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Molecular detection and phylogenetic analysis of Sacbrood virus in the Republic of Türkiye

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ABSTRACT

Sacbrood virus (SBV), infecting both larva and adult honeybees, is one of the most common honeybee viruses encountered worldwide. This study aimed to investigate the prevalence and genetic diversity of SBV in honeybees, and to determine the role of *Varroa destructor* (*V. destructor*) in the transmission of this virus. Adult honeybee samples were collected from randomly selected apiaries (n=62) in the Hatay Province in Türkiye. The presence of the *V. destructor* was investigated using a stereo microscope, whereas one step real time RT-PCR method was used to detect SBV in honeybees and *V. destructor* samples. It was determined that 8.1% (5/62) of the apiaries were infected with SBV. Furthermore, *V. destructor* was detected in 17 (27.4%) apiaries. However, the presence of SBV could not be detected in *V. destructor* samples. The results of the phylogenetic analysis, based on the SBV polyprotein gene, indicated that the isolates detected in this study belonged to the Türkiye genotype, displaying a separate cluster from the European-South American, Korean and Asian genotypes. This result suggests that genetic differences between SBV isolates vary depending on geographical distribution. Thus, further research is required to determine the SBV genotypes in Türkiye, and to understand the role of *V. destructor* in SBV transmission.

Key words: honeybee; Sacbrood virus; *Varroa destructor*; genetic characterization; Türkiye

Introduction

Honeybees are a significant component of the natural ecosystem as they contribute to increasing biodiversity and agricultural production (GALLAI et al., 2008). However, there has been a decrease in the honeybee population worldwide caused by the colony collapses in recent years (TENTCHEVA et al., 2004; TLAK GAJGER et al., 2014a; TRUONG et al., 2023). Climate change, exposure to acaricides, *Varroa destructor* (*V. destructor*) and infection by pathogenic microorganisms are reported as causes of colony collapses (BAILEY and PERRY, 2001; TENTCHEVA et al., 2004; LE CONTE et al., 2010; RIBIÈRE et al., 2010; CRESSWELL et al., 2012). Infection with pathogenic microorganisms, in particular viruses, causes significant losses in beehives (TENTCHEVA et al., 2004; TLAK

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GAJGER et al., 2010; TLAK GAJGER et al., 2014a; TLAK GAJGER et al., 2014b; TRUONG et al., 2023).

Sacbrood virus (SBV) is one of the most common viruses infecting honeybees worldwide (TANTILLO et al., 2015). SBV is a member of the *Iflavirus* genus of the *Iflaviridae* family, and has a single positive strand RNA genome with positive polarity (VALLES et al., 2017). The capsid proteins of the virus (VP1, VP2, and VP3) have functions in determining viral host specificity and tissue tropism (PROCHÁZKOVÁ et al., 2018).

SBV infection can be detected in both larvae and adult honeybees (TANTILLO et al., 2015). While the colour of infected larvae changes from white to yellow, no obvious clinical sign may be observed in adult honeybees, but they may have a decreased lifespan (WEI et al., 2022). It has been reported that the prevalence of SBV in colonies infested with the *V. destructor* mite is significantly higher compared to colonies not exposed to mite infestation (CHANTAWANNAKUL et al., 2006; MONDET et al., 2014).

Türkiye is one of the world's largest honey producers (AGRICULTURAL ECONOMIC AND POLICY DEVELOPMENT INSTITUTE, 2021). However, honey production per colony is quite low compared to other countries (FAOSTAT, 2021). There is no monitoring programme implemented to track honeybee losses in Türkiye. Therefore, the reasons for honeybee losses are not clearly known. Available information is limited about the prevalence and genetic variations of SBV, and role of *V. destructor* in the transmission of SBV in Türkiye. Therefore, this study aimed to investigate the prevalence and genetic diversity of SBV in honeybees, and to determine the role of *V. destructor* in the transmission of this virus.

Materials and methods

Sample collection. This study was carried out in Hatay Province in Türkiye between March 2022 and April 2023 (Fig. 1). Hatay is one of the important regions for honey production in Türkiye. In this region, migratory beekeeping is most common. The native honeybee race of the region (Hatay) and the Caucasian Hybrid are the common honeybee races. For the purposes of this study, two-stage sampling was used to select the apiaries. First, the number of apiaries to be sampled was determined using the method specified by THRUSFIELD (2007). It was determined that 62 apiaries should be sampled with a 90% confidence interval, 10% margin of error and an expected prevalence of 50% at an apiary level. The list of apiaries was obtained from Hatay Province Beekeepers' Association. Sixty two apiaries were randomly selected from the list using the randomization tool in Microsoft Excel software (Microsoft Corporation, USA), and contact established with the owners of the selected apiaries. The owners of these apiaries agreed to participate in this study. A total of 100 adult worker honeybee samples were collected from randomly selected hives within each apiary. The collected honeybees were stored on ice and immediately transferred to the laboratory and kept frozen at -85ºC until analysis.

