OCCURRENCE AND PREVALENCE OF SIX HONEY BEE VIRUSES IN HAKKARI (TURKEY) AND THEIR GENOMIC DIVERGENCE

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ABSTRACT: A survey study was conducted to establish the occurrence and prevalence of *Black queen cell virus* (BQCV), *Deformed wing virus* (DWV), *Sacbrood virus* (SBV), *Chronic bee paralysis virus* (CBPV), *Kashmir bee virus* (KBV) and *Israeli acute paralysis virus* (IAPV) in traditionally beekeeping sites of Hakkari province (Turkey). Including the central Hakkari, the districts of Cukurca, Semdinli and Yuksekova were surveyed to assess the types of symptoms and the severity of the diseases of honey bees in a total of 90 apiaries. The presence of BQCV, DWV, SBV and CBPV infections were ascertained for the first time by RT-PCR. BQCV was found in three sampled districts and was the most frequently detected virus, found in 32% of bee samples. Less than 24% of asymptomatic bee samples were positive for DWV, SBV and CBPV with the infection ratio of 23, 12 and 9%, respectively. The samples showing deformed wing symptoms were always reacted positive for DWV. During this survey, KBV and IAPV were not detected in any of tested bee samples. Some double and triple infections were encountered: BQCV+DWV 7 (7.7%), BQCV+SBV 4 (4.4%), BQCV+CBPV 2 (2.2%), DWV+CBPV 2 (2.2%), DWV+SBV 1 (1.1%) and DWV+BQCV+SBV 1 (1.1%). The overall incidences of detected viruses (BQCV, DWV, SBV and CBPV) were lower than the other records of various sites of the world. Based on blast analysis at NCBI database, the cloned nucleotide sequences of Hakkari's isolates of BQCV, DWV, SBV, and CBPV have been showed varied nucleic acid similarities between 77-99%.

KEY WORDS: *Apis mellifera,* Hakkari province, molecular characterization, RNA viruses, RT-PCR

Single-stranded RNA viruses, infecting honeybee, have been considered one of the most important factors that may play a role in honeybee mortality as well as the presence of bacteria, fungi, protozoa, parasitic mites or exposure to pesticides used in agriculture (Bailey & Ball, 1991; Suchail et al., 2004; Ellis & Munn, 2005). Twenty-four viruses have been described that affect honeybee (*Apis mellifera* L.) including *Black queen cell virus* (BQCV), *Deformed wing virus* (DWV), *Sacbrood virus* (SBV), *Chronic bee paralysis virus* (CBPV), *Kashmir bee virus* (KBV) and *Israeli acute paralysis virus* (IAPV) (de Miranda et al., 2013)). Among them, the only three (DWV, SBV, and CBPV) would produce clinical symptoms that can be easily recognizable. Honeybee RNA viruses have been detected on a global scale and are common in many countries and continents including Europe (Tentcheva et al., 2004), South America (Antunez et al., 2005), Australia (Anderson and Gibbs, 1988), USA (Chen et al., 2004a), South Africa (Benjeddou et al., 2002) and Asia (Christian et al., 2005). In general, they cause inapparent, symptomless infections in honeybee and therefore, often go undetected (Bailey, 1967).

In Turkey, traditional beekeeping is one of the oldest practices carried out by beekeepers in Hakkari province and some other localities. Cylindric long hives are typically used to produce bee products (e.g. honey) in the region. Recently,

mortality of honeybees has been considered one of the most serious problems that Hakkari's beekeepers face periodically. There are very limited number of reports about viral honeybee diseases in Turkey. Up to date, the presence of *Acute bee paralysis virus* (Rüstemoğlu & Sipahioğlu, 2016), *Deformed wing virus* (Gülmez et al, 2009), *Chronic bee paralysis virus*, *Black queen cell virus* (Gümüşova et al., 2010) and *Israeli acute paralysis virus* (Özkırım & Schiesser, 2013) were reported to infect honeybees.

