Honey bee viruses in Serbian colonies of different strength

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Protection of honey bees is of great economic importance because of their role in pollination. Crucial steps towards this goal are epidemiological surveys of pathogens connected with honey bee losses. In this study deformed wing virus (DWV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV) and sacbrood virus (SBV) were investigated in colonies of different strength located in five regions of Serbia. The relationship between colony strength and virus occurrence/infection intensity were assessed as well as the genetic relationship between virus sequences from Serbia and worldwide. Real-time RT-PCR analyses detected at least one virus in 87.33% of colonies. Single infection was found in 28.67% colonies (21.33%, 4.00%, 2.67% and 0.67% in cases of DWV, ABPV, SBV and CBPV, respectively). In the majority of colonies (58.66%) more than one virus was found. The most prevalent was DWV (74%), followed by ABPV, SBV and CBPV (49.30%, 24.00% and 6.70%, respectively). Except for DWV, the prevalence of the remaining three viruses significantly varied between the regions. No significant differences were found between colony strength and either (i) the prevalence of DWV, ABPV, SBV, CBPV and their combinations, or (ii) DWV infection levels. The sequences of honey bee viruses obtained from bees in Serbia were 93-99% identical with those deposited in GenBank.
Honey bee viruses in Serbian colonies of different strength

Running title: *Apis mellifera* viruses in Serbia

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Abstract

Protection of honey bees is of great economic importance because of their role in pollination. Crucial steps towards this goal are epidemiological surveys of pathogens connected with honey bee losses. In this study deformed wing virus (DWV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV) and sacbrood virus (SBV) were investigated in colonies of different strength located in five regions of Serbia. The relationship between colony strength and virus occurrence/infection intensity were assessed as well as the genetic relationship between virus sequences from Serbia and worldwide. Real-time RT-PCR analyses detected at least one virus in 87.33% of colonies. Single infection was found in 28.67% colonies (21.33%, 4.00%, 2.67% and 0.67% in cases of DWV, ABPV, SBV and CBPV, respectively). In the majority of colonies (58.66%) more than one virus was found. The most prevalent was DWV (74%), followed by ABPV, SBV and CBPV (49.30%, 24.00% and 6.70%, respectively). Except for DWV, the prevalence of the remaining three viruses significantly varied between the regions. No significant differences were found between colony strength and either (i) the prevalence of DWV, ABPV, SBV, CBPV and their combinations, or (ii) DWV infection levels. The sequences of honey bee viruses obtained from bees in Serbia were 93-99% identical with those deposited in GenBank.

Introduction

Honey bees (Apis mellifera) are well-known beneficial insects for their popular products, and much more for their important role in pollination (Venturini et al., 2017). Unfortunately, huge
losses of managed honey bee colonies were reported worldwide (van Engelsdorp et al., 2008; van Engelsdorp et al., 2009, Bacandritsos et al., 2010; van Engelsdorp et al., 2012; Lee et al., 2015; Antúnez et al., 2016; Kulhanek et al., 2017; Brodschneider et al., 2018), but no single factor was confirmed to be a certain cause of colony mortality (van Engelsdorp et al., 2009), although the mite *Varroa destructor* and associated viruses have most often been cited (Francis, Nielsen & Kryger, 2013; McMenamin & Genersch, 2015; Steinhauer et al., 2018).

More than 22 honey bee viruses have been identified and described so far (Genersch, 2010;), which exist or co-exist in individual bees or colonies, but may remain unnoticed (Chen & Siede, 2007; Brutscher, McMenamin & Flenniken, 2016). However, several viruses transferred by *V. destructor* considered to pose increasing risk to colonies’ health (Martin et al., 2012) including deformed wing virus (DWV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV) and the sacbrood virus (SBV), all of them seeming to have worldwide occurrence and distribution (Genersch, 2010; Simeunović et al., 2014a; Brutscher, McMenamin & Flenniken, 2016).

In Southeastern Europe, the presence and prevalence of bee viruses have been investigated in Hungary, Slovenia and Croatia (Bakonyi et al., 2002; Forgách et al., 2008; Toplak et al., 2012; Tlak Gajger et al., 2014; Tlak Gajger, Bičak & Belužić, 2014). In Serbia, four honey bee viruses were reported: ABPV, Egypt bee virus J strain (EBV), cloudy wing virus (CWV) and the black queen cell virus (BQCV) by Kulinčević, Ball & Mladjan (1990), and DWV and ABPV by Simeunović et al. (2014a). However, due to the lack of information on the prevalence of SBV and CBPV, as well as the long time which passed since the previous investigations necessity demands newer research.

There is limited information about the relation between colony strength and presence of bee viruses. The present study was aimed at (1) surveying the prevalence of DWV, SBV, ABPV and CBPV in honey bee colonies of different strength in Serbia; (2) exploring the differences between virus prevalence/intensity of infection and colony strength; (3) phylogenetic analyses to reveal the relationship between viruses found in Serbia and those deposited in GenBank.

**Materials and methods**
One hundred and fifty colonies were sampled from 32 apiaries (approximately five colonies per apiary) located in five administrative regions of Serbia (Fig. 1) in autumn (in period from September 25 to October 5) 2017. On each apiary two strong, one medium and two weak colonies were chosen. Colony strength assessment and classification were done as in Cavigli et al. (2016). The selected colonies were without visible signs of any disease. About a hundred workers, both foragers and house bees were chosen for each sample, placed in sterile test tubes on dry ice, and stored at -20°C until being processed. Thirty randomly selected specimens taken from each bee sample were pulverized and homogenized in 5 mL of PBS solution. After centrifugation, from 140 μL of the supernatant the RNA was extracted with ZR Viral RNA Kit™ (Zymo Research, Orange, CA).

