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The first molecular detection and phylogenetic assessment of six honeybee viruses in *Apis mellifera* L. colonies in Bulgaria

Short title: The first report for honeybee viruses in Bulgaria

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**Key words:** Honey bee viruses, *Apis mellifera*, RT-PCR, Bulgaria
Introduction

The Western honey bee (*Apis mellifera* L., Hymenoptera: Apidae) is a species of crucial economic, agricultural and environmental importance. Many factors lead to dramatic reduction of bee populations, reduction of bee production, and thus to disruption of the process of pollination of farming crops and wild plants (including certain endemic ones). Among such factors are known diseases with economic significance affecting the bees and their brood, such as bacterial, parasitic and viral (Dainat et al., 2012).

Viral diseases affect honey bee colonies and lead to severe economic losses in the beekeeping industry (Ball and Bailey, 1997; Berthoud et al., 2010). The most commonly identified honey bee viruses are 30-nm isometric particles with a single-stranded positive RNA (ssRNA) (Tentcheva et al., 2004). The size of the genome of honey bee viruses ranges from 3700 bp for CBPV (Ribière et al., 2010) but usually from 8 000 up to 10 000 bp (Tantillo et al., 2015). With the exception of CBPV, the genome of all honey bee viruses consists of two open reading frames (ORFs) in the 5’ and 3’ ends direction, encoding two polyproteins. The first ORF region encodes non-structural proteins involved in virus replication and processing while the 3’ flanking region encodes the structural proteins found in the viral particle (Tantillo et al., 2015). Usually, different protocols based on 5’ and 3’ flanking ORF regions (ORF1 and ORF2) have been used for virus detection, (Tentcheva et al., 2004; Maori et al., 2007;…….). Due to this reason, most investigations use different sequencing sets concerning phylogenetic analyses and geographical dispersal.

The SBV and DWV are assigned to the Family *Iflaviridae*, Genus *Iflavirus*, while ABPV, KBV and BQCV are members of the Family *Dicistroviridae*, Genus *Cripavirus* (Mayo, 2002). Up to now CBPV has been the only virus not classified yet because there is evidence that this virus is distinct from the other ones with respect to particle symmetry and size, and genome composition
and organization (Ribière et al., 2010). All of those viruses are widely dispersed worldwide (Ellis and Munn, 2005; Reynaldi et al., 2010, 2011).

Most investigations based on phylogenetic analysis have revealed multiple, i.e. ABPV and DWV, or endemic East Asian and Australian (Pacific region), and Indian origin (BQCV and SBV) of most described honey bee viruses as well as their introduction in the West direction toward Europe and worldwide (Tapaszti et al., 2008; Bakonyi et al., 2002; Yang et al., 2012).

Despite the importance of viral diseases on honey bee colonies health, the sequencing and phylogenetic data related to the origin and spreading of viruses are scarce. Up to date, there has been no information about honey bee viruses on the Balkan Peninsula, except the study of Hatjina et al. (2011).

Bulgaria as a part of the Balkans and a bordering region between the Middle East and Europe is an interesting point for understanding the dissemination and introduction of honey bee viruses. Therefore, in this study we have reported the first survey of six honey bee viruses (DWV, ABPV, CBPV, SBV, KBV and BQCV) in honey bee colonies in Bulgaria. A phylogenetic analysis was performed to reveal the geographic distribution of different virus strains.

**Materials and Methods**

**Honey bee sampling.** Samples of adult worker bees were collected from 50 colonies originating from 3 apiaries in 3 locations in Bulgaria: Rousse district (North Bulgaria, n=20), Sofia district (West Bulgaria, n=20) and Smolyan district (South Bulgaria, n=10), (Table 1) in May-June 2017. Five adult bees were collected individually from each colony, following the method described by Chen et al. (2004). All colonies were checked for clinical signs. The obtained samples
were put in a cooler bag and immediately sent to the laboratory, where they were frozen at -20°C.

**Total RNA extraction and RT-PCR amplification.** The frozen samples were crushed in a mortar and were homogenized in a RL lysis buffer (GeneMATRIX Universal RNA Purification Kit, Cat. No. E3598, EURx Ltd., Poland). After homogenization, the samples were centrifuged for 3 min at 15 000 x g to remove unhomogenized particles. An aliquot of supernatant was used for extraction of total RNA according to the manufacturer’s recommendations. The quality of the extracted total RNA was checked by electrophoresis and spectrophotometry. An average of 2 µg of the total RNA was used for copy DNA (cDNA) synthesis using Oligo(dT)$_{20}$ primers (NG dART RT-PCR kit, E0802, EURx Ltd., Poland) according to the manufacturer’s instructions.

