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RESEARCH

PCR-based detection of the honeybee tracheal mite (*Acarapis woodi*) in Türkiye

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Abstract

Acarapis woodi (Rennie 1921) (Acari: Tarsonemidae) is one of the mites that settles in the respiratory system of honeybees (*Apis mellifera* L. (Hymenoptera, Apidae)) and distributed throughout the world. It causes significant economic losses on honey production. In Türkiye, studies on the existence of *A. woodi* are very limited and so far, no studies on the molecular diagnosis and phylogenetic of it have been reported in Türkiye. This study was conducted to investigate the prevalence of *A. woodi* in Türkiye, especially in areas where beekeeping is intense. Diagnosis of *A. woodi* was performed using both microscopic and molecular methods using specific PCR primers. Adult honeybee samples were collected from 1.193 hives in 40 provinces of Türkiye between 2018 and 2019. Based on identification studies, the presence of *A. woodi* was detected in a total of 3 hives (0.5%) in 2018 and 4 hives (0.7%) in 2019. This is the first report for determination of *A. woodi* in Türkiye.

Keywords *Acarapis woodi* · Diagnosis · Honeybee · Trachea · Türkiye

Introduction

Beekeeping is the activity of obtaining various products such as honey, pollen, royal jelly by using honeybees (*Apis mellifera* L.) and plant sources. In addition, beekeeping activities play an indispensable role in meeting the world's food needs with its role in vegetable-fruit pollination. While Türkiye ranks 3rd in the world with 8.179.085 million in terms of the number of honeybee colonies, it ranks second with 104,077 tons with respect to honey production. While the average honey production per hive in the world is 20,6 kg, this number is 12,7 kg in Türkiye (TÜİK 2019). When both our share in world honey trade and honey production per colony are considered, it becomes clear that it cannot be sufficiently benefited from the current beekeeping potential of Türkiye. One of the most important reasons for this is bee diseases and pests that cause economic losses and high colony deaths. Tracheal acariosis, which has an important place among honeybee diseases, is a disease caused by *A. woodi* parasitizing in the respiratory tracts of adult honeybees. The adult, nymph and larval forms of the mite settle in the respiratory tracts (trachea) of bees, feed by sucking the hemolymph, where they mate, lay eggs, and defecate. While clinical symptoms are not noticed in adult bees in the early stages of the infection, it can cause a decrease in the ability to fly, weakening, death of the bee, decrease in colony productivity and even extinction of the hive in the last stage of the disease because of irregular breathing (Girişgin 2021). There are three species (*A. woodi, A. externus* and *A. dorsalis*) in the genus *Acarapis*. Although these three species are similar to each other in general, they have some morphological differences and differs in settlement part on the host

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bee. For settlement parts, *A. woodi* settles in the trachea (tracheal acarapiosis agent); *A. externus* parasitizes at the junction of the head and thorax (neck mite) and *A. dorsalis* parasitizes on the thorax, in the canal between the mesoscutum and the mesoscutellum, sometimes in the root of the wing and anterior part of the abdomen. The density of the genus *Acarapis* in the hive varies seasonally. Although *A. woodi* is more active in winter, *A. dorsalis* is more common in summer (Eckert 1961; Shaw et al. 1961; Ibay and Burgett 1989). Since *A. woodi* was first identified in England by Rennie in 1921, it has been spread to the other parts of the world such as Europe, Africa, Asia, and the Americas in a short time and has been caused serious losses on honeybee colonies and honey production. However, the rates and damage of tracheal acarapiosis in honeybees have started to decrease worldwide with the use of acaricides and international control programs against varroasitis since the 1980s. (Fernández 1999; OIE 2018). Although studies on the presence of *A. woodi* in Türkiye are very limited, this pest was detected in Türkiye in the study of Keskin et al. (2006). In their study, they didn't use molecular techniques (especially PCR-based techniques) for identification. But it was reported that tracheal acarapiosis has not been found in later studies conducted throughout Türkiye (Başar 1990; Şimşek 2005; Öztürk et al. 2016).

