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Molecular detection and phylogenetic analysis of the honeybee (*Apis mellifera*) sacbrood virus in Turkey

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Abstract: Sacbrood virus (SBV) is widely distributed in honeybees. It is an infectious and contagious disease, which affects both the larvae and adult stages of honeybees (*Apis mellifera*). This study reports the detection of sacbrood virus in Turkey in 2017. Phylogenetic analysis based on partial sequences encoding the polyprotein gene was carried out in SBV-infected bees from four different apiaries. Phylogenetic analysis revealed noteworthy results. Although European-South American, Asian, and Korean genotypes have a maximum of 5.3% variance with each other, the variation rates between them and the Turkish genotype were 10.4%, 11.4%, and 12.5%, respectively. Sequences of Turkish isolates did not fit into the previously reported genotype groups; furthermore, they created a completely separate branch according to phylogeny. This new branch was therefore named the Turkish genotype.

Key words: Genotype, honeybee, phylogeny, sacbrood virus, Turkey

Sacbrood virus (SBV) is one of the most widely distributed honeybee viruses. This virus can infect both larvae and adult stages of honeybees, but 2-day-old larvae are far more susceptible than adult bees (1). Infected larvae begin to turn from white to yellow and fail to pupate while ecdysial fluid rich in SBV accumulates beneath their unshed skin, forming the sac for which the condition is named (2). Worker bees play an important role in the transmission of the disease to the colonies (3). Infection has also been associated with *Varroa* infestation (4,5). Infection shows a seasonal pattern and is more common in spring and summer than autumn (4).

The virus belongs to the family Iflaviridae and genus *Iflavirus*; it is a positive-sense single-stranded RNA virus (6). The virus is 28 nm in diameter, nonenveloped, and round (7). The viral genome is 8832 bp in length and contains a large open reading frame encoding a polyprotein consisting of 2858 amino acids. The viral genome is monopartite monocistronic, with the structural genes (*VP2*, *VP3*, *VP1*) at the 5'-end and the nonstructural genes (RNA helicase, 3C cysteine protease, and RNA-dependent RNA polymerase) at the 3'-end. The VP4 protein has not been detected (3,8).

The aim of this study was to determine the phylogeny of the SBV isolates detected in Turkey and to investigate the phylogenetic relationship between SBV genotypes of different geographic origins.

A total of four SBV isolates from different apiaries analyzed from Muğla, which is located in the Aegean Region of Turkey, were examined in this study in March 2017.

A pool consisting of 15 adult bees or larvae from each apiary was homogenized with 9 mL of Eagle's minimum essential medium (Sigma, United Kingdom), followed by centrifugation for 30 min at 5000 rpm. For RNA extraction 200 µL of supernatant was used. Total RNA extraction was carried out by using the High Pure Viral RNA Kit (Roche, Germany) following the manufacturer's instructions.

Acquired RNA was used directly for one-step RT-PCR amplification. For RT-PCR amplification, primers were synthesized according to a previous report (9). The Transcriptor One-Step RT-PCR Kit (Roche, Germany) was used for amplification. The reaction was performed in a reaction volume of 50 µL. The primers (SBVf: 5'-CGTAATTGCGGAGTGGAAAGATT-3' and SBVr: 5'-AGATTCCTTCGAGGGTACCTCATC-3', corresponding to a conserved region of the polyprotein of SBV) were used in a final concentration of 0.4 mM. The thermal cycle profile was as follows: 50 °C for 30 min for reverse transcription, and predenaturation and inhibition

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of reverse transcriptase at 95 °C for 7 min; followed by 45 cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 20 s, and extension at 72 °C for 30 s, with final elongation at 72 °C for 10 min. PCR products were run on 1.5% agarose gel, which was stained with ethidium bromide in TAE buffer and photographed using equipment from Vilber Lourmat (France).

PCR products were sequenced in forward and reverse directions by Microsynt (Balgach, Switzerland). Nucleotide sequence results were assembled and edited using DNADynamo DNA Sequence Analysis Software. Consensus nucleotide sequences were verified by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (10).