With this study, we screened the apiaries in Hakkari for the presence of DWV, KBV, IAPV, CBPV, BQCV and SBV by reverse transcription-PCR (RT-PCR). For each detected virus species, one isolate was selected for cloning and sequencing to investigate the virus genetic diversity.

MATERIALS AND METHODS

Survey and sample collection

From surveyed 90 apiaries, a total of two hundred seventy honeybee samples from different districts of Hakkari province (central Hakkari, Yuksekova, Semdinli and Cukurca) of Turkey were randomly collected during May to August 2014. The honeybee samples exhibiting deformed wing, shortened abdomen, discoloration, flightless, blackening, paralysis etc. were also collected for virus detection. The honeybee samples were transported on ice, then frozen at -86 ºC until processed. Honeybees known to be infected with BQCV, DWV, SBV and CBPV from preliminary tests were used as positive controls for diagnosis of viruses. Genomic RNA from a healthy honeybee sample was served as negative control.

RNA isolation

A modified silica-capture procedure was adapted for the isolation of honeybee total RNA (Foissac et al., 2001). Honeybee samples were homogenized in a pre-cooled sterile mortar in the presence of 1 ml of grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc, 25 mM EDTA, 1.0 M KOAc, 2.5% PVP-40, 1% 2-mercaptoethanol). Aliquots of 500 µl of the extract were mixed with 100 µl of 10% sodium lauryl sarcosyl solution in a new set of sterile microfuge tubes and centrifuged at 14,000 rpm with a refrigerated centrifuge for 10 min. Then, 300 µl of the supernatant were transferred to a new sterile tube containing 150 µl of ethanol, 100 µl of resuspended silica and 300 µl of 6 M sodium iodide. After centrifugation at 6,000 rpm for 1 min, the supernatant was discarded and the pellet washed twice with washing buffer. The pellet was resuspended with 150 µl of RNase-free water and incubated for 4 min at 70 ºC, followed by a centrifugation at 14,000 rpm for 3 min. The supernatant was transferred to a new sterile tube and stored at –20 ºC until use.

Construction of gene specific primers and RT-PCR

Coat protein (CP), RNA dependent RNA polymerase (RdRp) or non-structural protein gene specific primers for DWV, BQCV, CBPV, SBV, IAPV, and KBV were designed based on the published nucleotide sequences in GenBank (GenBank Accession No. NC_004830.2, NC_003784, NC_010711, AY626247, KC690270, NC_004807, respectively). A web based program (Primer 3: http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) or Vector NTI software (Invitrogen) were implemented to select the best primer pair for each target honeybee virus. The designed primers used in the study are shown in Table 1. Reverse transcription of purified RNA was performed using a commercial cDNA kit (Fermentas) according to the manufacturer's recommendations. PCR was performed in a final volume of 25 µl containing 2.5 µl of 10x buffer (100 mM Tris-

HCl (pH 8.8.), 500 mM KCl, 0.8% Nonidet (octyl phenoxypolyethoxylethanol) P40), 1.5 µl 2.5 mM of MgCl2, 0.5 µl 20 mM of each primer, 0.5 µl of 10 mM dNTP mix (containing 10 mM of each), 0.2 µl of enzyme, 1 µl of cDNA, 18.3 µl of sterile RNAse free water. The RT-PCR cycling program of each virus are shown in Table 1. The all reactions were performed using an Eppendorf Mastercycler and products were visualized by electrophoresis in 1% (w/v) agarose gels stained with ethidium bromide (Sambrook et al., 1989).