The PCR mixture contained 25 µL of NZY Taq 2× Colourless Master Mix (Cat. No. MB04002, Nzytech, Portugal), 0.4 µM of each virus specific primer (FOR/REV), 1 µL of template cDNA in a total volume of 50 µL. All RT-PCR amplifications were carried out using a Little Genius thermocycler (BIOER Technology Co., Ltd) under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles (denaturation at 94 °C for 30 s; primer annealing at 56 °C for 30 s; extension at 72 °C for 1 min) and final extension at 72 °C for 10 min. PCR products were visualized on 1 % agarose gel with GreenSafe Premium (Cat. No. MB13201, Nzytech, Portugal). The fragment size was determined using Gene-Ruler™ 100 bp Ladder Plus (Cat. No. SM0323, ThermoFisher Scientific Inc.). The primers used for detection of the viruses (DWV, ABPV, CBPV, SBV, KBV and BQCV) had been described by Tentcheva et al. (2004). The successfully amplified products were purified by a PCR purification kit (Gene Matrix, PCR clean-up kit, EURx, Poland) and sequenced in both directions by a PlateSeq kit (Eurofins Genomics Ebersberg, Germany).
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Statistical and phylogenetic analysis. All obtained DNA sequences were manually edited and aligned with reference complete viral genomes (ABPV, Acc. No. AF150629, Govan et al., 2000; DWV, Acc. No. NC_004830, Lanzi et al., 2006; SBV, Acc. No. NC_002066, Ghosh et al., 1999; BQCV Acc. No. NC_00378, Leat et al., 2000) by using the MEGA7 program (Kumar et al., 2016). The obtained sequences (DWV - 388 bp, SBV -417 bp, ABPV – 435 bp and BQCV – 486 bp) were deposited in the GenBank database National Biotechnology Information Center (NCBI) under accession numbers MG599458-MG599464 and MG649495-MG649502. All obtained sequences covered the 3’ end of the ORF region of the viral genome. This part of the viral genome sequences was chosen for analysis based on the availability of most similar sequences as well as complete viral genomes of other countries’ viral isolates. After retrieving appropriate sequences from GenBank, all sequences were aligned using MUSCLE (Edgar, 2004), and then the best-fit substitution model was selected for constructing each viral phylogeny. The phylogenetic tree was constructed using the maximum likelihood method and a bootstrap value of 1000 replicates with MEGA7 program (Kumar et al., 2016) in each case. The phylogenetic trees were visualized by using FigTree v 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).
Results

Virus frequencies in honey bee colonies. The data for RT-PCR detection of six bee viruses in the 50 hives are shown in Table 1. Except for CBPV and KBV, where no virus was detected, the other 4 viruses were found with different frequencies in the investigated region of the country. DWV was found to be with the highest frequencies (10, 20 %). It was observed in all regions of the country – South Bulgaria (Smolyan; 2, 20 %), West Bulgaria (Sofia 3, 15 %) and North Bulgaria (Rousse; 5, 25%). The fact that this virus is present in honey bee colonies both in the flat and in the mountainous parts of the country shows that there are no significant differences between the different regions. SBV was the second most frequent one of those studied (6, 12 %). This virus was found only in West Bulgaria (Sofia; 6, 30 %) and nowhere in the other regions. In contrast to SBV, ABPV was detected only in North Bulgaria (2, 10 %), but with a low frequency (2, 4 %) in all 50 investigated colonies. This virus appears to show prevalence in the flat regions of the country. BQCV was with the lowest frequency, recorded only in West Bulgaria (Sofia; 1, 5 %) or only 2 % from all samples.

Discussion

DWV. DWV is one of the larger widely distributed honey bee viruses worldwide (Allen and Ball, 1996). The main vector for this virus is Varroa destructor, which is why this virus is absent in honey bee colonies in regions where this mite is not available (Australia and Oceania, Roberts et al., 2017). Our results demonstrate that this virus is present in all investigated regions (Table 1). A total of seven samples was successfully amplified and sequenced (GenBank Acc. No. MG599458-MG599464). On the Balkans, this virus was found only in Greece, where all investigated samples were positive (Hatjina et al., 2011). The intensity of infection in our samples
was lower compared to other regions in Europe where infection rate is over 90% (Tentcheva et al., 2004; Baker and Schroeder, 2008).

DWV includes three closely related types of viruses, which differ in terms of their pathogenic effect and genetic diversity (Brettell et al., 2017). These strains are named formally as A, B and C. Type A consists of DWV and Kakugo virus (KV), and type B, which is the most pathogenic (Lanzi et al., 2004; Ongus et al., 2004), is genetically similar to type C. Due to this reason there are recombinant forms between them. Type C is non-pathogenic for bees (Mordecai et al., 2016).