Due to its size and cryptic lifestyle, it is difficult to diagnose *A. woodi*. Specifically, the tracheal mites are very small and spend most of their time hiding within the tracheal networks of adult bees. This feature leads to missed diagnosis of the tracheal mites (Evans et al. 2007). Traditional methods such as the direct imagining of the mite and the lesions in the trachea for diagnosis of *A. woodi* is very time consuming. In addition, the methods such as Enzyme-linked immunosorbent assays (ELISA) and the visualization of guanine which is the main product of nitrogen metabolism in mites under ultraviolet light are of little use in routine diagnosis. Recently, techniques based on amplification and sequencing of the *COI* gene have been frequently used for the identification of *A. woodi*. In this context, molecular techniques give more sensitive and specific results, especially in case of low parasite loads (Garrido-Bailón et al. 2012; Pietropaoli et al. 2022).

In this study, it was aimed to investigate the presence of *A. woodi* in 1.193 hives in 400 beekeeping enterprises from 40 provinces of Türkiye, using both traditional microscopic diagnostic methods (Colin et al. 1979; Ritter 1996; Wilson et al.1997; Sammataro et al. 2013; OIE 2018) and molecular (using specific PCR primers) diagnostic methods (Cepero et al. 2015). As a result of this study, the presence of *A. woodi* was detected and reported by molecular techniques for the first time in Türkiye.

Material and methods

Collection of adults honeybees

Within the scope of this study, a total of 40 provinces (5 provinces affiliated to 8 Veterinary Control Institute Directorates) in Türkiye were selected, samples were randomly collected from 5 apiaries in each province and 3 different beehives were selected in each apiary. The sampling process was repeated in 2018 and 2019, and samples taken from 1.193 hives in 400 different bee enterprises in 40 provinces of Türkiye were brought to Samsun Veterinary Control Institute for diagnosis. The number of samples taken by the institutes and provinces is presented in Table 1.

Examination of honeybee samples

Identification of the agent

Dissection and grinding methods were used for direct diagnosis of *A. woodi* (Milne 1948; Lorenzen and Gary 1986). A pool of approximately 50 bees was created for the dissection method. In the dissection method (Ritter 1996; Wilson et al. 1997), the head was separated from the thorax and the tracheal respiratory airways were came into open. The prothoracic trachea, where the first stigmas were opened and the mite was primarily seen, was examined under the microscope (100×). Separately, thoraxes were separated from bodies, and they were kept in 8% KOH for approximately overnight at 37 ºC. After that, tracheas were taken on a microscope slide, water or glycerin was dripped on them and examined under the light microscope at 100× magnification. When the female mite finds a suitable host to lay eggs (queens, workers, or drones), it enters the trachea of the host via the spiracle (Sammataro et al. 2013). Therefore, attention was paid to the presence of oval, transparent eggs that can be easily seen in the trachea and melanization in the trachea during the diagnosis.

In the Grinding Method (Colin et al. 1979), after the legs and wings of about 200 bees were separated, their thoraxes were placed in a 100 ml tube filled with ¼ water, crushed three times in a homogenizer (Bead Ruptor Elite, Bead Mill Homogenizer, SKU 19-042E, OMNI International, USA) at 10,000 rpm. After that, body suspensions were filtered through 0.8 mm mesh sieve, rinsed with water, and diluted to 50 ml with sterile ddH_2O. The resulting suspensions were centrifuged at 1,500 g for 5 min and the supernatants were discarded. Pure lactic acid was added to the sediment, the muscular structures of the bee were dissolved, and the mites were examined under

Table 1 The number of hives examined for the presence of *A. woodi* between 2018 and 2019 and the provinces where they were taken