The obtained sequences were amplified in Rotor-Gene Q 5plex (Qiagen, Germany) and the target viruses detected with the Rotor-Gene Probe RT-PCR Kit (Qiagen, Germany), in separate single-step reactions. The primer pairs and probes for DWV, ABPV and SBV (Table 1) were the same as used by Chantawannakul et al. (2006) and for CBPV those deployed by Blanchard et al. (2007). The final primer concentration 800 nM and probe concentration of 400 nM proved optimum. The analyses were done in conditions defined in the work of Simeunović et al. (2014a). With each set of sample reactions standard dilutions of the control sample were run and a threshold level set according to the standard curve obtained.

Selected RNA isolates were subjected to endpoint RT-PCRs using primer pairs and following the recommendations from ANSES (2011). The sequencing of each amplicon was done in both orientations in ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

The obtained partial nucleotide (nt) sequences of honeybee viruses were identified by the BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank database. Sequences encoding a partial coding sequence (cds) of polyprotein gene of DWV, a capsid protein gene of ABPV, a partial cds of RNA-dependent RNA polymerase (RdRp) gene of CBPV, and a partial cds of polyprotein gene of SBV were recovered. They were used for phylogenetic analyses along with related sequences deposited in GenBank. The best models of sequence evolution according to the Bayesian Information Criterion assessed MEGA version 6 (Tamura et al., 2007) were as follows: T92 + G for DWV and SBV, T92 + I for ABPV, and K2 + G + I for
CBPV. Evolutionary relations assessed using these models of sequence evolution and the Neighbor-Joining (NJ) algorithm were shown as phylograms. Statistical support was tested with 1000 nonparametric bootstrap (BS) replicates, with 50% ≥ BS ≤ 74% considered moderate support, and BS ≥ 75% considered good support.

Statistical analysis

Depending on data characteristics (testing for normality), the results were presented through the mean and standard deviation, or the median and interquartile range were used. Differences were tested using ANOVA, t-test, or, where appropriate, non-parametric Mann-Whitney U test and Kruskal-Wallis test. Pearson chi-square analysis (or Fisher's exact test) were applied where necessary. Data analysis was performed using IBM SPSS Statistics ver. 21.0 software (IBM, USA).

Results

All of the four viruses in examined samples of adult bees were detected. Their prevalence differed depending on the region of sampling (Fig. 1). In 150 honey bee samples (colonies), the prevalence of these four viruses was as follows: 74% of DWV, 49.30% of ABPV, 24.00% of SBV and 6.70% of CBPV. Samples negative for all four viruses comprised 12.67% of the colonies investigated (Fig. 2).

In 87.33% of samples analysed at least one virus was detected. Single infection was found in 28.67% of colonies (DWV, ABPV, SBV and CBPV in 21.33%, 4.00%, 2.67% and 0.67% colonies, respectively, Table 2). The majority of colonies (58.66%) were found to be infected with more than one virus. DWV had the highest prevalence in all regions (66.70-83.30%), while the least prevalent virus was CBPV (0-19%). Except for DWV, the prevalence of the remaining three viruses was significantly different between different regions (χ² test: DWV $P=0.554$, ABPV $P=0.001$, SBV $P<0.001$, CBPV $P=0.030$).

The prevalence of each virus in weak, medium and strong colonies is shown in Fig. 3. DWV was most prevalent in strong colonies (78%), followed by weak (72.90%) and medium colonies (68.80%). The highest number of ABPV-positive samples was recorded in medium colonies (62.50%), followed by strong (49.20%) and weak colonies (42.40%). SBV was found in 25.40%
of weak colonies, 25.00% of medium ones and in 22.00% of strong colonies. CBPV were found in 9.40% of medium colonies, 8.50% weak and 3.40% strong colonies. No significant differences were recorded in the prevalence of DWV, ABPV, SBV and CBPV infections (and their combinations) between weak, medium and strong colonies were recorded with the \( \chi^2 \) test (Fig. 3). The significance of differences in virus infection levels (expressed through Ct values) between colonies of different strength were also tested. In order to avoid any confounding factor originated from the presence of other viruses in multiple infections, only single infections were taken into consideration in the data analysis. The numbers of samples with single ABPV, SBV and CBPV infections were not statistically valid for the comparison of their Ct values in strong, medium and weak colonies; therefore, only DWV infection intensity was eligible for testing in respect to colony strength. No significant differences were found between DWV infection levels in colonies of different strength (Fig. 4; ANOVA, \( F=0.681, P=0.513 \)). In addition, the results presented in Fig. 5 show that DWV Ct values significantly differ between single DWV infection and double infections caused by DWV and ABPV, SBV or CBPV (ANOVA, \( F=7.510, P<0.001 \)).

**Phylogenetic analyses**

Phylogenetic trees showing evolutionary relations between Serbian and worldwide honeybee viruses DWV, ABPV, SBV and CBPV are shown in Figs. 6-9, respectively.

Nine DWV sequences detected in Serbian honeybees were deposited in GenBank: Serbia D1 (GenBank Access. No. KM001902); Serbia D2 (Access. No. KM001903); Serbia D3 (Access. No. KM001904); Serbia D4 and D5 (Access. No. KM001905, these two sequences were identical and thus they were deposited in the GenBank under the same accession number); Serbia D6 (Access. No. KM001906); Serbia D7 (Access. No. KM001907); Serbia D8 (Access. No. KM001908); and Serbia D9 (Access. No. KM001909). BLAST search found 99 to 98% nucleotide identities with DWV sequences in the database. Eighteen additional DWVs sequences from the GenBank were used for phylogenetic analysis, and VDVs (AY251269 and JF440525) were used as outgroups to root the tree. The length of the aligned matrix was 420 nt. Evolutionary relations of studied DWVs are shown in Fig. 6. Five Serbian DWVs organized into
two moderately supported clusters, comprising three and two sequences, respectively, were closely related to DWVs from the United Kingdom, while others were dispersed throughout the tree.