Molecular cloning and sequencing of partial CP, non-structural protein and RdRp genes

For each detected virus species, an isolate was randomly selected for molecular cloning and sequencing. PCR amplified fragments were separated on 1% agarose gel and recovered using a GeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer's recommendations. The purified DNA fragments were ligated into the pGEM®-T Easy vector (Promega). The constructed plasmid was used to transform *Escherichia coli* JM 109 competent cells to ampicillin resistance by electroporation (BioRad, USA). The transformants harboring the DNA of the isolates were selected by blue-white selection on X-gal medium plate and screened as positive clones by colony PCR. The clones containing the four viruses related genes were selected for propagation. For each virus one clone was chosen for DNA sequencing. The cDNA clones were sequenced bidirectionally by automated DNA sequencer at Refgen Research and Biotechnology Company (Turkey).

Phylogenetic analysis of honeybee viruses

To create phylogenetic trees, sequences corresponding to amino acids of the RdRp, non-structural protein and CP domain were used to establish phylogenetic relationships of honeybee viruses. Based on non-structural protein, RdRp and CP gene sequences, the phylogenetic relationships among strains of the honeybee viruses and other close groups available in NCBI (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi) were initially assessed using BLAST. The sequences of viruses were aligned by using MEGA7 and CLC Main workbench 6.6.1 software and the cladistic analyses were performed by using MEGA7. The relationships were assessed using 1000 bootstrap replicates. The phylogenetic tree was created using Neighbor-Joining method from sequences of honeybee viruses and sequences of other 4 distinct honeybee viruses studied in this work.

RESULTS

Out of 90 apiaries examined, symptomatic individuals were encountered showing deformed wing induced by DWV and shortened abdomens, discoloration, flightless, blackening, paralysis induced by other bee viruses (Figs. 1a,b). The presence of DWV was easily distinguished by clinical symptoms. Almost all symptomatic individuals were reacted positive for DWV or co-infected with more than one virus in RT-PCR tests. However, DWV was also detected in individuals without symptoms.

Singular viral infections

Here, we demonstrate the successful use of RT-PCR to detect honeybee viruses in the apiaries of Hakkari. The method used in this study for detection of honeybee viruses was rapid, reliable and useful for large scale epidemiological studies. In total, 90 apiaries were examined for the presence of six viruses (DWV, BQCV, CBPV, IAPV, KBV and SBV). Gene specific primers of DWV (non-structural protein), BQCV (CP gene), IAPV (CP gene), SBV (CP gene), CBPV (RdRp gene) and KBV (CP gene) were used in uniplex-RT-PCR tests targeting to

generate 488, 567, 402, 429, 434 and 339 bp respectively. Based on uniplex-RT-PCR results, singular and mixed virus infections were encountered in collected bee samples. The viruses detected were DWV, BQCV, CBPV, and SBV. However, KBV and IAPV were not detected in any of the samples tested. The frequencies of the detected viruses were varied. During our survey, the most prevalent virus in tested bee populations was BQCV. The virus occurred in only three surveyed areas including central Hakkari, Semdinli and Yuksekova and in 32% of all samples (Fig. 2). This was followed by DWV (23%), SBV (12%), and CBPV (9%) (Table 2).

Multiple virus infections

Based on RT-PCR test results, multiple infections were commonly detected in a single bee sample. In Table 2, the number and combinations of multiple infections are shown. Almost 18% of the samples were infected by dual infections. Nearly 8% of the samples were infected by BQCV and DWV, 4% were BQCV and SBV, 2% were BQCV and CBPV, 1% were DWV and SBV and 2% were DWV and CPBV. One triple infection (DWV+BQCV+SBV) was also recorded. Among dual infections, the combination of BQCV and DWV infections was observed with the highest frequency (8%).

Genetic variation

The nucleotide sequences of virus-encoded RdRp, CP and non-structural protein gene were used to create multiple alignments. As shown in phylogenetic trees the compared individual virus species were exhibited high levels of sequence similarity for compared genes (Figs. 3A,B,C,D). For RdRp and CP genes, DWV formed a group with *Varroa destructor virus* 1 (VDV-1) and SBV with bootstrap values of 73% and 81%, respectively. The all compared viral sequences exhibited high levels of sequence similarity for all genes. SBV and DWV formed a separate group along with VDV-1, IAPV, KBV and ABPV.