Institutes	Provinces	Number of hives		Climate	Coordinates	
		2018	2019		Latitude	Longitude
Adana VKEM	Adana	15	15	Mediterranean	36.9914	35.3308
	Hatay	15	15	Mediterranean	36.3524	36.2935
	Maraş	15	14	Mediterranean	37.5753	36.9228
	Mersin	15	15	Mediterranean	36.8121	34.6415
	Osmaniye	15	15	Mediterranean	37.0748	36.2466
Etlik VKMAEM	Ankara	15	15	Terrestrial	39.9334	32.8597
	Bolu	15	14	Terrestrial	40.7325	31.6082
	Çankırı	15	15	Terrestrial	40.6002	33.6162
	Çorum	15	15	Terrestrial	40.5499	34.9537
	Kastamonu	15	15	Black Sea and Terrestrial	41.3766	33.7765
Bornova VKEM	Aydın	15	12	Mediterranean	37.8380	27.8456
	Denizli	14	15	Mediterranean	37.7830	29.0963
	İzmir	15	15	Mediterranean	38.4237	27.1428
	Manisa	15	15	Mediterranean	38.6140	27.4296
	Muğla	15	15	Mediterranean	37.2154	28.3636
Konya VKEM	Aksaray	15	15	Terrestrial	38.3686	34.0297
	Antalya	15	15	Mediterranean	36.8969	30.7133
	Burdur	15	15	Mediterranean	37.7182	30.2813
	Karaman	15	15	Mediterranean	37.1810	33.2222
	Konya	15	15	Terrestrial	37.8746	32.4932
Elazığ VKEM	Bingöl	15	15	Terrestrial, Harsh Continental	38.8855	40.4966
	Diyarbakır	15	15	Humid Terrestrial	37.9250	40.2110
	Elazığ	15	15	Terrestrial	38.6748	39.2225
	Hakkari	15	14	Terrestrial	37.5774	43.7368
	Van	15	15	Terrestrial	38.5012	43.3730
Pendik VKEM	Balıkesir	15	15	Mediterranean, Humid Terrestrial	39.6533	27.8903
	Çanakkale	15	15	Mediterranean	40.1467	26.4086
	Edirne	15	15	Mediterranean, Terrestrial	41.6771	26.5557
	İstanbul	15	15	Black Sea and Mediterranean	41.0082	28.9784
	Kırklareli	15	15	Black Sea and Terrestrial	41.7355	27.2245
Erzurum VKEM	Ağrı	15	15	Terrestrial	39.7191	43.0506
	Ardahan	15	15	Terrestrial	41.1130	42.7023
	Artvin	15	15	Black Sea	41.1809	41.8208
	Erzincan	15	15	Terrestrial	39.7469	39.4910
	Erzurum	15	15	Terrestrial, Harsh Continental	39.9056	41.2658
Samsun VKEM	Giresun	15	15	Black Sea	40.9175	38.3927
	Ordu	15	15	Black Sea	40.9862	37.8797
	Samsun	15	15	Black Sea	41.2797	36.3361
	Sivas	15	15	Terrestrial, Harsh Continental	39.7505:	37.0150
	Trabzon	15	15	Black Sea	41.0027	39.7168
Total		599	594			

VKMAEM; Veterınary Control Central Research Instıtute, VKEM; Veterınary Control Instıtute,

the light microscope at 18–20 × magnification. With this method, we were able to investigate not only *A. woodi*, but also *A. externus* and *A. dorsalis*. Therefore, this method might be chosen when a rough estimation of the degree of invasion in an area is required (Delfinado-Baker and Baker 1982).

Molecular identification of mites

Total DNA extraction was carried out from the homogenates prepared from the thorax of approximately 30 honeybees in each hive. DNA extraction was performed using the PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's recommendations.*A. woodi* samples were further characterized using the partial sequencing of cytochrome c oxidase subunit I (*COI*) gene.