Two Serbian ABPV sequences, KL4 and KL5, were identical, and thus, they were deposited in the GenBank under the same accession number, KM001899. They showed 97 to 93% nucleotide identities to ABPV sequences in the database. Ten additional European ABPV sequences from the GenBank were used for phylogenetic analysis, and KBV (AY452696) was used as outgroup to root the tree shown in Fig. 7. The length of the aligned matrix was 398 nt. Serbian ABPVs were closely related to the Hungarian ones while Western and Northern European viruses formed separate clusters.

Three identical Serbian SBV sequences, S1, KL2 and KL25, deposited in the GenBank under the same accession number, KM001901, showed 99 to 94% sequence identity rates with other SBVs in the database. Seventeen additional SBVs from the GenBank were used for phylogenetic analysis. The length of the aligned matrix was 570 nt, and the recovered tree is shown in Fig. 8. Three Serbian SBVs cluster together with SBVs from the continental Europe.

Two identical Serbian CBPV sequences, CBPV-1 and CBPV-3 deposited in the GenBank under the same accession number, KM001900, show 96 to 93% sequence identity with other CBPVs in the GenBank. The length of the aligned matrix comprising Serbian and ten additional CBPVs was 429 nt. The relations of studied CBPVs are shown in Fig 9.

**Discussion**

In the era of intensive agriculture and serious decline in pollinator populations worldwide, primarily honey bees (Goulson et al., 2015), it is of great importance to gain an insight into the distribution and prevalence of factors most often connected with bee losses in any geographic region (van Engelsdorp et al., 2008; van Engelsdorp et al., 2009; van Engelsdorp et al., 2012; Cavigli et al., 2016). In this study, samples from clinically healthy colonies in Serbian apiaries were analysed by real-time RT-PCR in order to detect honey bee viruses (DWV, ABPV, SBV and CBPV) and determine their prevalence patterns and prevalence. The results revealed DWV
to be the most prevalent virus in Serbian apiaries, not unlike in many other countries: Hungary (Bakonyi et al., 2002), France (Tentcheva et al., 2004; Mouret et al., 2013), Austria (Berenyi et al., 2006), Slovenia (Toplak et al., 2012) and Uruguay (Giacobino et al., 2016). High prevalence of DWV (74%) and ABPV (49.3%) recorded in Serbian apiaries are not surprising, knowing their close relation to *V. destructor* mite infestation and their persistence as subclinical infection in apparently healthy colonies (Gauthier et al., 2007; Mouret et al., 2013; Wells et al., 2016). The average prevalence of SBV in Serbian samples was 24%, and none of the investigated colonies exhibited signs of sacbrood disease. The absence of disease signs in all recorded SBV-positive colonies may be the result of prominent hygienic behaviour (Swanson et al., 2009), previously confirmed for honey bees throughout Serbia (Stanimirović et al., 2002; Stanimirović, Stevanović & Ćirković, 2005; Stanimirović et al., 2008; Stanimirović et al., 2011). The frequency of SBV in Serbia is similar with 40.24% recorded in Croatia (TlakGajger et al., 2014), considerably lower than 86% from France (Tentcheva et al., 2004) and 100% from Uruguay (Antúnez et al., 2006), but several times higher than 1.1%, 1.4% and 2% reported in Spain (Antúnez et al., 2012), England (Baker & Schroeder, 2008) and Hungary (Forgach et al., 2008), respectively. Low prevalence of CBPV in the samples is typical for asymptomatic colonies (Tentcheva et al., 2004). The rate of 0-19% CBPV-positive samples affirmed in Serbia is in accordance with the results obtained in the majority of Austrian federal states (Berenyi et al., 2006), Chinese provinces (Ai, Yan, & Han, 2012), Korea (Choe et al., 2012), Slovenia (Toplak et al., 2012), and the apiaries from Denmark (Nielsen, Nicolaisen & Kryger, 2008) and France (Tentcheva et al., 2004).

Among monitored honey bee viruses in Serbia, the highest incidence was recorded for DWV (66.7-83.3%). No significant differences in its prevalence among Serbian regions is not surprising knowing its global occurrence (Wilfert et al., 2016) and its dominance over other viruses in variable environmental conditions (Giacobino et al., 2016).

The second most common virus in Serbian apiaries was ABPV, but its incidence (16.7-68.2%) significantly varied between the regions. The prevalence of SBV and CBPV also displayed dissimilar patterns in environmentally different regions. Additional investigations are necessary to explain the observed significant differences. It can be assumed that these results may reflect the beekeepers’ negligence of apicultural measures (Stanimirovic et al., 2007a), but also may have risen from different means of *V. destructor* control (Nielsen, Nicolaisen & Kryger, 2008),
which may be the reason only in ABPV infection, since not all viruses are transmitted by varroa mites (Glenny et al., 2017).

Nevertheless, differences in orographic factors and forage quality between regions should be also considered as the environment was suggested as a key factor interacting with local bee populations and ecogenotypes (Stanimirović, Stevanović & Ćirković; 2005; Giacobino et al., 2016). Our results concerning 87.33% samples with at least one virus and 58.66% with two or more are similar to those observed in Austria (Berenyi et al., 2006), France (Tentcheva et al., 2004; Gauthier et al., 2007) and Slovenia (Toplak et al., 2012).