In comparing CP gene, DWV, SBV, Slow bee paralysis virus (SBPV) and VDV-1 formed a separate group in the phylogenetic tree. In the group, DWV and VDV-1were more related one another than to SBV and SBPV (Fig. 3A). The major functional domains associated with the CP, RdRp and non-structural protein gene can be readily identified.

DISCUSSION

In this study, we have screened the honeybee apiaries in traditional beekeeping areas of Hakkari, South East corner of Turkey, using reverse transcription-PCR (RT-PCR) method, for the presence of 6 honeybee viruses and analyzed selected isolates for viral genetic diversity. We have demonstrated that virus infections in honey bees are widespread among honeybees in nature of Hakkari, as we detected BQCV, DWV, SBV and CBPV in Hakkari's apiaries. This is the first report of these four viruses in Hakkari province. Almost all symptomatic samples from surveyed localities were infected with at least one virus or co-infected with more than one. In many cases, these viruses were also detected in bee samples without symptoms. In total, 35.5% of apiaries were positive for a single infection, approx. 18% a double infection, approx. 1% a triple infection and 54% at least with one infection.

BQCV was the most prevalent virus, with 32.2% of colonies screened being positive for the presence of this virus. It was present in every district of Hakkari, except in Cukurca. Total frequency of BQCV has been less then France 86% (Tentcheva et al., 2004), Uruguay 91% (Antunez et al., 2006), Brazil 37% (Weinstein-Teixeira et al., 2008) but, more than Austria 30% (Berenyi et al., 2006), the Eastern Black Sea Region of Turkey 21.4% (Gümüşova et al., 2010),

Spain 10.4% (Antunez et al., 2012), England 1.4% (Baker & Schroeder, 2008) and Denmark 1% (Nielsen et al., 2008). Although DWV was the most abundant virus in the other places of the world, it was the second abundant virus in Hakkari, being found in 23.3% of the apiaries. It was detected in every surveyed district of Hakkari. The virus was highly prevalent particularly in samples from central Hakkari. In the world, DWV was recorded in varying incidences i.e. 33% in Thailand (Sanpa and Chantawannakul, 2009), 20.3% in Brazil (Weinstein-Teixeira et al., 2008), 18.6% in Spain (Antunez et al., 2012), 100% in Uruguay (Antunez et al., 2006), 97% in France (Tentcheva et al., 2004) and England (Baker and Schroeder, 2008), 92% in Jordan (Haddad et al., 2008), 91% in Austria (Berenyi et al., 2006) and 55% in Denmark (Nielsen et al., 2008). SBV was detected in 12.2% of apiaries which is less than Uruguay 100% (Antunez et al., 2006), France 86% (Tentcheva et al., 2004), Denmark 78% (Nielsen et al., 2008), Austria 49% (Berenyi et al., 2006). CBPV was found in 8.8% apiaries of Hakkari. This result is less than Uruguay 47% (Antunez et al., 2006), France 28% (Tentcheva et al., 2004) and the Eastern Black Sea Region of Turkey 25% (Gümüşova et al., 2010). In general, the frequencies obtained from this study are less than the frequencies found for the same viruses from other parts of the world. The real reason of differences in prevalence of bee viruses worldwide are not fully known. It may probably be related to bee management practices, topographic isolation and sample selection.