The phylogenetic analysis on the newly sequenced Bulgarian fragment 3’ ORF 2 shows that all belong to type A DWV. From all seven obtained sequences, six split in numerous different branches (Fig. 1). The heterogeneity of our samples was calculated to be 0.021 (Kumar et al., 2016). The highest homology of Bulgarian virus sequences was found in six UK branches (Baker and Schroeder, 2008), two branches in Turkey (Acc. No. KU521781- KU521779; unpublished data), and three branches in Spain (Acc. No. DQ385499, DQ385501, DQ385502; unpublished data). The European strains formed numerous different genetic clades but there is no clear regional distribution. This means that virus strains dispersal has occurred in all Eurasia (Fujiyuki et al., 2004; Lanzi et al., 2004; Tentcheva et al., 2004; Baker and Schroeder, 2008; Moore et al., 2011; Reddy et al., 2013; Wang et al., 2013; Carrillo-Tripp et al., 2016; Lamp et al., 2016). The phylogenetic analysis based on different region (encoding capsid protein from 3’ ORF2) has also revealed numerous different geographically determined clades (US, Japan, China), but not European (Yang et al., 2013).

It may be concluded that Bulgarian DWV sequences shared common origin with the Turkish virus strains as well as with those in Mediterranean countries.
SBV. Sacbrood can infect either larvae or adult honeybees, with a higher sensibility of larvae to the infection. SBV primarily affects the brood of the honey bees that causes significant morphological alterations resulting in larval death (Berenyi et al., 2006). We found six cases in our samples (12 %), (Table 1). Five of them were successfully sequenced and deposited in GenBank under Acc. No. MG649495-MG649499. To date, on the Balkans, this virus has been detected once in Greece (Hatjina et al., 2011).

Phylogenetic analysis of Bulgarian samples showed the highest homology with samples from Papua (New Guinea) and Australia (Acc. No. KJ629183, Roberts and Anderson 2014; Acc. No. KY465679, Roberts et al., 2017). The estimated average divergence within this cluster was calculated to be 0.163 (Kumar et al., 2016). SBV from Europe formed a different clade including available sequences from West toward East direction (Fig. 2) (Ghosh et al., 1999; Tentcheva et al., 2004; Lomakina and Batuev, 2012). The genetic distance between the Bulgarian and the European clade was calculated to be 0.216 (Kumar et al., 2016). The European clade analyzed by 5’ ORF1 also includes additional data (not shown) (Grabensteiner et al., 2001). Based on this sequence data, the European clade has possibly originated from Nepal, while the Bulgarian clade is potentially with Australian origin. The US clade of SBV otherwise has a high homology and possible introduction with Chinese virus strains (Yang et al., 2013).

Taking together phylogenetic data for SBV worldwide showed high diversity split in different clades from the Pacific region (Australia, China, South Korea, India, Vietnam, etc.) (Roberts et al., 2017; Ma et al., 2011; Xia et al., 2015; Reddy et al., 2016; Nguyen and Le, 2013).

The phylogenetic analysis of the Balkan SBV revealed new European genotypes, introduced in a different way in Europe.
BQCV. BQCV is one of the most abundant honey bee viruses, about 80% after DWV and SBV (Tapaszti et al., 2009). Previous studies from the Balkan region had shown that in Greece honey bee colony infection was also the most abundant in all investigated apiaries (Hatjina et al., 2011). In our study, we found only one case of infection (Table 1). Phylogenetic analysis of the obtained sequenced fragment (Acc. No. MG649502) showed the highest homology with European BQCV strains from the Czech Republic (95%) and Hungary (93%) (Tapaszti et al., 2009, Spurny et al., 2017), (Figure 3). These data support three centers of virus diversity in Europe – Central and South-Eastern (CSE cluster), Central-North (CN cluster, Poland) and West Europe (WE cluster, France and UK) (Tentcheva et al., 2004; Tapaszti et al., 2009; Roberts et al., 2017; Spurny et al., 2017). The last two clusters – CN and WE are split from the CSE clade together with the Pacific group (Australia, Tasmania, South Africa and South Korea) (Roberts et al., 2017). The results for the 3’ ORF flanking region were also supported with virus phylogeny and diversity based on the more variable ORF1 region (Roberts et al., 2017). Considering this data, the CSE BQCV clade also includes regions in Austria and Germany (Tapaszti et al., 2009; Reddy et al., 2013).

In all clusters there exist different BQCV genotypes from China and Korea. This may be explained with the fact that there are at least three virus strains that infected honey bees in Europe imported from Central Asia (Figure 3).

It is obvious that the genetic diversity of viruses in Europe is supported by the geographic spreading in Europe, possibly by independent transfer of infection without co-infections. Our data enlarge the geographic spreading of CSE BQCV strains in South-Eastern direction.