Interestingly, no significant differences were found in the presence of DWV, ABPV, SBV and CBPV infections (and their combinations) in colonies of various strength. In addition, no significant differences were affirmed between single DWV infection levels (expressed through a Ct value) in colonies of different strength. These results may speak in favour of crucial influence of predisposing factors - pathogens, parasites, poor-quality nutrition, pesticides, and unfavourable climate conditions - on bee vitality (Stanimirović et al., 2007a; Simeunović et al., 2014b, Abbo et al., 2017; Annoscia et al., 2018, Glavinic et al., 2017, Stevanović et al., 2016).

Special emphasis should be put on the negative influence of infestation with *V. destructor*, a biological and mechanical vector of at least two viruses, DWV and APBV, (Ryabov et al., 2014; Abbo et al., 2017) and a possible factor that could contribute *Nosema ceranae* spreading (Glavinić et al., 2014:). In addition, we may assume that bees highly infected with viruses do not return from the field committing "altruistic suicide“ to regulate colony virus load as in cases of *V. destructor* and/or *N. ceranae* infected bees (Kralj & Fuchs, 2006; Higes et al., 2008). In our study, the presence of another virus(es), ABPV, SBV or CBPV, in co-infections significantly influenced the intensity of DWV infection. The observed differences in DWV Ct values between co-infections and single DWV infections could be explained with the influence of simultaneous replication of the another present virus, wherein the influence may be stimulatory or suppressive. However, we should have in mind recent characterization of DWV master variants (DWV-A, DWV-B, and DWV-C) and their impact on bee health (McMenamin & Flenniken, 2018).

Very small percentages of multiple infections in comparison with single infections found in this study point out the possibility that the former are related with severe *V. destructor* infestations commonly observed in Serbian apiaries (Stanimirović et al., 2007a, 2017).
High identity rates among relatively short studied nucleotide sequences of DWVs account for the poorly supported and unresolved phylogenetic tree (Fig.6). However, the observed close genetic distance between all DWVs is concordant with the hypothesis of their relatively recent evolutionary diversification and worldwide spread, potentially connected to the geographic expansion of their main vector, *V. destructor* (Berenyi et al., 2007; Wilfert et al., 2016). On the other hand, Serbian and Hungarian ABPVs are closely related, and this may be explained by the geographical vicinity and trade between beekeepers of the two countries. Both Serbian and Hungarian ABPVs are relatively distant from those from the Western and Northern Europe, and this finding is in accordance with the report of Bakonyi et al. (2002) that Hungarian ABPVs are not closely related with Western and Northern European ABPVs. Although Serbian SBVs are closely related with SBVs from the continental Europe, further analysis, involving sequences from neighbouring countries, are required for determining whether similar separation exists with SBVs, as it has been affirmed in case of Serbian ABPV. CBPVs from Serbia, France, Belgium and Spain are monophyletic but Serbian CBPVs occupy a rather long branch indicating a non-negligible genetic distance between Serbian and mentioned CBPVs. These findings may indicate that CBPV (which is taxonomically and genetically very different from the other three honey bee viruses analysed in this study), may have different epizootiological character, and hence, is less intensively involved in the geographical spread of honey bee virus strains (Ribière, Olivier & Blanchard, 2010). Alternatively, unique genetic properties (higher mutation rate or segment rearrangements) may explain the genetic seclusion of the Serbian CBPVs. However, for better understanding of viral diversity in honey bee colonies, additional analyses are needed. This is in accordance with the opinion of Galbraith et al. (2018), who also emphasized the importance of virus development dynamics and its possible impact on honey bees. Studies on bee pathogens causing colony decline in Serbia were mainly focused on *Nosema sp.* (Stanimirović et al., 2007b; Stevanovic et al., 2011, Stevanović et al., 2013; Glavinić et al., 2014; Simeunovic et al., 2014b) and *V. destructor* (Stanimirović et al., 2002, Stanimirović, Stevanović & Ćirković, 2005; Stanimirović et al., 2005; Stevanovic et al., 2008; Stanimirović et al., 2011; Radakovic et al., 2013; Gajic et al., 2013; Glavinić et al., 2014; Stanimirović et al., 2017) with only one study dealing with bee viruses (Simeunović et al., 2014a). Therefore, our work represents an important contribution towards better understanding of bee pathogens in Serbia.
Conclusions

This work represents the first thorough investigation aimed at the constitution of the epidemiological baseline regarding molecular identification, prevalence patterns and prevalence of honey bee viruses in Serbia. The geographic origin and strength of honey bee colonies in Serbia proved to be insufficient to induce significant differences in the prevalence of the investigated viruses. Infection intensity of DWV presented through Ct value greatly depends on the presence of co-infection with other viruses. However, single ABPV, SBV and CBPV infection were not frequent enough to allow the comparison of their Ct values. In addition, the sequence analyses of Serbian honey bee viruses confirmed their identity and enabled an insight into their phylogenetic relationship with those found worldwide.

References

Abbo PM, Kawasaki JK., Hamilton M, Cook SC, DeGrandi-Hoffman G, Li W F, Liu J, Chen YP. 2017. Effects of Imidacloprid and Varroa destructor on survival and health of European honey bees, Apis mellifera. Insect Science 24:467-477 DOI 10.1111/1744-7917.12335.

Ai H, Yan X, Han R. 2012. Occurrence and prevalence of seven bee viruses in Apis mellifera and Apis cerana apiaries in China. Journal of Invertebrate Pathology 109:160-164 DOI 10.1016/j.jip.2011.10.006.