For each identified virus species, a virus isolate was randomly selected, and their partial genomes were characterized. For BQCV and SBV partial coat protein (CP) genes and for DWV and CBPV partial RdRp genes were characterized, cloned and sequenced. Virus sequence data generated were deposited in GenBank, (Accession No. KP835212 for SBV, KP835213 for BQCV, KP835214 for DWV, KP835215 and KP835216 for CBPV). Based on blast analysis at NCBI database, the sequences of Hakkari's isolates of BQCV, DWV and SBV have been showed varied nucleic acid similarities as 89-90%, 96-99%, and 77-90%, respectively. However, the nucleotide sequences of the PCR product of CBPV-Hakkari isolate was only 85-86% identical to the world CBPV isolates. Even though mono infections were more common than dual and triple infections in our study, a high level of dual infections was observed in sampled apiaries. Dual, triple and even quadruple infections of BQCV, DWV, SBV, and CBPV have been recorded by others (Anderson & Gibbs, 1988; Evans, 2001; Chen et al., 2004b).

The phylogenetic trees were created mainly from sequences obtained from recent isolates of bee viruses from different countries (Fig. 3). DWV was clustered with the members of the Iflavirus genus (Dicistroviridae). The basic genome organization of DWV, SBV, SBPV, and VDV-1 is typical for the Iflavirus genus. The particles of the members contain a single molecule of linear, positive sense, ssRNA and the 3'-end of the viral RNA is polyadenylated. All the obtained BQCV, SBV and CBPV sequences were exhibited high levels of variation, but each clustered into one cluster, involving the genus members which they belong to (Figs. 2B,C,D).

In the present work, we have provided evidence for four out of six different honeybee viruses as singular or multiple-infections in Hakkari, Turkey. The low incidence of infection levels of bees in Hakkari probably resulted from high *mountains* constitute the *several* ecozones for bees in the surveyed areas, restricting the flying of bees. Based on genomic sequences, the diversity of viruses in Hakkari was found high, in particular, CBPV. For better understanding of viral introduction, transmission, and viral fluctuations in bee populations, further studies need to be made, covering different types of insects around the apiaries

and virus-vector mites in Hakkari. To control bee viruses, monitoring their prevalence and spread is vital.

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Table 1. Primers used in this study and the amplification target.

Primers (5'–3')	Product size (bp)/ Position at genome/
Deformed wing virus DWV-F (5'-TTGGTATGCTCCGTTGACTG-3') DWV-R (5'-ATTCCTCAGAAGTTGGTTTCG-3')	488/ Non-structural protein
Black queen cell virus BQCV-F (5'-GACAGCGTGCCAAAGAGAG-3') BQCV-R (5'-GCGAACCCGTCCAATACTTA-3')	567/ CP gene
Israeli acute paralysis virus IAPV-F (5'-TTGGCGTGCAACTATGTGTT-3') IAPV-R (5'-TCTTCTGCCCACTTCCAAAC-3')	402/ CP gene
Sacbrood virus SBV-F (5'-TATTCAGGGGGACGCTACAC-3') SBV-R (5'-AGTGCTGCTTGAAACCCTGT-3')	429/ CP gene
Chronic bee paralysis virus CBPV-F (5'-GCAAACTGCCCACCAATAGT-3') CBPV-R (5'-TGGTACGGAAGGTGTGTCAA-3')	434/ RdRp gene
Kashmir bee virus KBV-F (5'-CACATTCCGAACAATAA-3') KBV-R- (5'-GCGATAGGAATTTTGCGGTA-3')	339/ CP gene

Table 2. Virus infections encountered in honeybee samples of Hakkari.

Type of infection	**Detected virus(es)**	**Number of infected sample (Incidence of viral infection %)**
Mono infection	BQCV, DWV, SBV and CBPV	32 (35.5%)
	BQCV	15 (16.6%)
	DWV	10(11.11%)
	SBV	5 (5.5%)
	CBPV	4 (4.4%)
Dual infection	BQCV+DWV, BQCV+SBV, BQCV+CBPV, DWV+CBPV, DWV+SBV	16(17.7 %)
	BQCV + DWV	7 (7.7%)
	BQCV + SBV	4 (4.4 %)
	BQCV + CBPV	2 (2.2%)
	DWV + CBPV	2 (2.2%)
	DWV + SBV	1 (1.1%)
Triple infection	DWV + BQCV + SBV	1 (1.1%)

a) b) c)

Figure 1. Deformed wing symptom encountered in the field surveys a) under microscope and b) in the field, c) swollen abdomen induced by BQCV.