ABPV. ABPV is also frequently found in honey bee colonies. Apparently, this virus plays a role in cases of a sudden collapse of the honey bee and is transmitted by the parasitic mite Varroa destructor (Bakonyi et al., 2002). On the Balkans (Greece) ABPV was detected as a common
Infection pathogen (Hatjina et al., 2011). In our study this virus was found in two honey bee colonies (2, 4 %) (Table 1) and sequencing fragments were deposited in GenBank under Acc. No. MG649500-MG649501. The phylogenetic analysis revealed high homology (97 %) with ABPV genotypes from Hungary (Figure 4), (Bakonyi et al., 2002) and form a clade with Slovenian viruses (Jamnikar et al., 2009, unpublished data). The estimated average divergence within this clade was calculated to be 0.018 (Kumar et al., 2016). Up to now genetic analyses of ABPV in Europe have clearly distinguished three main clades (Bakonyi et al., 2002). There are two clades concerning Central Europe that are split into two branches – Hungary and Austria-Germany, and a third one which is more distinct in UK. In Poland ABPV shows mixed infection between these clades (Bakonyi et al., 2002). The UK clade is closely related with the US strains (Govan et al., 2000; Li et al., 2010; Baker and Schroeder, 2008). To date, there have been no data about ABPV outside Europe and North America (USA and Canada) despite the presence of the main transmission agent *Varroa destructor* (Yang et al., 2013). This virus is also not detected in Australia due to the absence of *Varroa* (Roberts et al., 2017).

Our findings have enlarged the existing data on the geographic spreading of ABPV up to South-Eastern Europe, where this virus was detected for the first time.

**Conclusion**

The present study has described new data for four honey bee viruses (DWV, SBV, ABPV and BQCV) in Bulgaria. Moreover, these have been the first sequenced data reported for the Balkan Peninsula. We try to introduce phylogenetic data about geographic distribution and genetic diversity including new data sets in the Balkans. The phylogenetic analysis of ABPV and BQCV has shown a close relation to Central-European virus strains that differ from West-European and
Polish genotypes. The Bulgarian samples of SBV seem to form a new distinct clade within the European strains with high homology to viruses from the Pacific region. Finally, DWV is present with multiple different genetic genotypes and is closely related to the Mediterranean and Turkish strains.

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References


recombination. *Journal of General Virology* 88:3428-3438. DOI: 10.1099/vir.0.83284-0.


Figures caption

**Figure 1.** Molecular phylogenetic analysis of deforming wing virus (DWV) isolates from Bulgaria and other countries. Each isolate is indicated by country of isolation and GenBank accession number. Bulgarian isolates identified by this study are represented by green diamonds.

**Figure 2.** Molecular phylogenetic analysis of Sacbrood virus (SBV) isolates from Bulgaria and other countries. Each isolate is indicated by country of isolation and GenBank accession number. Bulgarian isolates identified by this study are represented by green diamonds.

**Figure 3.** Molecular phylogenetic analysis of black queen cell virus (BQCV) isolates from Bulgaria and other countries. Each isolate is indicated by country of isolation and GenBank accession number. Bulgarian isolate identified by this study is represented by green diamond. C/SE Europe – Central/South-Eastern Europe; W Europe – Western Europe; CN Europe – Central-Northern Europe.

**Figure 4.** Molecular phylogenetic analysis of acute bee paralysis virus (ABPV) isolates from Bulgaria and other countries. Each isolate is indicated by country of isolation and GenBank accession number. Bulgarian isolates identified by this study are represented by green diamonds.
Table 1. Distribution of DWV, SBV, ABPV, BQCV, CBPV, and KBV viruses in three different regions in Bulgaria (from 50 apiaries).

<table>
<thead>
<tr>
<th>Region(^a)</th>
<th>No. of hives</th>
<th>DWV</th>
<th>SBV</th>
<th>ABPV</th>
<th>CBPV</th>
<th>BQCV</th>
<th>KBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smolyan (SB)</td>
<td>10</td>
<td>++(^c)</td>
<td>- (^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sofia (WB)</td>
<td>20</td>
<td>+++</td>
<td>+++++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rousse (NB)</td>
<td>20</td>
<td>+++++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

| No. (%) of positive colonies | 10 (20) | 6 (12) | 2 (4) | 0 | 1 (2) | 0 |

\(^a\) SB – South Bulgaria; WB – West Bulgaria; NB – North Bulgaria;

\(^b\) (+) = Positive; (-) = Negative;

\(^c\) number of detected viruses in each studied region.
Figure 1
Figure 2
Molecular phylogenetic analysis of black queen cell virus (BQCV) isolates from Bulgaria and other countries. Each isolate is indicated by country of isolation and GenBank accession number. Bulgarian isolate identified by this study is represented by green diamond. C/SE Europe – Central/South-Eastern Europe; W Europe – Western Europe; CN Europe – Central-Northern Europe.