Annoscia D, Brown S, Di Prisco G, De Paoli E, Del Fabbro S, Zanni V, Galbraith D, Caprio E, Grozinger CM, Pennacchio F, Nazi, F. 2018. Haemolymph removal by the parasite Varroa destructor can trigger the proliferation of the Deformed Wing Virus in mite infested bees (Apis mellifera), contributing to enhanced pathogen virulence. bioRxiv p.257667 DOI 10.1101/257667.

ANSES - French Agency for Food, Environmental and Occupational Health and Safety. 2011. Screening for several honeybee viruses using PCR (in-house method) (ABPV, IAPV, KBV, DWV, SBV, BQCV, CBPV)) Available at http://www.anse.fr/en (accessed 5 July 2017).
Antúnez K, D’Alessandro B, Corbella E, Ramallo G, Zunino P. 2006. Honeybee viruses in Uruguay. *Journal of Invertebrate Pathology* 93:67–70 DOI 10.1016/j.jip.2006.05.009.

Antúnez K, Anido M, Garrido-Bailon E, Botias C, Zunino P, Martínez-Salvador A, Higes M. 2012. Low prevalence of viruses in Spain during 2006 and 2007. *Research in Veterinary Science* 93:1441-1445 DOI 10.1016/j.rvsc.2012.03.006.

Antúnez K, Invernizzi C, Mendoza Y, Zunino P. 2016. Honeybee colony losses in Uruguay during 2013–2014. *Apidologie* 48:364-370 DOI 10.1007/s13592-016-0482-2.

Bacandritsos N, Granato A, Budge G, Papanastasiou I, Roinioti E, Cordon M, Falcaro C, Gallina A. Mutinelli F. 2010. Sudden deaths and colony population decline in Greek honey bee colonies. *Journal of Invertebrate Pathology* 105:335-340 DOI 10.1016/j.jip.2010.08.004.

Baker A, Schroeder DD. 2008. Occurrence and genetic analysis of picorna-like viruses infecting worker bees of *Apis mellifera* L. populations in Devon, South West England. *Journal of Invertebrate Pathology* 98:239–242 DOI 10.1016/j.jip.2008.02.010.

Bakonyi T, Farkas R, Szendroi A, Dobos-Kovacs M, Rusvai M. 2002. Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* field samples: rapid screening of representative Hungarian apiaries. *Apidologie* 33:63-74 DOI 10.1051/apido:2001004.

Berenyi O, Bakonyi T, Derakhshifar I, Köglberger H, Nowotny N. 2006. Occurrence of six honeybee viruses in diseased Austrian apiaries. *Applied and Environmental Microbiology* 72:2414–2420 DOI 10.1128/AEM.72.4.2414-2420.2006.

Berenyi O, Bakonyi T, Derakhshifar I, Köglberger H, Topolska G, Ritter W, Pechhacker H, Nowotny N. 2007. Phylogenetic analysis of deformed wing virus genotypes from diverse
geographic origins indicates recent global distribution of the virus. *Applied and Environmental Microbiology* 73:3605–3611 DOI 10.1128/AEM.00696-07.

Blanchard P, Ribiere M, Celle O, Lallemand P, Schurr F, Olivier V, and Faucon JP. 2007. Evaluation of a real-time two-step RT-PCR assay for quantitation of Chronic bee paralysis virus (CBPV) genome in experimentally-infected bee tissues and in life stages of a symptomatic colony. *Journal of Virological Methods* 141:7-13 DOI 10.1016/j.jviromet.2006.11.021.

Brodschneider R, Gray A, Adjlane N, Ballis A, Brusbardis V, Charrière JD, Chlebo R, Coffey MF, Dahle B, de Graaf DC, Dražić MM, Evans G, Fedoriak M, Forsythe I, Gregorc A, Grzęda U, Hetzroni A, Kauko L, Kristiansen P, Martikkala M, Martin-Hernández R, Medina-Flores CA, Mutinelli F, Raudmets A, Ryzhikov VA, Simon-Delso N, Stevanovic J, Uzunov A, Vejsnæs F, Wöhl S, Zammit-Mangion M, Danihlik J. 2018. Multi-country loss rates of honey bee colonies during winter 2016/2017 from the COLOSS survey. *Journal of Apicultural Research* 57:452-457, DOI: 10.1080/00218839.2018.1460911.

Brutscher LM, McMenamin AJ, Flenniken ML. 2016. The buzz about honey bee viruses. *PLoS Pathogens* 12:e1005757 DOI 10.1371/journal.ppat.1005757.

Cavigli I, Daughenbaugh KF, Martin M, Lerch M, Banner K, Garcia E, Brutscher LM, Flenniken ML. 2016. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. *Apidologie* 47:251-266 DOI 10.1007/s13592-015-0395-5.

Chantawannakul P, Ward L, Boonham N, Brown M. 2006. A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Tai honeybee (Apis mellifera) apiary. *Journal of Invertebrate Pathology* 91:69–73 DOI 10.1016/j.jip.2005.11.001.

Chen YP, Siede R. 2007. Honey bee viruses. *Advances in Virus Research* 70:33-80 DOI 10.1016/S0065-3527(07)70002-7.
Choe SE, Nguyen LTK, Noh JH, Koh HB, JeanYH, Kweon CH. Kang SW. 2012. Prevalence and distribution of six bee viruses in Korean *Apis cerana* populations. *Journal of Invertebrate Pathology* **109**:330-333 DOI 10.1016/j.jip.2012.01.003

Forgách P, Bakonyi T, Tapaszti Z, Nowotny N. Rusvai M. 2008. Prevalence of pathogenic bee viruses in Hungarian apiaries: situation before joining the European Union. *Journal of Invertebrate Pathology* **98**:235-238 DOI doi.org/10.1016/j.jip.2007.11.002.