Questionnaire survey. A semi-structured survey was used with the owners to collect data on their honeybee apiaries. The survey included questions to collect data on the type of beekeeping practised (migratory or stationary beekeeping), the honeybee race raised (domestic or foreign honeybee race), the number of hives (5-20, 21-50, 51-100, 101-150 and ≥151), the use of the same beekeeping equipment (yes or no) and data on the use of drugs against *V. destructor* (yes or no).

Investigation of the presence of the V. destructor mite. The presence of the *V. destructor* mite in live honeybee samples was investigated morphologically using a stereo microscope (WOAH, 2021). *V. destructor* mites found in the morphological examination were separated in relation to the apiaries where they were detected.

RNA extraction. Pools consisting of 100 adult honeybees from each apiary were placed in sterile falcon tubes containing PBS (pH 7.4). Furthermore, *V. destructor* samples (pools varying in mite number per each apiary) were used for RNA extraction. Samples were crushed using the TissueRuptor device (Qiagen, Hilden, Germany). The homogenates obtained were centrifuged for 30 minutes at 5000 rpm and 4°C, and 200 µl of

Fig. 1. Geographic location of Hatay Province (orange star symbol) where the study was conducted

supernatant was taken for RNA extraction. Total nucleic acid extraction was performed using a commercial kit (High Pure Viral Nucleic Acid Kit, Roche, Germany) in accordance with the manufacturer's instructions. The extracts were then stored at -20°C until analysis.

One-Step real-time RT-qPCR analysis for detection of SBV. Probe and primer pairs, targeting the 70 bp region identified by BLANCHARD et al. (2014), located in the N-terminal part of the polyprotein domain, were used for detecting the SBV virus. The PCR master mix was prepared using a commercial kit (AgPath-ID One-Step RT-PCR, ThermoFisher Scientific, MA, USA). The prepared mix contained 320 nM of each primer, 200 nM of probe and 5µl of extracted RNA. Real time RT-PCR analysis was performed using a LightCycler 2.0 real time PCR device (Roche Applied Science, Indianapolis, USA). Amplification comprised reverse transcription for 10 minutes at 45°C, initial PCR activation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. Nuclease-free water was used as a negative control in all analyses.

RT-PCR amplification, sequencing and phylogenetic analysis. Samples found to be SBV positive at the end of the one-step real time RT-PCR analyses were analysed again with the one-step RT-PCR method using primer pairs, as reported by SGUAZZA et al. (2013). A 50 µl master mix was prepared containing 400 nM of each primer and 5 µl of extracted RNA. The amplification conditions used were: reverse transcription at 60°C for 15 minutes, initial PCR activation at 98°C for 30 seconds, followed by 20 seconds at 95°C, 20 seconds at 56°C, 45 cycles of 30 seconds at 72°C, and a final extension step of 10 minutes at 72°C. The PCR products were run on 1.5% agarose gel electrophoresis. The PCR products were then purified from the gel using a commercial purification kit (HibriGen Gel Extraction Kit, Kocaeli, Türkiye), and sequence analysis was performed in the laboratory to which services were outsourced (BM Laboratory, Ankara, Türkiye). The data obtained as a result of forward and reverse sequence analysis were evaluated using the Bioedit program (version 7.0.5.3) and sequence data were compared with other virus isolates available in GenBank. Phylogenetic analysis was carried out with the sequence data obtained in the study and the sequence data obtained from GenBank. The phylogenetic tree was constructed with the MEGA program (version 11.0) using the maximum-likelihood method with the Kimura 2-parameter model, and a bootstrap value of 1.000.

Statistical analysis. Fisher's exact test was used to compare the data collected statistically. A level of $P<0.05$ was considered statistically significant. SPPS software (version 22, IBM, Armonk, NY, USA) was used for statistical analysis.

