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Molecular survey of endosymbiotic bacteria in the honeybee ectoparasite *Varroa destructor* in Türkiye

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ABSTRACT

Varroa destructor is recognized as the predominant ectoparasite affecting Western honey bees (*Apis mellifera* L.) globally, representing a significant threat to the sustainability of bee colonies. The bacterial community of the digestive system and body tissues of *Varroa* mites has been documented in previous studies, however, the diversity and prevalence of detected endosymbiotic bacteria remain limited. In this study, the existence of four commonly found endosymbiotic bacteria including *Wolbachia*, *Cardinium*, *Spiroplasma*, and *Rickettsia* was investigated in various *Varroa* mite populations collected from Turkish apiaries. Almost half of the sampled population was infected with at least one endosymbiotic bacteria. *Wolbachia* endosymbiont was detected as the most prevalent genus, observed in six populations followed by *Cardinium* present in three populations. Furthermore, *Spiroplasma* and *Rickettsia* endosymbionts were each detected in one sample. To our knowledge, this study provides the first molecular characterization of *Cardinium* endosymbionts in V. destructor. The identity of 16S rDNA sequences of *Cardinium* was 98.9% of the sequence of *Cardinium* reported from another mite species, *Brevipalpus papayensis*, in the NCBI database. The study contributes new insights into the endosymbiotic bacterial community of *Varroa* mites. Understanding the diversity and prevalence of endosymbiotic bacteria in *Varroa* mites could facilitate the development of targeted management strategies to control *Varroa* infestations and improve honeybee health.

Türkiye'de bal arısı ektoparaziti Varroa destructor'un endosimbiyotik bakterilerinin moleküler araştırması

ÖZET

Varroa destructor, dünya genelinde bal arılarının (*Apis mellifera* L.) bir ektoparazit olarak kabul edilmekte ve arı kolonilerinin sürdürülebilirliği için ciddi bir tehdit oluşturmaktadır. *Varroa* akarlarının sindirim sistemi ve vücut dokularındaki bakteri topluluğu daha önceki çalışmalarda büyük ölçüde ortaya çıkarılmış olsa da, tespit edilen endosimbiyotik bakterilerin çeşitliliği ve yaygınlığı oldukça sınırlı kalmıştır. Bu çalışmada, Türkiye arıcılık kovanlarından toplanan çeşitli *Varroa* popülasyonlarında yaygın bulunan dört endosimbiyotik bakterinin (*Wolbachia, Cardinium, Spiroplasma* ve *Rickettsia*) varlığı araştırılmıştır. Örneklenen popülasyonların neredeyse yarısı en az bir endosimbiyotik bakteri ile enfekte bulunmuştur. *Wolbachia* endosimbiyotik bakterisi, altı popülasyonda tespit edilerek en yaygın cins olarak kaydedilmiş ve ardından üç popülasyonda bulunan *Cardinium* yer almıştır. Ayrıca, *Spiroplasma* ve Rickettsia endosimbiyotik bakterileri her biri bir örnekte tespit edilmiştir. Bu çalışma, *Cardinium* endosimbiyotik bakterisinin *V. destructor*'de ilk moleküler karakterizasyonunu sunmaktadır. Elde edilen 16S rDNA dizileri, NCBI veritabanında bulunan *Brevipalpus papayensis*'ten rapor edilen *Cardinium* dizisi ile %98.9'u ile benzerlik göstermektedir. Bu çalışma, *Varroa* akarlarında tespit edilen endosimbiyotik bakteri çeşitliliğinin genişlemesine katkı sunmaktadır. *Varroa* akarlarında bulunan endosimbiyotik bakterilerin çeşitliliği ve yaygınlığını anlamak, *Varroa* enfestasyonlarını kontrol etmek ve arı sağlığını iyileştirmek için hedefe yönelik kontrol stratejilerinin geliştirilmesine katkı sağlayacaktır.

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1. Introduction

The primary constituent of honeybee populations is predominantly the species *Apis mellifera* L. (Hymenoptera: Apidae), which holds a global distribution. It has been used to produce honey, wax, and various other products associated with apiculture as well as play a crucial role in the pollination of plant species (1). Türkiye is known as the world's second-largest honey-producing country, with 8 million beehives and a honey yield of 110 thousand tons annually (2). However, the Turkish beekeeping industry currently suffers from considerable losses in honey production that are caused by a multitude of factors.

