A Preliminary Molecular Study on the Haplotypes of *Varroa destructor* in Turkey

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ABSTRACT

Varroosis is one of the most important diseases of honeybees. Four species, *Varroa jacobsoni, Varroa destructor, Varroa underwoodi* and *Varroa rindereri* are present in the genus of *Varroa*, the most prevalent one being *V. destructor*. *V. destructor* has different haplotypes which have different pathogenic effects on the honeybees. Korean (K) haplotype is more prevalent and pathogenic than the Japanese (J) haplotype. The aim of this study was to determine the haplotypes of *V. destructor* in Turkey. Female mites from ten different provinces of Turkey were examined. After DNA extraction partial sequences of mitochondrial *COX1* gene was amplified with PCR and subsequently sequenced. At the conclusion of molecular and phylogenetic analysis, we determined that all isolates were identical to each other and corresponded with the K haplotype of *V. destructor*, with 100% identity. This study supports preliminary data regarding the haplotypes of *V. destructor* from Ankara, Cankiri, Corum, Elazig, Kirsehir, Kirikkale, Hakkari, Hatay, Samsun and Tekirdag provinces of Turkey. Considering that Turkey is a bridge between Asia, Europe and Africa, more detailed studies should be conducted for the determination of different haplotypes or variants of *V. destructor*.

Key words: *Varroa destructor*; Haplotype; Phylogenetic; Turkey.

INTRODUCTION

Beekeeping is one of the oldest professions in the human history and has an important place in agriculture. As a result of beekeeping activities, valuable products such as honey, beeswax, pollen, propolis, royal jelly, bee venom, apilarnil and bee bread are produced. These invaluable products are widely used for nutrition, medical purposes and in industry (1).

Bees are the most important pollinators, and the global flora is strictly related to this activity. In this context, bees are one of the most fundamental elements for the continuity of plant and animal production as well as for the formation of a healthy ecosystem (1). Breeding errors, environmental pollution, global climate changes, improper and uncontrolled use of pesticides and diseases, cause production and colony losses in beekeeping (2).

Bee diseases may be grouped as bacterial, viral, fungal, and parasitic (protozoan, helminth and arthropods) (3). Varroosis is the most important parasitic disease of honeybees with different haplotypes displaying different pathogenic effects on the honeybees. The disease was first detected in the Indian bees (*Apis cerana*) on the island of Java in 1904, and its causative mite was named as *Varroa jacobsoni*. Then it spread from Southeast Asia worldwide, with mobile beekeeping, escaped swarms and bee trade among countries (4). The mite, whose

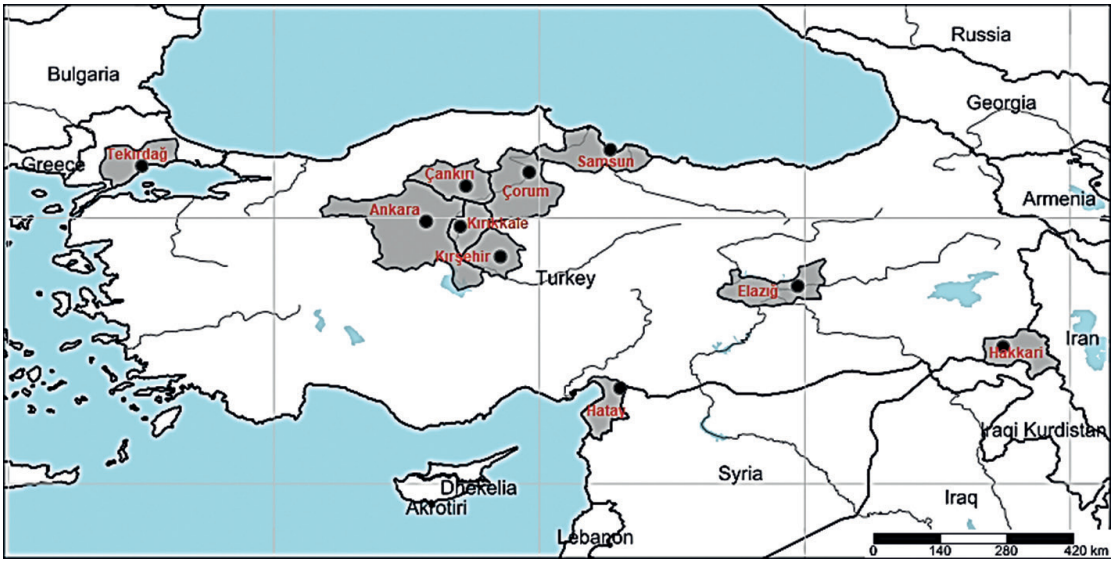

Figure 1: Map of Turkey showing study locations where the *V. destructor* isolates were collected (https://www.simplemappr.net/).