Results

Overview of the selected apiaries. Of the 62 sampled apiaries, 16 (25.8%) were engaged in stationary beekeeping and 46 (74.2%) were engaged in migratory beekeeping. While the percentage of apiaries that raised the local honeybee race (Hatay) was 27.4% (17/62), the percentage of those where raised Caucasian hybrid honeybee race was 72.6% (45/62). The number of hives in 7 of the sampled apiaries was 50-100, in 16 of the sampled apiaries it was 100-150, and 150 and above in 39 apiaries. The same beekeeping equipment was used in 82.3% of the sampled apiaries. The owners of all sampled apiaries reported that they were using drugs contain amitraz, which is a formamide exhibiting both acaricidal and insecticidal activity, against *V. destructor.*

Frequencies of V. destructor. In this study, a total of 18 apiaries were sampled in 2022, whereas 44 apiaries were sampled in 2023. As a result of morphological examination, *V. destructor* was detected in 4 of the 18 apiaries, and 13 of the 44 apiaries. There was no significant difference between *V. destructor* detection rate in 2022 and 2023 (P=0.76).

One-Step real-time RT-qPCR results for honeybees. SBV specific RNA was determined in honeybee samples from 3 (16.7%) of the 18 apiaries and 2 of the 44 apiaries in 2022 and 2023, respectively (P=0.14) (Table 1). Out of the 5 SBV-positive apiaries, 3 apiaries were also positive for *V. destructor*. SBV positivity was not significantly different in the presence of *V. destructor* in the sampled apiaries (P=0.12).

Two of the five apiaries where SBV was detected were raising native breeds (Hatay), and 3 were raising Caucasian hybrid honeybee races. No statistically significant difference was found between races in terms of SBV positivity (P=0.61). Furthermore, two of the five apiaries where SBV was detected were engaged in stationary beekeeping, and three were engaged in migratory beekeeping. No statistically significant difference could be found between stationary and migratory beekeeping (P=0.60). All apiaries where SBV was detected were using same beekeeping equipment.

Detection of SBV in V. destructor samples. In this study, SBV-specific RNA was not detected in the *V. destructor* samples.

Genetic variability and phylogenetic analysis of SBV isolates. Sequence analysis of the SBV polyprotein gene revealed that the nucleotide homology between the five isolates in this study ranged between 99.6% and 100%, whereas they had 88.1% - 100% nucleotide homology with other SBV isolates from different countries. The lowest nucleotide homology was found to be with South Korean isolates (HQ916834, HQ916828, HQ916830 and HQ916836) whereas the highest homology was with Turkish isolates (MH251272 and MT467561).

Table. 1. Number of investigated apiaries and SBV positive apiaries in the various districts of Hatay Province

Name of district	No. of sampled apiaries	No. of SBV positive apiaries
Antakya	14	1
Arsuz	7	-
Dörtyol	30	1
Kırıkhan	4	2
Samandağ	7	1

It was determined that the isolates identified in this study had 100% amino acid homology between themselves, whereas the similarities with SBV isolates from different regions varied between 92.5% and 100%.

Two strains, represented by two and three isolates each, were recorded in the GenBank database under accession numbers OQ736750 and OQ736751.

A phylogenetic tree was constructed on the basis of the nucleotide sequences of the 244 bp polyprotein gene of SBV. The sequences in this study that were 100% identical were not included in that analysis. SBV isolates identified in this study and the SBV isolates previously identified in Türkiye were determined to belong to the Türkiye genotype, displaying a separate cluster from the European-South American, Korean and Asian genotypes (Fig. 2).