Varroa destructor Anderson & Trueman (Acari: Varroidae) holds a preeminent status as the principal obligatory ectoparasite affecting the Western honey bee, *Apis mellifera*, on a global scale (3). *Varroa* species directly harm honeybee colonies by consuming hemolymph and fat body tissues, which leads to decreased body weight and reduced honeybee lifespan (1, 4). Hence, effective treatment approaches are required to enhance animal welfare and performance. Among them, tau-fluvalinate, flumethrin, coumaphos, and amitraz are commonly preferred due to their in-hive selectivity (5, 6). However, with inappropriate usage, the beekeeping industry is facing the development of resistance and also the existence of chemical residues in bee products such as honey and beeswax (7, 8).

Arthropods serve as hosts for a multitude of facultative symbiotic bacteria (9). These bacteria can influence the host in commensal, mutualistic, or even parasitic ways, thereby having profound implications for several crucial aspects of the host's nutritional physiology (10), reproduction (11), vector capability (12), or defense mechanisms (13). Given the robust interdependency between symbiont and host, the absence of symbionts could potentially lead to fitness defects (14). In addition, these bacteria can lead to cytoplasmic incompatibility, which was previously suggested as a promising tool for pest control (15). In previous studies focusing on the microbial community of *V. destructor*, various taxonomic groups have been identified as prevalent. Specifically, *Morganella* sp. and *Enterococcus* sp. were found to be the most common taxa, as reported by Hubert et al. (16). Additionally, Enterobacteriaceae were detected in 50–88% of *Varroa* mites in Poland (17). Furthermore, a significant proportion of the sequences retrieved from *V. destructor* samples were attributed to actinomycete bacteria, as indicated by Cornman et al. (18). Notably, certain oxalotrophic bacteria, classified within the Proteobacteria and Actinobacteria phyla, were also identified in *V. destructor* (19). In addition to these findings, endosymbiotic bacteria, namely *Wolbachia* and *Spiroplasma*, have been confirmed to inhabit *V. destructor* (16, 20, 21).

Despite these previous studies, little is known about the endosymbiotic bacterial community of *V. destructor*. This study aims to fill this gap by examining the occurrence of four commonly found endosymbiotic bacteria (*Wolbachia*, *Cardinium*, *Rickettsia*, and *Spiroplasma*) across 23 different *V. destructor* populations in Türkiye.

2. Material and Methods

Mite collection and DNA extraction

The populations of *V. destructor* were sampled from 23 different apiaries using the powdered sugar method in Türkiye in 2022. Mite samples were transported to the laboratory in 90% ethanol for further processing.

To avoid surface contamination in genomic DNA, a surface sterilization procedure was employed prior to the genomic DNA extraction. The sterilization process followed established protocols (22) and aimed to eliminate external contaminants and potential microbial interference. Initially, the mites were subjected to a dual 5-minute wash in a 0.1% (w/v) benzalkonium chloride solution, which was followed by two separate 5-minute rinses in 100% (v/v) ethanol. Subsequently, the cleaned mites were left to air-dry on sterile filter paper. Following the sterilization process, the genomic DNA was isolated from pools of 10 mites using the Qiagen DNeasy Blood & Tissue Kit (Hilden, Germany) according to the manufacturer’s instructions. The extracted DNAs were kept at -20 °C for subsequent analyses.

Screening of bacteria using PCR

The presence of four common symbiotic bacteria across 23 *Varroa* populations was investigated through the application of the conventional polymerase chain reaction (PCR). Specific primers targeting the 16S ribosomal RNA (rRNA) of each bacterium, along with the conditions for PCR reactions, are detailed in Table 1.

Table 1: Primers, fragment sizes, sequences, and annealing temperature of used primers in this study
Table 1: *Çalışmada kullanılan primerler, sekans dizilimleri ve bağlanma sıcaklıkları*

Bacteria	Gene	Fragment size	Primers	Sequence (5–3)	T_A (ºC)	References
Cardinium	16S rDNA	450	CLO_F1 CLO_R1	GGAACCTTACCTGGGCTAGAATGTATT GCCACTGTCTTCAAGCTCTACCAAC	54	(39)
Wolbachia	*wsp*	600	Wsp _F Wsp _R	TGGTCCAATAAGTGATGAAGAAACTAGCTA AAAAATTAAACGCTACTCCAGCTTCTGCAC	58	(40)
Spiroplasma	16S rDNA	450	Spoul-F Spoul-R	GCTTAACTCCAGTTCGCC CCTGTCAATGTTAACCTC	55	(41, 42)
Rickettsia	16S rDNA	800	Rb_F Rb_R	GCTCAGAACGAACGCTATC GAAGGAAAGCATCTCTGC	58	(43)