In DNA barcoding studies, scientists often use mitochondrial gene cytochrome c oxidase subunit I (*COI*) to identify invertebrate species, and this method helps effectively determine the species-level taxonomic identity (Hebert et al. 2003a, 2003b). To distinguish *Acarapis woodi* from other *Acarapis* species (*A. externus* and *A. dorsalis*), a new PCR protocol was developed using specific primers designed by Cepero et al (2015). Approximately 180 bp fragment of the *COI* gene was targeted and amplified. The primer pairs of AwF (5′- GGAATATGATCTGGTTTAGTTGGTC-3′ as forward) and AwR (5′- GAATCAATTTCCAAACCCACCAAT C -3′ as reverse) were used for PCR amplifications. PCR reactions were performed in 50 µl reaction volume. The PCR mixture contained 0.4 pmol/µl of each primer, 200 mM each dNTP, 1 × PCR reaction buffer, 2 mM $MgCl_2$, 1.5 U *Taq* DNA polymerase and 50 ng genomic DNA. After mixing all components, the final volume was adjusted to 50 µl with sterile distilled water. The PCR program consisted of 95 °C (10 min) for the initial denaturation, followed by 35 cycles of 95 °C (30 s) for denaturation, 59 °C (30 s) for annealing, 72 °C (45 s) for extension, and a final extension of 72 °C (7 min). After performing PCR, 5 µl of PCR products was analyzed by electrophoresis on 2% agarose gel containing ethidium bromide to check the sizes and amounts of the amplicons. Finally, the accurate products were sent to BM Labosis (Türkiye) for sequencing. The obtained sequences were subjected to the nucleotide BLAST searches in the NCBI GenBank database to get the percentage similarity of the samples to the most related species (Altschul et al. 1990; Cepero et al. 2015).

Phylogenetic analysis

Phylogenetic analysis of *A. woodi* samples and their closely related species was performed for molecular characterization of the mites. The sequences were edited using Bioedit, and multiple sequence alignments were created using *COI* sequences belonging to our samples and different species from the NCBI GenBank database for the purpose of developing a phylogenetic tree. The multiple sequence alignment was performed with ClustalW in Bioedit (Hall 1999). Finally, the sequences were subjected to neighbor-joining analysis with p-distance correction, gap omission, and 1.000 bootstrap pseudoreplicates using MEGA 6.0 (Tamura et al. 2013).

GenBank accession numbers

The GenBank accession numbers of the *COI* gene sequences belonging to *A. woodi* samples are MW748228, MW748229, MW748230, MW748231, MW748232, MW748233, and OM688864 for sample L49/1, L50/1, L147/3, LS7/1, LS38/3, LS58/2, and LS38/2 respectively. All gene sequences were deposited in NCBI GenBank database.

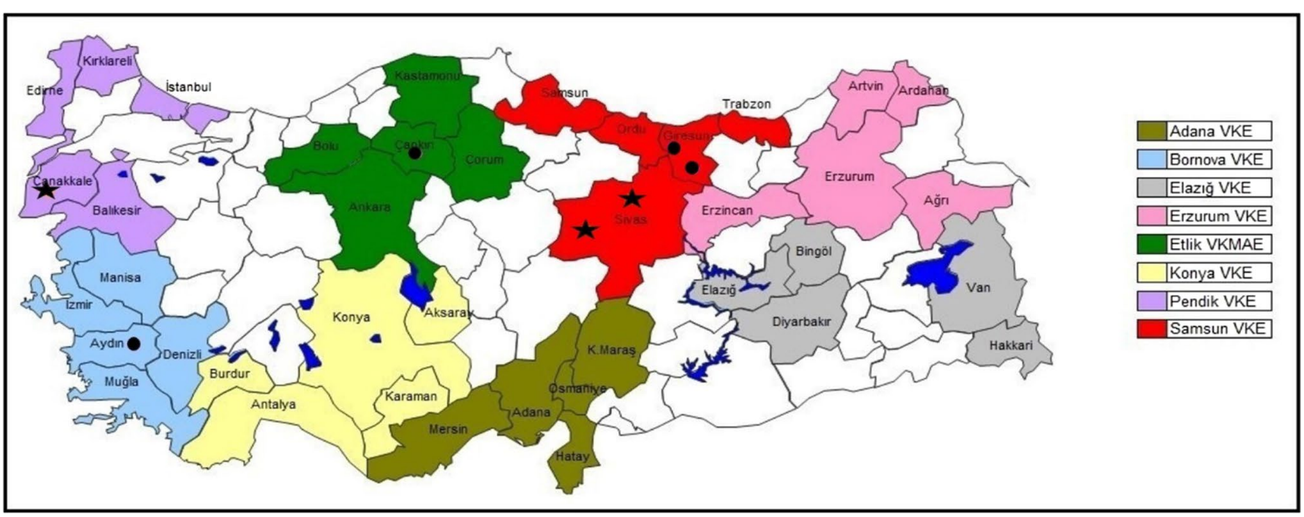

Fig. 1 Sampling for *Acarapis woodi* in Türkiye was carried out in 40 provinces affiliated to 8 Veterinary Control Institutes. Provinces marked with star were found positive for *A.woodi* in 2018, and provinces marked with circle were found positive for *A.woodi* in 2019