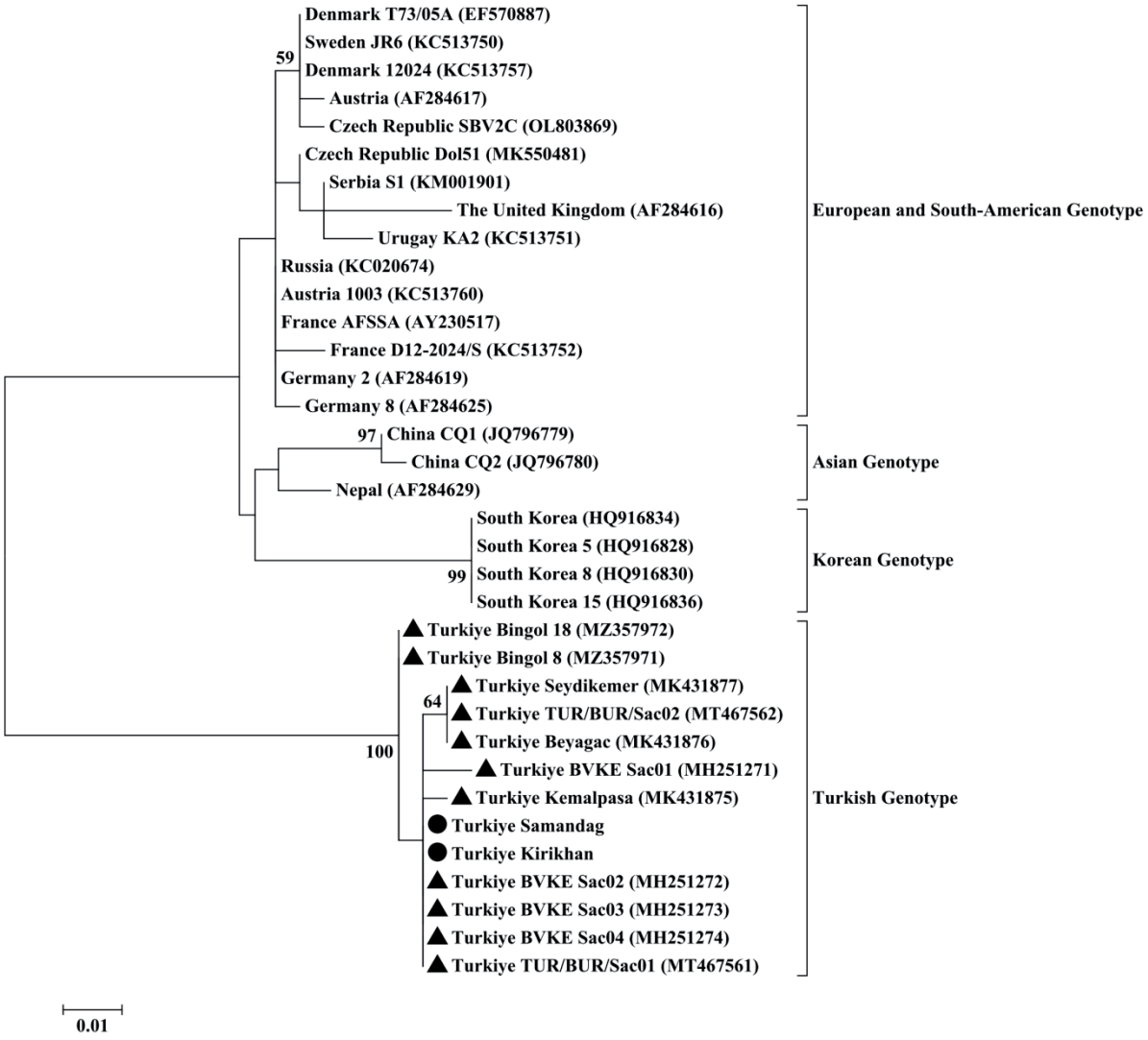

Fig. 2. Phylogenetic tree of the SBV polyprotein gene

Only values above 50% were reported. Sequences identified in this study were marked with a round black dot, whereas previous Turkish SBV isolates were marked with a black triangle

Discussion

Viral diseases are one of the significant factors threatening the beekeeping industry worldwide. SBV is one of the honeybee viruses frequently encountered in the world, and is associated with colony collapse disorder (LI et al., 2019; WEI et al., 2022). The *V. destructor* mite is reported to play an important role in the transmission of SBV (LE CONTE et al., 2010; WEI et al., 2022). Therefore, this study focused on SBV prevalence and the role of the *V. destructor* mite in the transmission of SBV.

The presence of *V. destructor* mites was detected in 17 (27.4%) of the 62 apiaries. In this study, *V. destructor* mites were detected in apiaries where acaricides were used. This is expected because there is no anti-*V. destructor* treatment with 100% effectiveness. It is only possible to attempt with treatment to keep the infestation rate below the threshold level. Furthermore, over time, the mites have become resistant to acaricides (LE CONTE et al., 2010).

The rate of *V. destructor* detected in Hatay Province (27.4%) was lower than the rate detected in different regions of Türkiye. The rate of *V. destructor* detected in different studies conducted in Türkiye was reported to vary between 35.0% and 90.0% (CAKMAK et al., 2003; GÜLMEZ et al., 2009; GÜMÜŞOVA et al., 2010). The differences between the *V. destructor* rates detected may be explained by the time of sampling, climatic conditions, and the beekeepers' use of biotechnical and biosecurity measures to combat *V. destructor* (COBEY, 2001). In this study, the owners of all the sampled apiaries reported that the most recent *V. destructor* treatment had been conducted one to two months before sampling. This fact could explain the low rate of detection of *V. destructor* in the sampled apiaries. Furthermore, the lower detection rate observed may be explained by the relatively small samples of adult honeybees per hive for *V. destructor* mite laboratory examination, and the diagnostic method used in this study.