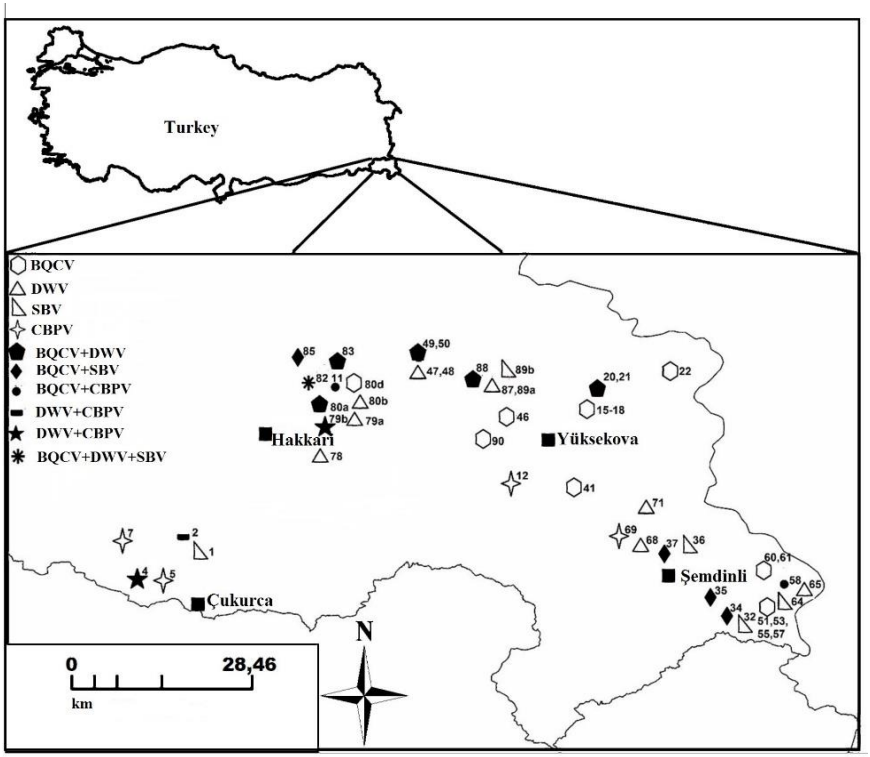
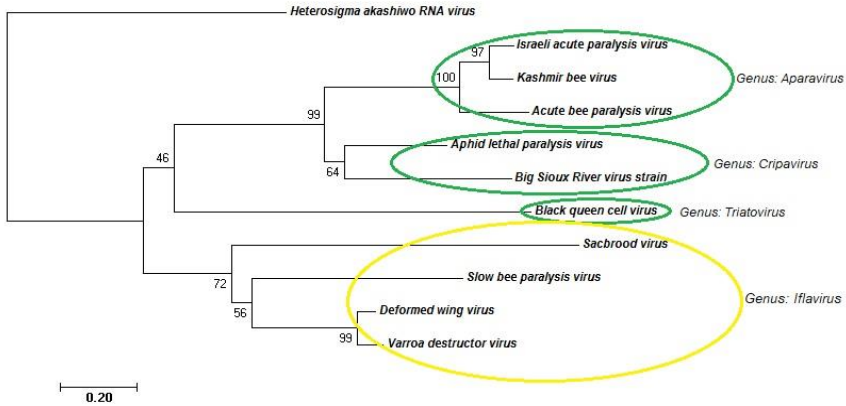
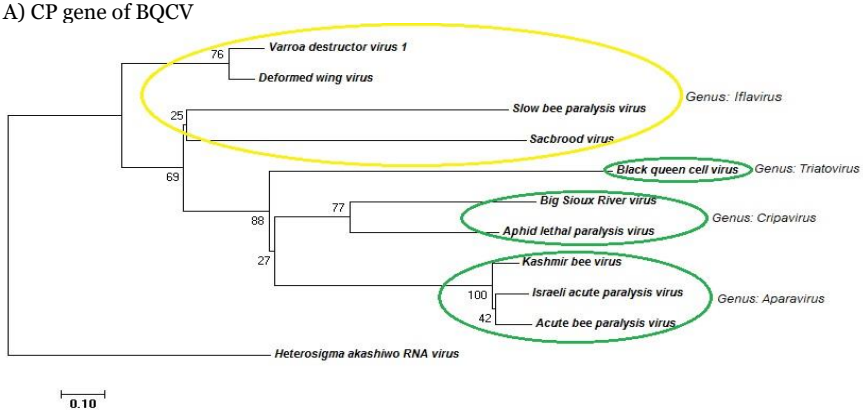