Results

No *A. woodi* was found in microscopic examinations in honey bee colonies collected from various regions of Türkiye between 2018–2019. However, based on PCR analyzes, *A. woodi* was detected in 2 apiaries in Sivas (one beehive in each enterprise) and in one apiary (one beehive) in Çanakkale for 2018. For 2019, it was detected in one apiary in Çankırı and Aydın (one beehive for each) and one apiary (two beehives) in Giresun. Study area, provinces in Türkiye where samples were taken—Signs indicate positivity in sampled provinces (Fig. 1).

The presence of *A. woodi* was detected in 3 hives (0.5%) in 2018 and in 4 hives (0.7%) in 2019, in total 7 hives (0.6%). An approximately 180 bp fragment of *COI* gene region was also sequenced for further characterization of samples and to construct a dendrogram using closely related species of *A. woodi* samples. According to the identification studies, all samples (L49/1, L50/1, L147/3, LS7/1, LS38/3, LS58/2, and LS38/2) were identified as *Acarapis woodi* (Table 2). This identification was also supported by the phylogenetic analysis (Fig. 2).

Discussion

Acarapisosis is an important disease of the adult honeybees *Apis mellifera* L. and other *Apis* bees worldwide, and its causative agent is *Acarapis woodi* which is a parasite affecting the

Table 2 Proposed identification of the isolates with their closely related species based on the BLAST searches in the NCBI GenBank database

Isolate	Related species	Coverage (%)	Identity (%)	GenBank number
L49-1	*Acarapis woodi* isolate E2	100	98,31	HF945446.1
	Acarapis woodi	100	98,31	LC512730.1
	Acarapis woodi	100	98,31	KX790788.1
	Acarapis woodi isolate E2, clone 15	100	98,31	HG967529.1
	Acarapis woodi isolate A9	100	98,31	HF945444.1
L50-1	*Acarapis woodi* isolate E2	100	98,30	HF945446.1
	Acarapis woodi	100	98,30	LC512730.1
	Acarapis woodi	100	98,30	KX790788.1
	Acarapis woodi isolate E2, clone 15	100	98,30	HG967529.1
	Acarapis woodi isolate A9	100	98,30	HF945444.1
L147-3	*Acarapis woodi* isolate E2	100	98,31	HF945446.1
	Acarapis woodi isolate E2, clone 15	100	98,31	HG967529.1
	Acarapis woodi isolate 1953, clone 5	100	97,75	HG967550.1
	Acarapis woodi isolate 1431, clone 1	100	97,75	HG967531.1
	Acarapis woodi isolate 1953, clone 2	100	97,73	HG967549.1
LS7-1	*Acarapis woodi* isolate E2	100	98,88	HF945446.1
	Acarapis woodi	100	98,88	LC512730.1
	Acarapis woodi	100	98,88	KX790788.1
	Acarapis woodi isolate E2, clone 15	100	98,88	HG967529.1
	Acarapis woodi isolate A9	100	98,88	HF945444.1
LS38-2	*Acarapis woodi*	100	99,44	LC512730.1
	Acarapis woodi	100	99,44	KX790788.1
	Acarapis woodi isolate A9	100	99,44	HF945444.1
	Acarapis woodi	100	99,44	AB634837.1
	Acarapis woodi isolate B1G5	100	98,88	HQ162656.1
LS38-3	*Acarapis woodi* isolate E2	100	98,31	HF945446.1
	Acarapis woodi	100	98,31	LC512730.1
	Acarapis woodi	100	98,31	KX790788.1
	Acarapis woodi isolate E2, clone 15	100	98,31	HG967529.1
	Acarapis woodi isolate A9	100	98,31	HF945444.1
LS58-2	*Acarapis woodi* isolate E2	100	98,88	HF945446.1
	Acarapis woodi isolate E2, clone 15	100	98,31	HG967529.1
	Acarapis woodi	100	97,75	LC512730.1
	Acarapis woodi	100	97,75	KX790788.1
	Acarapis woodi isolate 1953, clone 5	100	97,75	HG967550.1