main host is the Indian bee (*A. cerana*), was first detected in the European honeybee (*Apis mellifera*) in China in 1960. In the following years, the disease spread to Europe and entered the Thrace region of Turkey through Bulgaria in 1976 and spread rapidly by means of uncontrolled mobile beekeeping. By 1983, the disease was detected in almost all provinces of Turkey (1-3).

There are four species in the *Varroa* genus: *Varroa jacobsoni, Varroa destructor, Varroa underwoodi* and *Varroa rindereri*. Among these, *V.destructor* is the most common species in the world. *V. jacobsoni* is seen in Southeast Asia-Java Islands, *V. underwoodi* in Nepal-South Korea and *V. rindereri* in Asia (1, 5).

Previous studies revealed that *V. destructor* is the etiologic agent of the varroosis in Turkey (6, 7). It is known that *V. destructor* has haplotypes with different pathogenic effects on honeybees in various parts of the world (8). Among these haplotypes, it has been stated that Korean (K) and Japanese (J) haplotypes parasitize *Apis cerana* in Asia, and the haplotype K, parasitizes *A. mellifera* worldwide. Korean haplotype is more pathogenic to *A. mellifera* than J haplotypes due to its higher reproductive capacity (9, 10).

Methods such as microsatellite analysis, RAPD-PCR, PCR-RFLP and DNA sequencing were used for the detection of different haplotypes of *V. destructor* (9, 11-13). The number of studies on the subject in Turkey is until now quite limited (6, 14, 15).

The aim of this study was to support preliminary data concerning the haplotypes of *V. destructor* from a wide geographic area of Turkey.

MATERIALS AND METHODS

Museum samples obtained from Ankara, Cankırı, Corum, Kirsehir, Kirikkale, Hakkari, Hatay, Samsun and Tekirdag provinces of Turkey were used in this study (Figure 1).

DNA extractions were carried out with Quick-DNA Tissue/Insect Kit (Zymo Research, USA) according to the manufacturer's protocol. Samples were washed with PBS with five times before the DNA extraction. No success was achieved in extraction from a single mite because the parasites remained in the alcohol for a prolonged period of time. Ten pools, each containing three parasitic mites, were prepared and then extraction was carried out again. COXF (5'GG(A/G)GG(A/T)GA(C/T)CC(A/T)ATT(C/T)T(A/T)TATCAAC3') and COXR (5'GG(A/T)GACCTGT(A/TA(A/T)AATAGCAAATAC3') primer pairs were used for the amplification (2720 Thermal Cycler, Applied Biosystems) of partial sequences of mitochondrial COX-I gene (16).

PCR was carried out in a final volume of 50 µL, containing 30.75 µL molecular grade sterile water (Vivantis, USA), 5 µL 10X Taq buffer (including 20 mM $MgCl_2$), 5 µL (100 µm) diluted dNTP mix (1 mM) (Thermo Scientific, DreamTaq, UK), 2 µL of each primer (40 ρmol)

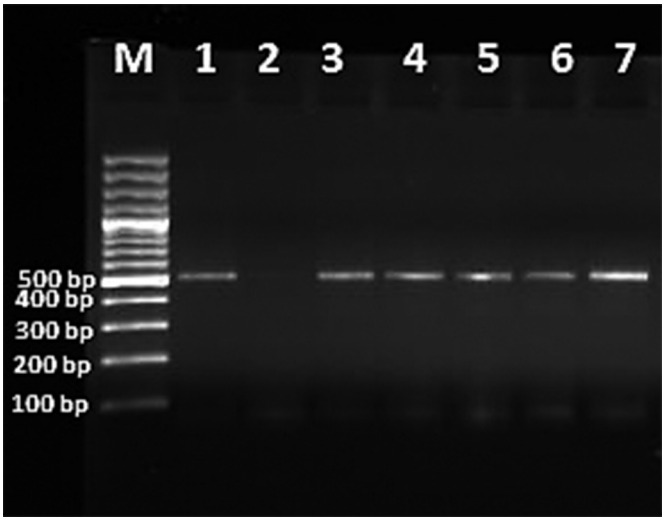

Figure 2. Agarose gel image of partial Mt-*COX1*-PCR products,
M: DNA ladder, **1:** Positive control, **2:** Negative control, **3-7:** *Varroa* sp. positive museum samples.