Francis RM, Nielsen SL, Kryger P. 2013. Varroa-virus interaction in collapsing honey bee colonies. *PLoS One* **8**:e57540 DOI 10.1371/journal.pone.0057540.

Gajic B, Radulovic Z, Stevanovic J, Kulisić Z, Vucicevic M, Simeunovic P, Stanimirovic Z. 2013. Variability of the honey bee mite *Varroa destructor* in Serbia, based on mtDNA analysis. *Experimental and Applied Acarology* **61**:97-105 DOI 10.1007/s10493-013-9683-9.

Galbraith DA, Fuller, ZL, Ray AR, Brockmann A, Frazier M, Gikungu MW, Martinez FI, Kapheim KM, Kerby JT, Kocher SD, Losyev O, Muli E, Patch HM, Rosa C, Sakamoto JM, Stanley A, Vaudo AD, Grozinger CM. 2018. Investigating the viral ecology of global bee communities with high-throughput metagenomics. *Scientific Reports* **8**:8879 DOI 10.1038/s41598-018-27164-z.

Gauthier L, Tentcheva D, Tournaire M, Dainat B, Cousserans F, Colin ME, Bergoin M. 2007. Viral load estimation in asymptomatic honey bee colonies using the quantitative RT-PCR technique. *Apidologie* **38**:426-435 DOI 10.1051/apido:2007026.

Genersch E. 2010. Honey bee pathology: current threats to honey bees and beekeeping. *Applied Microbiology and Biotechnology* **87**:87–97 DOI 10.1007/s00253-010-2573-8.
Giacobino A, Molineri AI, Pacini A, Fondevila N, Pietronave H, Rodriguez G, Palacio A, Bulacio Cagnolo N, Orellano E, Salto CE, Signorini ML, Merke J. 2016. *Varroa destructor* and viruses association in honey bee colonies under different climatic conditions. *Environmental Microbiology Reports* **8**:407-412 DOI 10.1111/1758-2229.12410.

Glavinić U, Stevanović J, Gajić B, Simeunović P, Đurić S, Vejnović B, Stanimirović Z. 2014. *Nosema ceranae* DNA in honey bee haemolymph and honey bee mite *Varroa destructor*. *Acta Veterinaria-Beograd* **64**:349-357 DOI 10.2478/acve-2014-0033.

Glavinic U, Stankovic B, Draskovic V, Stevanovic J, Petrovic T, Lakic N Stanimirovic Z. 2017. Dietary amino acid and vitamin complex protects honey bee from immunosuppression caused by *Nosema ceranae*. *PLoS ONE* **12**:e0187726 DOI 10.1371/journal.pone.0187726.

Glenny W, Cavigli I, Daughenbaugh KF, Radford R, Kegley SE, Flenniken ML. 2017. Honey bee (*Apis mellifera*) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. *PLoS One* **12**:e0182814 DOI 10.1371/journal.pone.0182814.

Goulson D, Nicholls E, Botias C, Rotheray EL. 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**:1255957 DOI 10.1126/science.1255957.

Higes M, Martín-Hernández R, Botías C, Bailón EG, González-Porto AV, Barrios L, Del Nozal MJ, Bernal JL, Jiménez JJ, Palencia PG, Meana A. 2008. How natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environmental Microbiology* **10**:2659-2669 DOI 10.1111/j.1462-2920.2008.01687.x.

Kralj J, Fuchs S. 2006. Parasitic mites influence flight duration and homing ability of infested *Apis mellifera* foragers. *Apidologie* **37**:577–587 DOI 10.1051/apido:2006040.
Kulhanek K, Steinhauer N, Rennich K, Caron DM, Sagili RR, Pettis JS, Ellis JD, Wilson ME, Wilkes JT, Tarpy DR, Rose R, Lee K, Rangel J, vanEngelsdorp D. 2017. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. *Journal of Apicultural Research* **56**:328-340 DOI 10.1080/00218839.2017.1344496.

Kulinčević J, Ball B, Mladjan V. 1990. Viruses in honey bee colonies infested with *Varroa jacobsoni*: first findings in Yugoslavia. *Acta Veterinaria Beograd* **40**:37–42.

Lee K, Steinhauer N, Rennich K, Wilson ME, Tarpy DR, Caron DM, Rose R, Delaplane KS, Baylis K, Lengerich EJ., Skinner JA, Wilkes JT, Sagili R, vanEngelsdorp D. 2015. A national survey of managed honey bee 2013–2014 annual colony losses in the USA. *Apidologie* **46**:292–305 DOI 10.1007/s13592-015-0356-z.

Martin SJ, Highfield AC, Brettell L, Villalobos EM, Budge GE, Powell M, Schroeder DC. 2012. Global honey bee viral landscape altered by a parasitic mite. *Science* **336**:1304-1306 DOI 10.1126/science.1220941.

McMenamin AJ, Genersch E. 2015. Honey bee colony losses and associated viruses. *Current Opinion in Insect Science* **8**:121-129 DOI [10.1016/j.cois.2015.01.015](http://dx.doi.org/10.1016/j.cois.2015.01.015).

McMenamin AJ, Flenniken ML. 2018. Recently identified bee viruses and their impact on bee pollinators. *Current Opinion in Insect Science* **26**:120-129 DOI 10.1016/j.cois.2018.02.009.

Mouret C, Lambert O, Piroux M, Beaudeau F, Provost B, Benet P, Colin ME, L'Hostis M. 2013. Prevalence of 12 infectious agents in field colonies of 18 apiaries in Western France. *Revue de Medecine Veterinaire* **164**:577–582.

