolates showed $\geq 98\%$ similarity with *A. apis*, except for LS125. The isolate LS125 showed 94.76% similarity with *A. apis* (Table 3). Finally, *A. apis* isolates were compared with reference species and isolates included in the study of Klinger et al. (2013) using phylogenetic analysis based on ITS gene sequence. According to the phylogenetic analysis, all isolates were found to be identical to *A. apis* (Fig. 2).

In total, the infection rate was determined to be 2.18%, seven of which were from 2018 (1.17%) and nineteen of which from 2019 (3.2%). It was detected that the temperature was between 9.4 and 27.4 °C when the collection area and climatic characteristics of the positive samples were considered. Relative humidity was between 28.2 and 90.8%. Other climatic characteristics of the locations where positive samples were taken are given in Table 2.

Discussion

Honey is one of the most important agricultural products in the world and Turkey is in an important position in terms of world honey production. Besides honey production, the production of beekeeping byproducts (such as royal jelly and bee venom) is an important issue with respect to health benefits such as wound healing, aging, gastrointestinal disorders and allergies and there is not much trend at this point (Burucu and Gülse Bal 2017; Pasupuleti et al. 2017). In addition to the benefits of beekeeping to the agricultural economy, honeybees pollinate agricultural and wild plants as they forage on flowers and this process is extremely important for humanity. In this way, they help plants to produce fruits and seeds (Sıralı and Cınbırtoğlu 2018). However, there are many diseases and pests that cause mass deaths in honeybee colonies, and these negatively affect the production of honey and beekeeping byproducts (Adjlane and Haddad 2017). Knowing the distribution and frequency of these disease factors in honeybee colonies is of great importance to control them. Therefore, in this study, the distribution and presence of *A. apis*, which causes chalkbrood disease in honeybees, was examined in larval samples collected between 2018 and 2019 throughout Turkey.


LS102
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GQ867785 Ascosphaera apis 7405
GQ867786 Ascosphaera apis 7406
GQ867765 Ascosphaera apis13786
GQ867764 Ascosphaera apis 13785
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U68325 Ascosphaera celerrima
GQ867788 Ascosphaera flava 5144
GQ867791 Ascosphaera pollenicola 62712
GQ867772 Ascosphaera variegata 62710
U68319 Ascosphaera variegata
U68320 Ascosphaera colubrina
GQ867774 Ascosphaera larvis 7945 C
GQ867775 Ascosphaera larvis 7946 D
GQ867773 Ascosphaera larvis 62708
GQ867776 Ascosphaera sp. ATCC 64254
GQ867789 Ascosphaera major 694
88
98
95
83
100
GQ867793 Ascosphaera acerosa 201316
U68322 Ascosphaera asterophora
U68324 Ascosphaera fusiformis
75
GQ867790 Ascosphaera osmophila 64269
100
GQ867781 Ascosphaera aggregata 690
100
GQ867784 Ascosphaera aggregata wild1
U68331 Ascosphaera subcuticulata
GQ867779 Ascosphaera proliperda wild2
GQ867792 Ascosphaera proliperda 28358
100
GQ867777 Ascosphaera proliperda 696(2)
86
GQ867777 Ascosphaera proliperda 696
97
U68327 Ascosphaera naganensis
U68326 Ascosphaera xerophila
85
HQ540523 Ascosphaera subglobosa voucher A.A. Wynns 5004(C)
U68316 Ascosphaera duoformis
99
GQ867794 Ascosphaera atra 693A
100
GQ867769 Ascosphaera atra 5147
GQ867787 Eremascus albus UCB50-026
GQ867782 Ascosphaera torchioi wild3
GQ867780 Ascosphaera torchioi wild4/WA01
100
GQ867763 Ascosphaera torchioi wild5
JX268539 Ascosphaera torchioi
MH862269 Bettsia alvei CBS 491.91
0.05

◀**Fig. 2** A phylogram showing phylogenetic position of *A. apis* isolates and the reference strains from the study of Klinger et al. (2013) based on the ITS sequence. The tree was constructed using neighbor-joining (N-J) analysis with p-distance correction. The bootstrap analysis was based on 1.000 pseudoreplicates and bootstrap values with > 70% were indicated. The solid black circle indicates *A. apis* isolates obtained from this study. The scale shown on the bottom of the phylogram indicates the degree of dissimilarity