health of honeybees worldwide. The prevalence of this mite in Europe is relatively low. In our country, it has been declared that this mite does not exist in Türkiye with limited research. This study is the most comprehensive study on the prevalence of *A. woodi* in Türkiye. In previous studies related to Türkiye, microscopic diagnostic methods were generally used for the diagnosis of *A. woodi*, but no study was performed at molecular level for diagnosis of *A. woodi*. When we look at researches all over the world with respect to the distribution of *A. woodi*, the prevalence of *A. woodi* can differ according to the collection place and season. In the study of Korpela (2002), it was found *A. woodi* prevalence as 9.2% in Finland. Bacandritsos and Saitanis (2004) reported that they found A. woodi at the rate of 5.43% in Greece. Durán et al. (2019) reported that positivity in terms of tracheal acariosis was reported for only 1.68% in their study on the presence of *A. woodi* in 298 beekeeping farms in Chile between 2012 and 2016. In this study, we searched the presence of *A. woodi* in 1,193 hives in 400 beekeepers, the overall prevalence of *A. woodi* was 0.6% in the hives. Also, Otis et al. (1988) reported that the most intense period of *A. woodi* prevalence was between November and February. Since the sampling took place in the spring months in our study, it suggests that one of the reasons for the low prevalence of *A. woodi* may be due to seasonal sampling. Studies on the prevalence of *A. woodi* in Türkiye are very limited. Cakmak et al. (2003) reported that they could not detect trachea mite in honeybees in the Southern Marmara region. Şimşek (2005) detected some parasitic and fungal diseases in honeybee colonies in the Elazig region and reported that *Acarapis woodi* was not encountered between 2002 and 2004. In another study conducted by Yalçınkaya (2008), the presence of *A. woodi* was investigated in adult bee samples from Adana and Hatay regions of Türkiye by dissection and guanine visualization technique. After the research carried out with classical and molecular methods, the mite itself, eggs or nymphs were not found in any of the samples. Beyazıt et al. (2012) reported that they could not detect *A. woodi* in any of 394 beekeeping in the provinces of Muğla, Aydın, Denizli, Manisa, Kütahya and Uşak in the Aegean region of Türkiye. In another study aiming to show the presence of tracheal mite, which is newly recognized and unknown to beekeepers in Türkiye, Öztürk et al. (2016) collected 150 adult bees from 13 provinces (Artvin, Rize, Ordu, Muğla, Aydın, Edirne, Kırklareli, Adana, Antalya, Gaziantep, Hatay, Hakkari, and Van). The presence of tracheal mite was examined by both 2 methods and tracheal mite could not be detected in any sample. However, Keskin et al. (2006) found *A. woodi* in honeybee colonies at the rate of 12.8% using the classical dissection method and 16.3% using the guanine stain method. The guanine method is generally not recommended because it was reported that *A. woodi* and other *Acarapis* spp. cannot be distinguished and low-level infestations cannot be detected using this method (Mozes-Koch and Gerson 1997). There is not any other study reporting the presence of *A. woodi* in Türkiye.