For the phylogenetic characterization of Turkish SBV isolates, partial 282 nt sequences encoding the polyprotein gene of SBV were determined for each isolate. Sequences adopted from other reports were obtained from GenBank. Data regarding a total of 44 sequences were used for analysis. The multiple sequence alignments of data were performed using the ClustalW algorithm for 282 bp followed by deciding on the eligible DNA/protein models for phylogeny. Phylogenetic trees were constructed by MEGA6 software using the maximum likelihood (ML), neighbor-joining (NJ), and minimum evolution (ME) methods with Tamura-3 parameter with invariable sites model (T92+I) for ML, Tamura-3 parameter with gamma distributed (T92+G) model for NJ and ME, and a bootstrap value of 1000 replicates for all methods (11).

Amino acid distances were calculated using the Jones–Taylor–Thornton method with gamma distributed model (JTT+G) and a bootstrap value of 1000 replicates (11,12). Similarity and identity of amino acids and nucleotides were determined by an online program (imed.med.ucm.es/Tools/sias.html).

All samples (one larva, three adult bees) were found to be positive for SBV. A positive band at 342 bp suggested the presence of SBV. The PCR product of each reaction was sequenced. Consensus nucleotide sequences were verified by BLAST (NCBI) (10) and confirmed as SBV.

Phylogenetic analysis was carried out by using the 362–644 nt positions of the partial polyprotein region. Blanchard et al. (13) reported that SBV had 3 genotypes (European-South American, Asian, and Korean) according to the partial sequence of the polyprotein gene. All three methods gave similar tree profiles. Results using the ML method are summarized in the Figure. Sequences of the Turkish isolates (Accession Numbers MH251271, MH251272, MH251273, and MH251274) did not fit into the previously reported genotype groups. Furthermore, they created a completely separate branch.

Amino acid and nucleotide distances between the genotypes were determined. Results showed that the distances of the Turkish genotype and other genotypes were 10.4%, 11.4%, and 12.5% for nucleotides and 6.1%, 6.1%, and 7.9% for amino acids for the European-South American, Asian, and Korean genotypes, respectively.

On the other hand, the distance between the three genotypes mentioned above were a maximum of 5.3% in nucleotides and 6% in amino acids. Within-mean-group distance was found to be 0.4%, 1.4%, 2.1%, and 0.4% for nucleotides and 0%, 1%, 2.9%, and 0.2% for amino acids in the Turkish, European-South American, Asian, and Korean genotypes, respectively.

SBV was first observed in the United States in 1913 (14) and then reported from different countries (4,5,15,16). According to previous reports, infection of SBV has been found on every continent but Antarctica and has caused epidemics (15,17). Outbreaks are often seen in spring and autumn (4). SBV attacks both brood and adult stages of bees, but larvae of about 2 days old are most susceptible to SBV infections (1). In this study, SBV infection was detected in larvae and adult bee samples sent from Muğla Province in March 2017.

The nucleotide sequences and genetic diversity of SBV have been addressed in previous studies with isolates originating from different countries (15,18,19). Grabensteiner et al. (15) mentioned three different genotypes, namely the European, Far East, and South African genotypes. According to Kukielka and Sanchez-Vizcaino (18), there were three genotypes: the European genotype of isolates from Spain, France, and Austria; the Chinese genotype from Chinese isolates; and the Uruguay-United Kingdom genotype from these countries' isolates. Genotyping as recommended by Blanchard et al. (13) was used in this study. According to this, isolates from France, Germany, Sweden, Denmark, Britain, and Uruguay form the genotype of Europe-South America; China, Nepal, and India form the Asian genotype; and Korean isolates form the Korean genotype. However, results from Turkey have not complied with the genotype isolates of the studies conducted earlier. These isolates emerged as a totally different branch in the phylogenetic tree and have been considered as a separate genotype.