(Alpha DNA, Qebec), 5 µL of template DNA, and 0.25 µL of TaqDNA polymerase (1.25 IU) (Thermo Scientific, DreamTaq, UK). The PCR conditions were: 5 min at 94˚C (initial denaturation), 35 cycles of 1 min at 94˚C, 1 min at 50˚C and 1 min at 72˚C and finally 5 min at 72˚C (final extension).

The PCR products were separated on agarose gels (1.5%), stained with ethidium bromide (Vivantis, USA) and visualized and photographed (Kodak, Gel Logic 112 imaging system, USA) on a UV transilluminator (Bioview, Israel) (Figure 2). Obtained amplicons were sequenced bidirectionally (Medsantek, Istanbul, Turkey).

Sequences were edited with Geneious Prime 2021.0.3 (https://www.geneious.com) software and aligned with the Clustal Omega Program (http://www.clustal.org/omega/). Sequence analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov). Before the phylogenetic analysis, best substitution models (HKY+G for Bayesian analysis and GTR+G for Maximum Likelihood) were determined by using jModelTest (17). MrBayes 3.2.6 (18) and PhyML software (19) were used for the construction of Bayesian and Maximum Likelihood phylogenetic trees. Phylogenetic trees in Newick format were graphed using the FigTree v1.4.4 program (Institute of Evolutionary Biology, University of Edinburgh, UK). *In silico* restriction (RFLP) analysis was performed by using Geneious Prime 2021.0.3 software (https://www.geneious.com).

RESULTS

Approximately 550 bp region of Mt-*COX1* gene was amplified at the end of PCR reactions (Figure 2).

After editing of obtained DNA sequences, 477 base paired regions were deposited to GenBank under the accession numbers of MW729721-MW729730 (n: 10). Sequence results corresponded to K haplotype of *V. destructor* (AF106899, GQ379056, KU196785, KU196784) from GenBank and all our isolates were 100% identical to each other.

Results of the *in silico* restriction analysis demonstrated that sequences of *Varroa underwoodi*, Korean (K) haplotype and Turkish isolates of *V. destructor* had one restriction site for *XhoI*, *V. rindereri. V. jacobsoni* had one restriction site for *SacI*, while J haplotypes of *V.destructor* had two restriction sites for *XhoI* and *SacI* enzymes (Figure 3).

The results of both phylogenetic trees (Bayesian and Maximum Likelihood) were compatible with each other, and the Turkish isolates were in the same clade with K haplotypes (Figure 4, 5).

DISCUSSION

Different molecular diagnostic techniques such as microsatellite analysis, RAPD-PCR, PCR-RFLP and DNA sequenc-

Figure 3. *In silico* restriction profiles of *Varroa* species.

ing were used for the detection of different haplotypes of *V.destructor* (9, 11-13).

In PCR-RFLP method, partial sequence of mitochondrial COX1 gene was amplified and digested with *SacI* and *XhoI* enzymes. *XhoI* digests both J and K haplotypes, while J haplotype is digested only with *Sac I* enzyme (13-16). In our *in silico* analysis, we found that there was only one restriction site for *XhoI* in Turkish isolates (Figure 3), which supported the presence of the K haplotype.

In studies conducted to determine different haplotypes of *V. destructor*, it has been observed that the genetic variation was very low (10,13). A microsatellite analysis of 11 loci of 45 mite populations from 17 countries showed the heterozygosity rate is less than 1.3% (10). This situation was probably associated with the reproductive strategy of the *Varroa* species. Fertilized female mites enter the broods just before sealing and lays about 4-6 eggs. Male mites emerge first form the eggs and then fertilize the females. Females deposit the sperm and do not need further fertilization. All siblings coming from a founder female carry the same genome (20, 21). According to different researchers, sex is determined by arrhenotoky (20) or pseudo-arrhenotoky systems in *Varroa* mites (21), hence, all males have the same genome as their mothers.