Nielsen SL, Nicolaisen M, Kryger P. 2008. Incidence of acute bee paralysis virus, black queen cell virus, chronic bee paralysis virus, deformed wing virus, Kashmir bee virus and
sacbrood virus in honey bees (*Apis mellifera*) in Denmark. *Apidologie* 39:310-314 DOI 10.1051/apido:2008007.

Radakovic M, Stevanovic J, Djelic N, Lakic N, Knezevic-Vukcevic J, Vukovic-Gacic B, Stanimirovic Z. 2013. Evaluation of the DNA damaging effects of amitraz on human lymphocytes in the Comet assay. *Journal of Biosciences* 38:53-62 DOI 10.1007/s12038-012-9287-2.

Ribière M1, Olivier V, Blanchard P. 2010. Chronic bee paralysis: a disease and a virus like no other? *Journal of Invertebrate Pathology* 103: S120-S131 DOI 10.1016/j.jip.2009.06.013.

Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, Mead A, Burroughs N, Evans JD. 2014. A virulent strain of deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after *Varroa destructor*-mediated, or in vitro, transmission. *PLoS Pathog.*, 10:e1004230 DOI 10.1371/journal.ppat.1004230.

Simeunović P, Stevanović J, Vidanović D, Nišavić J, Radović D, Stanišić Lj, Stanimirović Z. 2014a. A survey of deformed wing virus and acute bee paralysis virus in honey bee colonies from Serbia using real-time RT-PCR. *Acta Veterinaria-Beograd* 64:81-92 DOI: 10.2478/acve-2014-0009.

Simeunovic P, Stevanovic J, Cirkovic D, Radojicic S, Lakic N, Stanisic Lj, Stanimirovic Z. 2014b. *Nosema ceranae* and queen age influence the reproduction and productivity of the honey bee colony. *Journal of Apicultural Research* 53:545-554 DOI 10.3896/IBRA.1.53.5.09.

Stanimirović Z, Pejović D, Stevanović J, Vučinić M, Mirilović M. 2002. Investigations of hygienic behaviour and disease resistance in organic beekeeping of two honeybee ecogeographic varieties from Serbia. *Acta Veterinaria-Beograd* 52:169-180 DOI 10.2298/AVB0203169S.
Stanimirović Z, Stevanović J, Ćirković D. 2005. Behavioural defenses of the honey bee ecotype from Sjenica – Pester against Varroa destructor. *Acta Veterinaria-Beograd* **55**:69-82 DOI 10.2298/AVB0501069S.

Stanimirović Z, Stevanovic J, Jovanovic S, Andjelkovic M. 2005. Evaluation of genotoxic effects of Apitol® (cymiazole hydrochloride) *in vitro* by measurement of sister chromatid exchange. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **588**:152-157 DOI 10.1016/j.mrgentox.2005.10.003.

Stanimirović Z, Ćirković D, Pejin II, Pejović D. 2007a. Strategy for ecologic control in fighting Varroa destructor. *Veterinarski glasnik* **61**:11-35 [in Serbian].

Stanimirović Z, Stevanovic J, Bajic V, Radovic I. 2007b. Evaluation of genotoxic effects of fumagillin by cytogenetic tests *in vivo*. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **628**:1-10 DOI 10.1016/j.mrgentox.2006.09.014.

Stanimirović Z, Stevanović J, Mirilović M, Stojić V. 2008. Heritability of hygienic behaviour in grey honey bees (*Apis mellifera carnica*). *Acta Veterinaria-Beograd* **58**:593-601 DOI 10.2298/AVB0806593S.

Stanimirović Z, Aleksić N, Stevanović J, Ćirković D, Mirilović M, Djelić N, Stojić V. 2011. The influence of pulverised sugar dusting on the degree of infestation of honey bee colonies with Varroa destructor. *Acta Veterinaria-Beograd* **61**:309-325 DOI 10.2298/AVB1103309S.

Stanimirović Z, Glavinić U, Lakić N, Radović D, Ristanić M, Tarić E, Stevanović J. 2017. Efficacy of plant-derived formulation “Argus Ras” in Varroa destructor control. *Acta Veterinaria-Beograd* **67**:191-200 DOI 10.1515/acve-2017-0017.
Steinhauer N, Kulhanek K, Antúnez K, Human H, Chantawannakul P, Chauzat MP, vanEngelsdorp D. 2018. Drivers of colony losses. *Current Opinion in Insect Science* **26**:142-148 DOI 10.1016/j.cois.2018.02.004.

Stevanovic J, Stanimirovic Z, Radakovic M, Stojic V. 2008. In vitro evaluation of the clastogenicity of fumagillin. *Environmental and Molecular Mutagenesis* **49**:594-601 DOI 10.1002/em.20409.

Stevanovic J, Stanimirovic Z, Genersch E, Kovacevic SR, Ljubenkovic J, Radakovic M, Aleksic N. 2011. Dominance of *Nosema ceranae* in honey bees in the Balkan countries in the absence of symptoms of colony collapse disorder. *Apidologie* **42**:49–58 DOI 10.1051/apido/2010034.

Stevanovic J, Simeunovic P, Gajic B, Lakic N, Radovic D, Fries I, Stanimirovic Z. 2013. Characteristics of *Nosema ceranae* infection in Serbian honey bee colonies. *Apidologie* **44**, 522-536 DOI 10.1007/s13592-013-0203-z.

Stevanovic J, Schwarz RS, Vejnovic B, Evans J, Irwin RE, Glavinic U, Stanimirovic Z. 2016. Species-specific diagnostics of *Apis mellifera* trypanosomatids: a nine-year survey (2007-2015) for trypanosomatids and microsporidians in Serbian honey bees. *Journal of Invertebrate Pathology* **139**:6-11 DOI 10.1016/j.jip.2016.07.001.