PCR amplifications were performed in a total volume of 30 µl, comprising 2 µl of mite DNA (ranging between 40–50 ng/µL ng/µL), 1 µl of both the forward and reverse primers, 11 µl of PCR-grade water, and 15 µl of the Takara MasterMix (Takara, Japan), using the TProfessional thermocycler (Biometra, Germany). The nuclease-free water as a negative control, and DNAs originating from *Wolbachia, Cardinium, Rickettsia*, and *Spiroplasma* as a positive control were included in all reaction setups. Each individual PCR reaction was iterated three times to ensure the negativity. The resulting PCR products were subsequently subjected to gel electrophoresis on a 1.5% agarose gel in 0.5x TBE buffer. Visualization of the separated fragments was accomplished following staining with SYBRTM Safe DNA gel stain (Thermo Fisher Scientific, USA), utilizing a UV transilluminator. The PCR products were purified using the HighPrep PCR clean-up system (MagBio Genomics) and were sequenced using the aforementioned primers (Macrogen, Amsterdam, The Netherlands).

The *Cardinium* sequences obtained in the present study and found in other mite species from public GenBank were aligned using MAFFT v7 (23) with "Auto" strategy. A Maximum likelihood (ML) phylogenetic tree based on 16S rRNA sequences belonging to *Cardinium* endosymbionts was constructed with IQ-TREE web server (24) using the K2P+I model (identified to be the best-fit model by ModelFinder; 25) with 1000 ultrafast bootstraps. The resulting phylogenetic tree was visualized and annotated using the Interactive Tree of Life software (iTOL v6) (https://itol.embl.de).

3. Results

A total of 23 DNA samples originating from Varroa mites were subjected to investigation for the presence of four distinct endosymbiotic bacteria (*Wolbachia*, *Cardinium*, *Rickettsia*, and *Spiroplasma*) (Table 2). The initial identification of bacterial presence was conducted through agarose gel analysis, employing a comparison of product sizes according to a positive reference sample. Subsequently, all identified positive samples were sequenced and acquired sequences were submitted to the public GenBank database (accession numbers: *Wolbachia;* OR992605-OR992610, *Canditatus* Cardinium; OR982396-OR982398, *Spiroplasma*; OR982394, *Rickettsia;* OR982395). Upon conducting a BLASTn search using the acquired sequences, notable similarities were found, with >99%, 98.9%, 99.7%, and 99% corresponding to the deposited sequences of *Wolbachia* (KX146861), *Canditatus* Cardinium (MH806395), *Spiroplasma* (CP029202), and *Rickettsia* endosymbiont (MF156623) respectively, within the NCBI database.

Table 2: Presence of endosymbiotic bacteria in sampled *Varroa destructor* populations
Table 2: *Örneklenen Varroa destructor popülasyonlarındaki endosimbiyotik bakteri varlığı*

No	Population	Location	Wolbachia	Cardinium	Spiroplasma	Rickettsia
1	VAR1	Ankara/Ayaş	-	-	-	-
2	VAR2	Ankara/Bala	+	-	-	-
3	VAR3	Ankara/Beypazarı	+	-	-	-
4	VAR4	Ankara/Çankaya	-	-	-	-
5	VAR5	Ankara/Çubuk	+	+	-	-
6	VAR6	Ankara/Güdül	-	-	-	-
7	VAR7	Ankara/Gölbaşı	-	-	-	-
8	VAR8	Ankara/Gölbaşı	+	-	-	-
9	VAR9	Ankara/Gölbaşı	-	-	-	-
10	VAR10	Ankara/Haymana	-	-	-	-
11	VAR11	Ankara/Kalecik	-	-	-	-
12	VAR12	Ankara/Kalecik	-	-	-	-
13	VAR13	Ankara/Kazan	-	-	-	-
14	VAR14	Ankara/Kazan	-	-	-	-
15	VAR15	Ankara/Kızılcahamam	+	-	-	-
16	VAR16	Ankara/Kızılcahamam	-	-	+	-
17	VAR17	Ankara/Kızılcahamam	-	-	-	-
18	VAR18	Ankara/Nallıhan	+	-	-	-
19	VAR19	Ankara/Polatlı	-	-	-	-
20	VAR20	Hatay	-	+	-	-
21	VAR21	Muğla	-	-	-	-
22	VAR22	Ordu	-	-	-	+
23	VAR23	Zonguldak	-	+	-	-

Among the 23 adult *Varroa* populations, *Wolbachia* emerged as the prevailing genus, being identified in six populations. *Cardinium* was detected in three populations, representing the first documented in *V. destructor*. The phylogenetic analyses revealed that the obtained *Cardinium* sequences clustered together with the closest sequence belonging to *Brevipalpus papayensis* (Figure 1). Moreover, *Rickettsia* and *Spiroplasma* endosymbionts were each encountered in one sample. Each bacterium is isolated within separate populations, while a population (VAR5) demonstrates the co-presence of both *Wolbachia* and *Cardinium*.