In our study, all isolates from different parts of Turkey were identical to each other and had 100% identity with GenBank results of K haplotypes. We detected two substitutions in the aligned sequences (at positions 140 and 284) of the J and K haplotypes. At these positions, J haplotype has adenine and thymine, while K haplotypes and Turkish isolates have guanine and cytosine bases, respectively. We accepted these substitutions as silent mutations as there were no differences at the amino acid level.

Two phylogenetic trees were obtained with Maximum Likelihood and Bayesian methods. Both trees were compatible with each other and the Turkish isolates were found to be in the same clade with K haplotypes (Figure 4 and 5).

To the best of our knowledge, there are only three studies concerning the determination of different haplotypes of *V. destructor* in Turkey using DNA sequencing and PCR-RFLP methods (6, 14, 15). According to these researchers, K haplotypes of *V. destructor* were detected in Kastamonu, Eregli, Sinop, Samsun, Ordu, Gumushane, Bayburt (6), Aydın and Siirt (14, 15) provinces of Turkey. However, the sequence data regarding the K haplotypes were stated to be found in these studies could not be detected in the GenBank. Therefore, these studies may be sufficient to demonstrate the presence of the K haplotype but insufficient to provide phylogenetic data due to the lack of sequence information. In this study, we determined K haplotypes and deposited the sequences in the GenBank for further research.

Before 1976, the presence of *Varroa* mites in honeybee colonies was unknown in Turkey. After its appearance, parasites spread quickly and by the year 1983, the disease were detected in almost all provinces of Turkey and the consequences were devastating for beekeepers and the Turkish economy (1). From 1976 to 2004, these mites were thought to be *V. jacobsoni*, however, with two studies conducted after 2004, it was found that the parasite was *V. destructor* (6, 7).