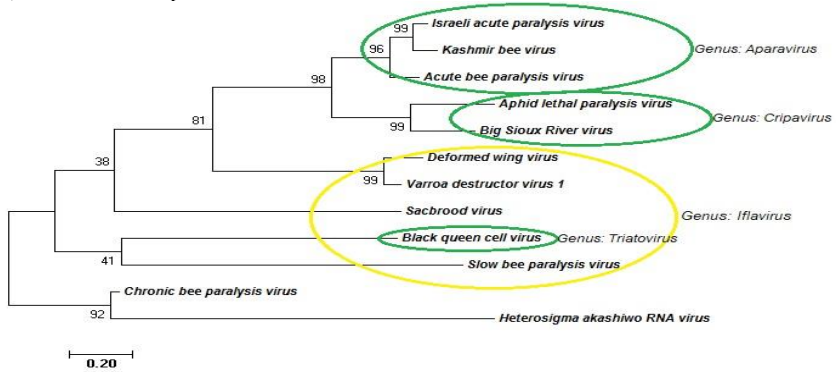
Figure 2. Hakkari province, located at the eastern Anatolia (Turkey), surveyed for the presence of bee-infecting viruses. Each symbol shows single and multiple virus infections and their combinations.



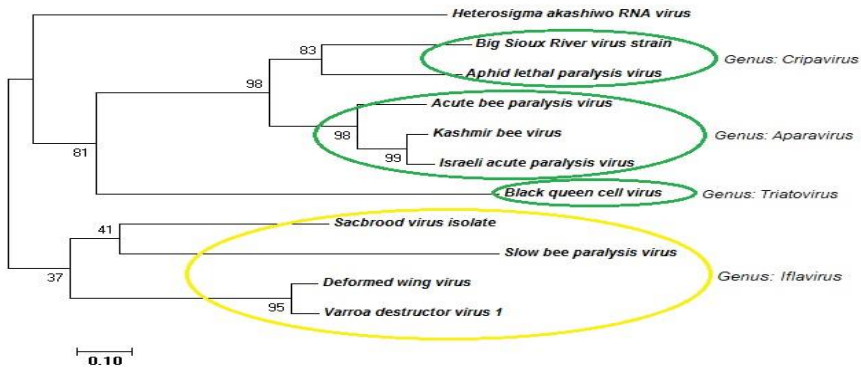
A) CP gene of BQCV



B) Non-structural protein of DWV



C) RdRp gene of CBPV



D) CP gene of SBV

Figure 3. Phylogenetic trees derived from the CP (A) BQCV, non-structural protein (B) DWV, RdRP (C) CBPV and CP (D) SBV sequences of the viruses. The sequences were aligned using the MEGA 7 software program. Neighbor-Joining method was used to create the phylogenetic relationship of viruses. *Heterosigma akashiwo RNA virus* was used as an outgroup to root the trees. Green and yellow hoops show Dicistroviridae and Iflaviridae respectively. Numbers at each node represent bootstrap values as percentages of 1000.