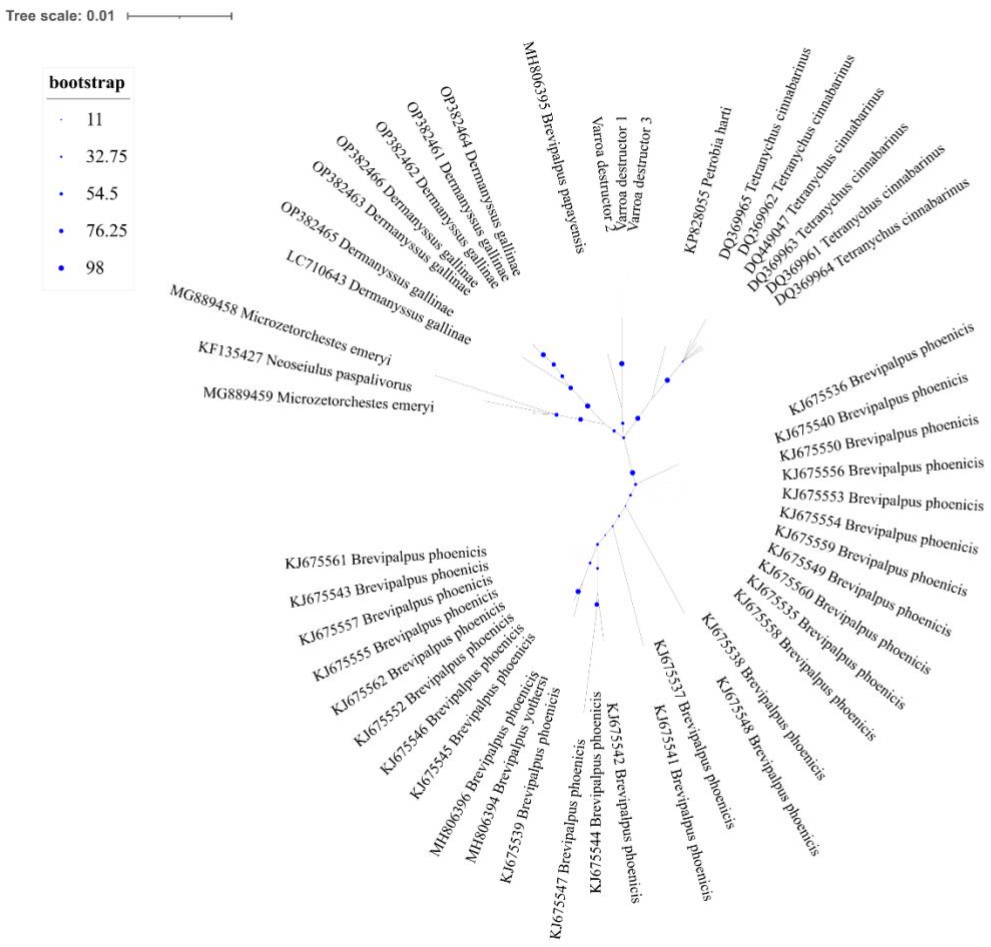

Figure 1: A phylogenetic tree of *Cardinium* sequences belonging to mite species. The sequences obtained in the present study are shown in bold.

Şekil 1: *Akar türlerine ait Cardinium dizilerinin filogenetik ağacı. Bu çalışmada elde edilen diziler koyu renkle gösterilmiştir.*

4. Discussion and Conclusion

The bacterial community of the digestive system and body tissues of *Varroa* mites had been determined in previous studies (16, 26, 27). In many cases, the bacterial diversity of the microbiome of *Varroa* samples was determined two times less when compared to the honeybee sample. The lower bacterial diversity observed in *Varroa* mites may be explained by the transmission of bacteria from honeybees to mites rather than vice versa (27). Furthermore, this symbiotic bacteria community had lower diversity in *Varroa* mites than Honeybees. Although *Wolbachia* and *Spiroplasma* were identified within *V. destructor*, *Cardinium* and *Rickettsia* have not yet been observed (16, 20, 21). This study provides the occurrence of these two endosymbiotic bacteria in *Varroa* mites.