The current study provides further evidence that K hap-

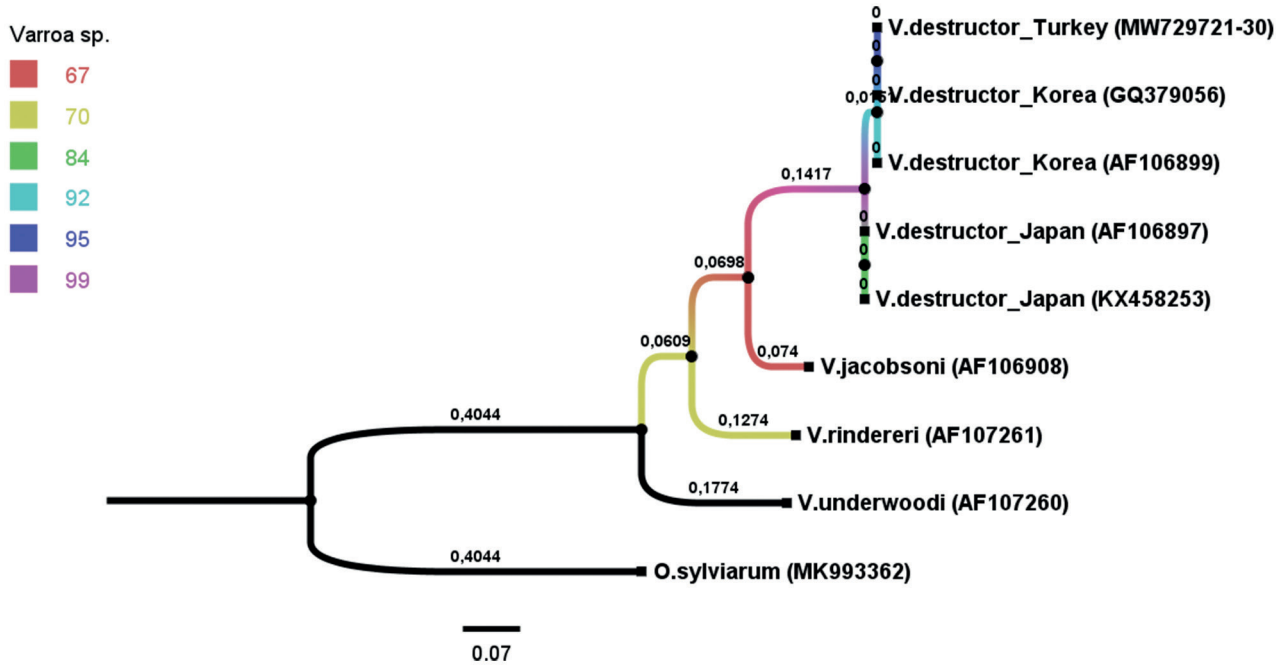

Figure 4: Maximum Likelihood phylogenetic tree based on the partial sequences of *COX1* gene, showed the relationships between the Turkish isolates and *Varroa* species/haplotypes. GenBank accession numbers are listed in parenthesis near the taxon names. *Ornithonyssus sylviarum* (MK993362) was used as an outgroup. Colors of branches represents the bootstrap support values (1000 replications).

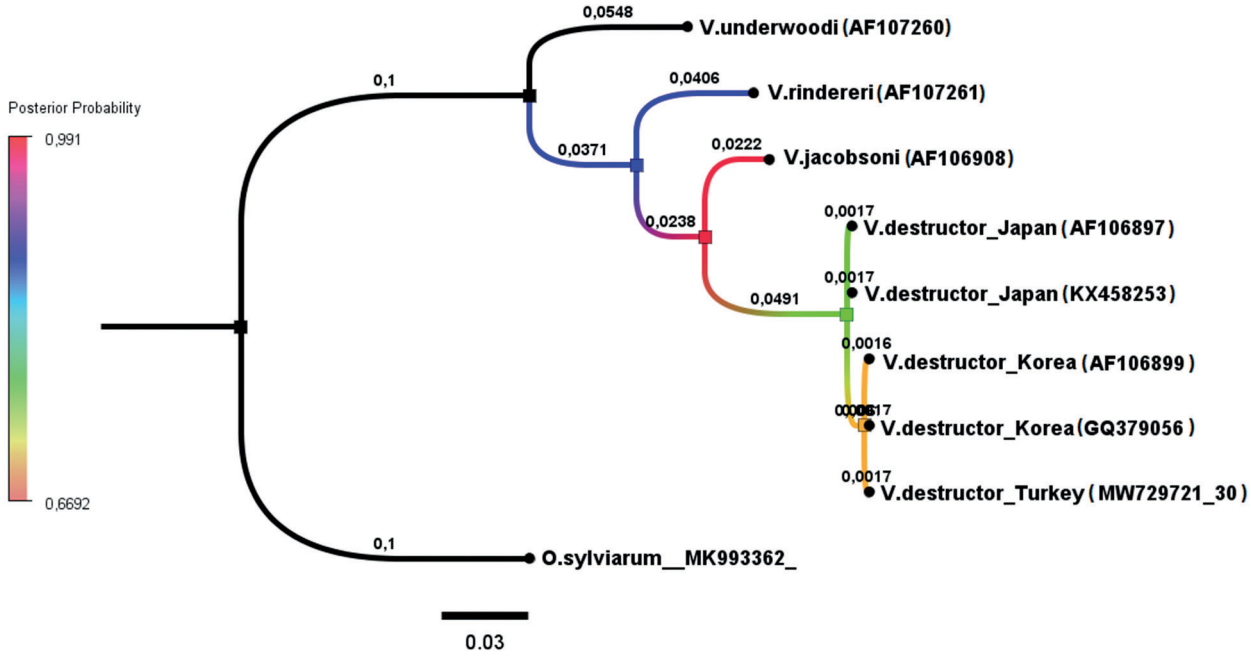

Figure 5: Bayesian phylogenetic tree based on the partial sequences of *COX1* gene, shows the relationships between the Turkish isolates and *Varroa* species/haplotypes. GenBank accession numbers are listed in parenthesis near the taxon names. *O. sylviarum* (MK993362) was used as an outgroup. Colors of branches represents posterior probability values (0-1).

lotype of *V. destructor* is present in Turkey. It is possible that different selection pressures (different disease control methods, differences in geographic and climatic factors, etc.) can produce different variants. Considering that Turkey serves as a bridge between Asian, European and African continents, more detailed studies should be conducted with different mitochondrial and nuclear markers for the determination of different haplotypes.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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