SBV, which infects honeybee larvae and adults, is a virus that is quite common all over the world (WEI et al., 2022). The prevalence of SBV was reported to be 13.6% in Argentina (SGUAZZA et al., 2013), 40.2% in Croatia (TLAK GAJGER et al., 2014a), 49.0% in Austria (BERÉNYI et al., 2006), 86.0% in France (TENTCHEVA et al., 2004), and 90.5% in South Korea (CHOE et al., 2012). It has been reported that SBV prevalence in apiaries varies between 2.7% and 22.3% in different regions of Türkiye (KALAYCI et al., 2020; CAGIRGAN and YAZICI, 2021). For the purpose of this study, the presence of SBV was only examined in adult honeybees, and the SBV rate was determined as 8.1% (5/62). Differences in SBV prevalence between different regions and countries may be attributed to the honeybees sampled (larvae or adult honeybee), types of beekeeping (migratory or stationary), the sampling method, the number of apiaries sampled, and the time of sampling. The foremost reason for the low SBV rate detected in this study is thought to be the collection of sample honeybees using random sampling. In most of the previous studies where a high prevalence was detected, sample collection was performed in apiaries that had suffered colony collapse.

The phylogenetic tree constructed on the basis of the polyprotein gene sequences revealed that the SBV field isolates identified in this study were clustered in the same cluster with the SBV isolates previously identified in Türkiye (Fig 2). This result is consistent with previous studies reporting that the Türkiye genotype is circulating in Türkiye (KALAYCI et al., 2019; YILDIRIM et al., 2020). This can be explained by the fact that SBV isolates circulating in the same country or between neighbouring countries are more likely to be similar to each other, and by the geographical distribution of the isolates (REDDY et al., 2016).

Although *V. destructor* has been reported to have a role in the transmission of SBV (CHANTAWANNAKUL et al., 2006; WANG et al., 2019), no virus was detected in this study in the *V. destructor* mites. Furthermore, in this study, no significant association was found between presence of *V. destructor* mites and SBV positivity (P=0.12). It was reported that SBV does not replicate in *V. destructor* mites, and that the mite assumes a mechanical role in the transmission of the virus (SHEN et al., 2005). The reason why SBV was not detected in the mite samples examined within the scope of this study may be explained by insufficient viral load detected in the mites examined (GIUFFRE et al., 2019).

Conclusions

The results of this study indicate that the Türkiye genotype is spread in Türkiye. It was determined that the number of hives in the apiaries where SBV infection was detected was 50 or more, and that same beekeeping equipment was widely used. As the number of hives increases, there may be further difficulties in maintenance and feeding conditions. Further studies covering a larger area and investigating the role of mites in the transmission of viruses will contribute to our understanding of the epidemiology of SBV infection in Türkiye.

Ethics approval

The study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (No. 2021/02-02).

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Authors Contributions

Aykut Zerek and Murat Şevik contributed to the conception and design of the experiments. Aykut Zerek and İpek Erdem performed sample collection. Aykut Zerek, Murat Şevik, İpek Erdem and Mehmet Yaman were involved in data collection and analysis, and helped drafting and reviewing the manuscript. All of the authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest related to this article.

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SAŽETAK

Virus Sacbrood (SBV), koji inficira i larvu i odrasle jedinke, jedan je od najčešćih virusa u pčela medarica diljem svijeta. Cilj je rada bio istražiti prevalenciju i gensku raznolikost SBV-a u medonosne pčele kako bi se ustanovila uloga nametnika *Varroa destructor* (*V. destructor*) u prijenosu virusa. Iz nasumično odabranih pčelinjaka u provinciji Hatay u Turskoj prikupljeni su uzorci odraslih pčela (n=62). Prisutnost nametnika *V. destructor* istražena je stereomikroskopijom, dok je za detekciju SBV-a u odraslih pčela i uzorcima *V. destructor* primijenjena metoda RT-PCR u jednom koraku. U 8,1% pčelinjaka (5/62) ustanovljena je infekcija SBV-om. Osim toga, nametnik *V. destructor* otkriven je u 17 (27,4%) pčelinjaka. Prisutnost SBV-a, međutim, nije pronađena u uzorcima *V. destructor*. Rezultati filogenetske analize temeljeni na strukturnom poliproteinu SBV pokazali su da izolati otkriveni u ovom istraživanju pripadaju turskom genotipu, koji se pojavljuje kao izolirani klaster u odnosu na europsko-južnoamerički, korejski i azijski genotip. Rezultati istraživanja upućuju na razlike izolata SBV-a s obzirom na geografsku raspodjelu. Potrebna su stoga daljnja istraživanja kako bi se ustanovili genotipovi SBV-a u Turskoj i razumjela uloga nametnika *V. destructor* u prijenosu SBV-a.

Ključne riječi: medonosne pčele; virus Sacbrood; *Varroa destructor*; genetska karakterizacija; Turska