Wolbachia is a widely distributed symbiotic bacteria in terrestrial arthropods, with approximately 20–70% of insect species, marking it as one of the most frequently encountered genera of endosymbiont bacteria discovered to date (28). *Wolbachia* symbionts primarily result in reproductive anomalies such as cytoplasmic incompatibility, induction of parthenogenesis, and feminization (29). While *Wolbachia* infections have been extensively studied in certain insect species, their presence in *Varroa* mites seemed to be less explored at that time and it has been found in *V. destructor* recently (20). Additionally, the vertical transmission of endosymbiotic bacteria occurs more frequently;

however, horizontal transmission of *Wolbachia* between honey bees and *Varroa* mites has been documented (20). In this study, we detected that six (26%) out of 23 adult *Varroa* populations were infected with *Wolbachia* endosymbiont with the identity of 99% in the NCBI database.

Candidatus Cardinium infections have been reported in a variety of arthropod species, with over 50% of chelicerates known to harbor this bacterium (27). Unexpectedly, despite its widespread occurrence, *Candidatus* Cardinium has not been documented in *Varroa* populations. Similar to *Wolbachia*, *Cardinium* is also a symbiotic bacterium capable of influencing reproductive systems (30). In the present study, the *Cardinium* was detected in three out of 23 *Varroa* populations, showing 99.8% similarity with deposited sequences of *Cardinium* obtained from *Brevipalpus papayensis* (MH806395). To our knowledge, this is the first report of *Cardinium* infection in *V. destructor*. Moreover, one of these populations was also infected with *Wolbachia*, consistent with the findings of previous studies by Zchori-Fein and Perlman (30), and Koç et al. (22) which documented co-infection in the mite species *Metaseiulus occidentalis* and *Dermanyssus gallinae* respectively.

Rickettsia are classified as maternally inherited Alphaproteobacteria, estimated to be found in approximately one of four terrestrial arthropods (27). In insect populations, *Rickettsia* spp. have been known to modify reproduction and fecundity (31, 32). An unidentified rickettsia-like organism was first reported in the rectum of *Varroa* by analyzing histological sections using transmission microscopy (33). In addition, Diplorickettsia (an obligatory intracellular parasite with a close relation to the genus *Rickettsia*) was found in *Varroa* (27). In this study, we found only one *Rickettsia* positive sample, and BLAST analysis showed that the obtained *Rickettsia* sequence had 99% similarity with *Rickettsia* endosymbiont of *Chrysoperla pallida* (MF156623) (34). The study represents the first molecular characterization of *Rickettsia* endosymbiont in *V. destructor* except for the above-mentioned cases. Some *Rickettsia* endosymbionts have mutualistic relationships with their hosts and may provide benefits. Following the first detection of *Rickettsia* endosymbionts in *Varroa* mites, further investigations are needed to delve into their biological roles in greater detail.

Spiroplasma is a genus of bacteria known to infect various arthropods, including insects and horizontal transmission between different insect species has been documented (35). Honeybees also serve as reservoirs for *Spiroplasma*, and these bacteria are currently considered occasional pathogens (36). A potential honeybee pathogen *Spiroplasma* was exclusively found in *V. destructor* sampled from winter debris (16). In this study, the use of specific primers in PCR confirmed the presence of *Spiroplasma* in a single sample with a 99.7% similarity to *S. melliferum* (CP029202). *Spiroplasma melliferum* has been identified as the potential cause of neurological disorders in honeybees but is also recognized as facultative (secondary) symbionts, with 33 and 54% prevalence in colonies in the USA and Brazil respectively (36). It has been reported that the prevalence of *S. melliferum* in the samples from the honeybee colony increased from 5% in February to 68% in May and subsequently declined to 25% in June and 22% in July (37). Supportingly, *Spiroplasmosis* was known as "May disease" in honeybees in southwestern France (38). There is a chance of encountering a limited number of positive cases owing to the duration of our sample collection.

The endosymbiotic bacteria in arthropods have been investigated for many years because of their crucial role in host biology. However, studies specifically regarding the endosymbionts of *Varroa* mites have not been extensively conducted. These studies are also important to clarify the horizontal transmission of endosymbiotic bacteria between honeybees and mites as they are both arthropods. This study demonstrated the presence of *Wolbachia*, *Cardinium*, *Rickettsia*, and *Spiroplasma* endosymbionts in adult female *V. destructor* with the first molecular identification of *Cardinium* and *Rickettsia* symbionts.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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Authors' Contributions

Motivation / Concept: Nafiye KOÇ İNAK
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Control/Supervision: Nafiye KOÇ İNAK
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Analysis and / or Interpretation: Nafiye KOÇ İNAK
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Ethical approval

An ethical statement was received from the authors that the data, information and documents presented in this article were obtained within the framework of academic and ethical rules and that all information, documents, evaluations and results were presented in accordance with scientific ethics and moral rules

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