Chalkbrood disease caused by *A. apis* in honeybees has been quickly spread to many countries after it was first reported in Germany (Maassen 1913). It is well-known that the incidence of this disease varies according to many factors such as geographical regions and climatic conditions (Castagnino et al. 2020). Rundassa (2001) inspected 276 colonies and found that 48 colonies (17.4%) were found to be positive in terms of *A. apis* in Ethiopia between 2000 and 2001. Faucon et al. (2002) reported the overall rate of chalkbrood disease as 36% in 41 honeybee colonies in different parts of France between 1999 and 2000. Yoshiyama and Kimura (2011) examined 112 European honeybee colonies collected from different regions of Japan with PCR technique, and they found the disease rate as 24.1%. According to the studies carried out in Turkey, it is seen that the incidence of chalkbrood disease varies according to provinces and regions throughout the country (between 79.59% – 0%) (Kösoğlu et al. 2000; Güzerin 2013). Chalkbrood disease was first diagnosed in Turkey in 1988, and 82.61% of beekeepers in Hatay province stated that honey yield decreased due to *A. apis* and 18% of them did not get any honey due to this pathogen (Şahinler and Şahinler 1996; Balkaya et al. 2016). In a survey study conducted throughout Turkey, the incidence of chalkbrood disease in honeybee colonies was reported as 39.61% (Çağlar and Öner 2001). In addition, the incidence of chalkbrood disease in main geographical regions of Turkey was determined to be 9% for Aegean Region (Özbilgin et al. 1999), 7.8% for Black Sea Region (Yaşar et al. 2002), 36.3% for Thrace region (Sıralı and Doğaroğlu 2005) and 11% for South Marmara region (Çakmak et al. 2003). In another study conducted in Thrace region of Turkey, the incidence of chalkbrood disease was reported as 26.4% (Sıralı 1993). In a study conducted by Borum and Ülgen (2010), they evaluated the prevalence of fungal infections in Bursa province and surrounding apiaries and examined a total of 84 hives. As a result, they determined the rate of chalkbrood disease as 23.8%. In the studies carried out in Adana and Hatay provinces, the disease rate was reported very low. While no disease was found in these regions in 2008, the disease rate in Hatay region was only 0.1% in 2005 (Yalçınkaya 2008; Şahinler and Gül 2005). Similarly, chalkbrood disease was not determined in a study conducted in Muğla province in 2006 (Şimşek 2008). In the studies performed in Tekirdağ, Tokat and Toros villages, the incidence of chalkbrood disease was determined as 20%, 8.33% and 14.6%, respectively (Soysal and Gürcan 2005; Parlakay and Esengün 2005; Özkök 1995; Özkırım 2000) determined the rate of chalkbrood disease as 47% in Ankara province and its environment. In a study conducted by Özkırım and Keskin (2002) in the same region (Ankara), the disease rate was determined as 3.84%. In this study, the rate of chalkbrood disease was determined to be 2.18% in the larval samples collected from throughout Turkey. These studies show that the incidence of chalkbrood disease varies according to the sampling years, region, and climatic conditions. So, when evaluating the distribution and spread of chalkbrood disease, many factors should be simultaneously considered in a study region. In this way, it is thought that the relationship between the incidence of the disease and various factors can be established.

Within the scope of this study, various climatic characteristics were considered for localities which were positive for *A. apis*. It was determined that the temperature range was between 9.4 and 27.4 °C and relative humidity range was 28.2 and 90.8%. Total amount of daily rainfall was between 0 and 21.7 mm. We found that there was little difference between the positive localities in terms of these climatic factors. It is known that chalkbrood disease is related to various climatic factors and is generally found in honeybee colonies in the spring months when there is an excessive humidity and sudden temperature changes in the hive. Some other factors such as viral or bacterial infection, the presence of *Varroa* and pesticide poisoning also influence the severity and prevalence of chalkbrood disease (Castagnino et al. 2020). In the localities which were determined to be positive with respect to the presence of chalkbrood disease in this study, the temperature and humidity measurements (not in the hive) were very variable, and it is thought that this may trigger the development of chalkbrood disease. In addition, it should be noted that other factors that were not investigated in this study may be effective on the rate of the disease. More detailed and statistical sampling is needed to establish a clear relationship between climate characteristics and the frequency of disease.

Fungal isolates were identified by ITS gene sequencing after *A. apis* isolation was performed from the suspected larval samples. After analyzing the gene sequence data, it was determined that there were no significant genetic variations among isolates. Only a significant variation has been detected in the isolate LS125 (Bingöl) sample. Recent studies showed that no significant variation has been detected between A. *apis* isolates according to ITS gene region (Anderson et al. 1998; Jensen et al. 2013). These results are compatible with our results. However, the isolate LS125 is needed to characterize in detail using further molecular techniques to verify it's a new species or not. Moreover, PCR-based DNA fingerprinting techniques such as BOX, REP and ERIC PCR should be used to determine the genetic diversity among isolates (Reynaldi et al. 2003). In this way, it is thought that epidemiological studies will be contributed to determine of clonal identity or relatedness of *A. apis* isolates.

In conclusion, the presence of *A. apis* was investigated by the culture-dependent methods throughout Turkey between 2018 and 2019 and the overall disease rate was determined to be 2.18% in larval samples. To establish a clear relationship between the incidence of the disease and various factors (biotic or abiotic), more detailed sampling strategy and molecular analyses should be considered.

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Authors' contributions Ali Sevim participated in almost all parts of the study such as the study conception, design, analysis and writing the manuscript. Rahsan Akpınar participated in larvae and data collection and performed initial morphological examination. Şengül Alpay Karaoğlu and Arif Bozdeveci performed culturing of the fungal isolates. Elif Sevim participated in gene sequencing.

Declarations

Conflicts of interest/Competing interests The authors have no conflicts of interest to declare.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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