In this study, both microscopic and molecular techniques for the diagnosis of *A. woodi* (PCR using specific primers) was used. While *A. woodi* was not found in microscopic examinations, it was detected according to PCR-based sequence analysis of *COI* gene. During the survey, we realized that beekeepers regularly use acaracides against varroa

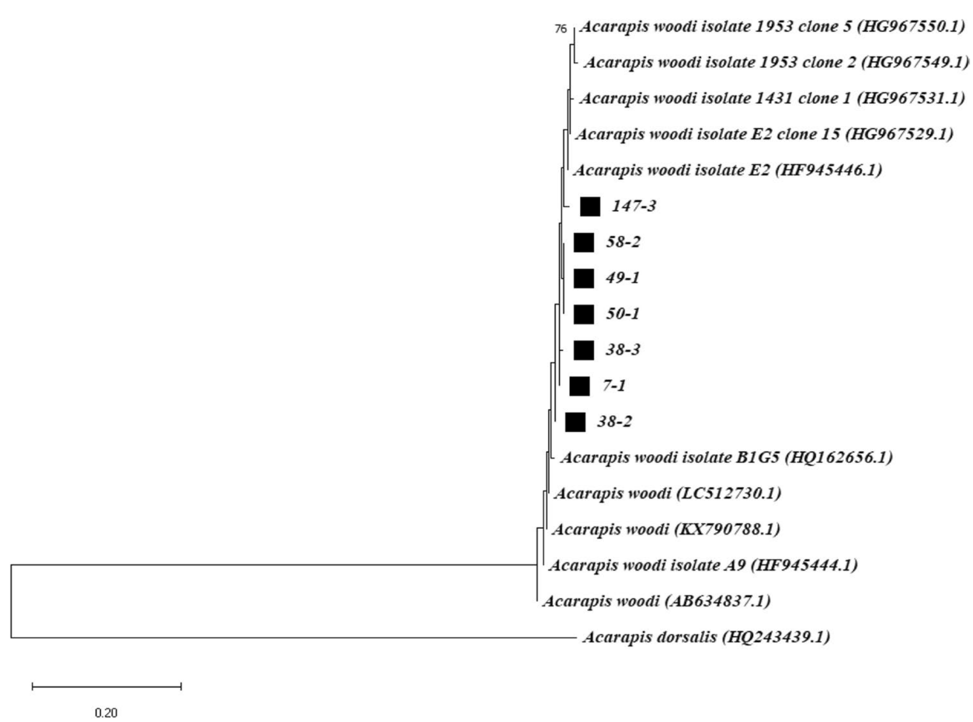

Fig. 2 Pyhlogenetic analysis of the *Acarapis woodi* isolates and their closely related species based on the partial sequence of the *COI* gene. Neighbor-joining analysis with p-distance method was used to construct the dendrogram. Bootstrap values shown next to nodes are based on 1,000 replicates. Bootstrap values C ≥ 70% are labeled. Isolates were indicated with black squares. The scale on the bottom of the dendrogram shows the degree of dissimilarity. *Acarapis dorsalis* was used as an external grup

mite in hives. We know that these acaricides can affect the tracheal mites including *A. woodi* but the body parts and cell debris of *A. woodi* can remain in respiratory systems of bees and these eruptions can be helpful for diagnosis of it by PCR (Garrido-Bailón et al. 2012; Ahn et al. 2015). Therefore, PCR-based methods could be more sensitive for detection of *A. woodi* even after acaricides use.

Conclusions

As a result of this study, it was determined that *A. woodi* is present in bee colonies in Türkiye. In recent years, PCR-based methods have been widely used in the diagnosis of *A. woodi*. In this study, *A. woodi* was not found in microscopic examinations, but *A. woodi* was detected according to the results of the PCR method. When diagnosing *A. woodi*, using both microscopic and molecular methods (PCR method using specific primers) at the same time will provide the most reliable results. In addition, in cases where a large number of colony scans will be made, it is recommended to use the PCR technique, which is a more specific method in the diagnosis of *A. woodi*, since microscopy detection methods are more difficult and take a lot of time.

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Authors contribution RKA, SK, ŞAK and AB designed the research;RKA, SK, ŞÖT, CA, ŞAK, AB and IA: Microscopic analyzes were performed,RKA, SNÇ, GG, BK and MY: PCR analyzes were performed,ES and AS: analyzes were performed.RKA, AS and ES: reviewed the final version of the manuscript.All authors have reviewed and approved the final version of the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval There is no animal or human study requires ethics permission.

Consent to participate Not applicable.

Consent to publish Not applicable.

Conflict of interest No potential conflict of interest was reported by the author(s).

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