Due to Turkey's climate, rich botanical diversity, and status as a transitional region between the European and Asian continents, it has one of the highest potentials in the world for apiculture with about 7.5 million colonies, but there are not sufficient data about honeybee viruses. According to our studies, Turkish isolates of SBV emerged as a completely different branch in the phylogenetic tree and were considered as a separate genotype. Therefore, we conclude that the new branch is the Turkish genotype.

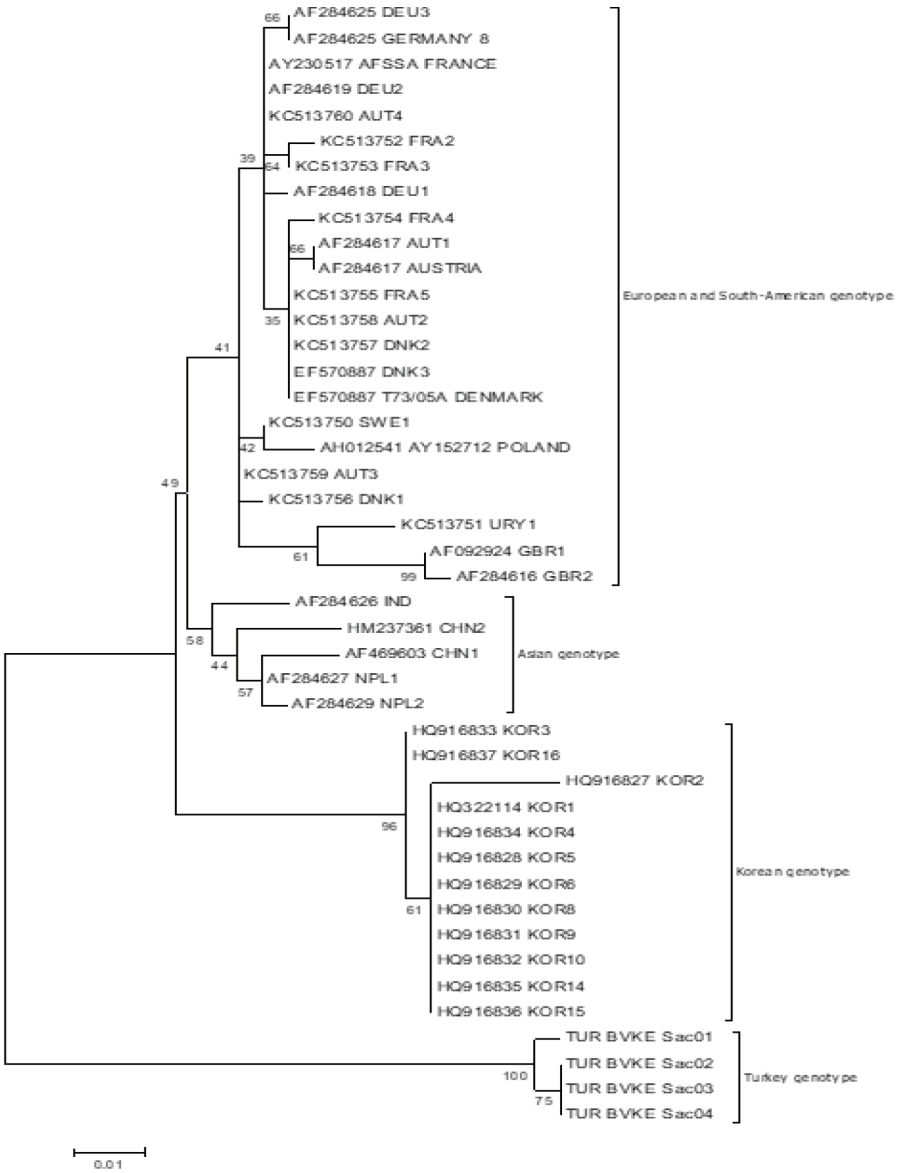

Figure. The tree was constructed using the maximum likelihood method and 1000 bootstrap replicates with Tamura-3 parameters were performed for each analysis to assess the reliability of the tree's construction.

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