Swanson JA, Torto B, Kells SA, Mesce KA, Tumlinson JH, Spivak M. 2009. Odorants that induce hygienic behavior in honeybees: identification of volatile compounds in chalkbrood-infected honeybee larvae. *Journal of Chemical Ecology* **35**:1108-1116 DOI 10.1007/s10886-009-9683-8.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**:1596-1599 DOI 10.1093/molbev/msm092.
Tentcheva D, Gauthier L, Zappulla N, Dainat B, Cousserans F, Colin ME, Bergoin M. 2004. Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. *Applied and Environmental Microbiology* 70:7185–7191 DOI 10.1128/AEM.70.12.7185-7191.2004.

Tlak Gajger I, Kolodziejek J, Bakonyi T, Nowotny N. 2014. Prevalence and distribution patterns of seven different honeybee viruses in diseased colonies: a case study from Croatia. *Apidologie* 45:701-706 DOI 10.1007/s13592-014-0287-0.

Tlak Gajger I, Bičak J, Belužić R. 2014. The occurrence of honeybee viruses in apiaries in the Koprivnica-Križevci district in Croatia. *Veterinarski Arhiv* 84:421-428.

Toplak I, Rihtarič D, Ciglenečki UJ, Hostnik P, Jenčič V, Barlič-Maganja D. 2012. Detection of six honeybee viruses in clinically affected colonies of Carniolan gray bee (*Apis mellifera carnica*). *Slovenian Veterinary Research* 49:89-96.

van Engelsdorp D, Hayes J, Underwood RM, Pettis J, 2008. A survey of honey bee colony losses in the US, fall 2007 to spring 2008. *PLoS ONE* 3:e4071 DOI 10.1371/journal.pone.0004071.

van Engelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruege E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y, Underwood R, Tarpy DR, Pettis JS. 2009. Colony collapse disorder: a descriptive study. *PLoS ONE* 4:e6481 DOI 10.1371/journal.pone.0006481.

van Engelsdorp D, Caron D, Hayes J, Underwood R, Henson M. 2012. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. *Journal of Apicultural Research* 51:115–124 DOI 10.3896/IBRA.1.51.1.14.
Venturini E, Drummond FA, Hoshide AK, Dibble AC, Stack LB. 2017. Pollination reservoirs in lowbush blueberry (Ericales: Ericaceae). *Journal of Economic Entomology* 110:333-346 DOI 10.1093/jee/tow285.

Wells T, Wolf S, Nicholls E, Groll H, Lim KS, Clark SJ, Swain J, Osborne JL, Haughton AJ. 2016. Flight performance of actively foraging honey bees is reduced by a common pathogen. *Environmental Microbiology Reports* 8:728-737 DOI 10.1111/1758-2229.12434.

Wilfert L, Long G, Leggett HC, Schmid-Hempel P, Butlin R, Martin SJM. Boots M. 2016. Deformed wing virus is a recent global epidemic in honeybees driven by *Varroa* mites. *Science* 351:594-597 DOI 10.1126/science.aac9976.
Figure 1

Prevalence patterns of investigated viruses in honey bee colonies in five regions of Serbia
Figure 2

Overall prevalence of CBPV, SBV, ABPV and DWV in Serbian bees (analyzed in 150 samples)
Figure 3

Prevalence of DWV, ABPV, SBV, CBPV and their combinations (A-K) in weak, medium and strong colonies
Figure 4

Intensity of DWV infection (Ct values) in weak, medium and strong colonies

ANOVA

\[ F = 0.681 \quad P = 0.513 \]
Figure 5

Intensity of virus infections (Ct values) in single DWV infection and in double infections (DWV+ABPV, DWV+CBPV and DVW+SBV)

ANOVA

\[ F = 7.510 \quad P < 0.001 \]
Figure 6

Neighbour-Joining tree of studied DWV sequences.

The tree was constructed using a 420 nt long aligned matrix of 18 sequences encoding a partial coding sequence (cds) of polyprotein gene of DWVs. VDVs (AY251269 and JF440525) were used as outgroups to root the tree. Viruses are indicated with GenBank Access. Nos. and the country of origin. Numbers at nodes represent bootstrap support. Bar on the left shows the number of nucleotide substitutions per site.
Figure 7

Neighbour-Joining tree of studied ABPV sequences.

The tree was constructed using a 398 nt long aligned matrix of 12 sequences encoding a capsid protein of ABPVs. KBV (AY452696) was used as outgroups to root the tree. Viruses are indicated with GenBank Access. Nos. and the country of origin. Numbers at nodes represent bootstrap support. Bar on the left shows the number of nucleotide substitutions per site.
Figure 8

Neighbour-Joining tree of studied SBV sequences.

The tree was constructed using a 429 nt long aligned matrix of 20 sequences encoding a partial coding sequence (cds) of polyprotein gene of SBVs. Viruses are indicated with GenBank Access. Nos. and the country of origin. Numbers at nodes represent bootstrap support. Bar on the left shows the number of nucleotide substitutions per site.
Figure 9

Neighbour-Joining tree of studied CBPV sequences.

The tree was constructed using a 570 nt long aligned matrix of 12 sequences encoding a partial coding sequence (cds) of RNA-dependent RNA polymerase (RdRp) gene of CBPV. FJ345326 was used as outgroup to root the tree. Viruses are indicated with GenBank Access Nos. and the country of origin. Numbers at nodes represent bootstrap support. Bar on the left shows the number of nucleotide substitutions per site.
**Table 1** (on next page)

Primers and probes (TaqMan Probe®) used for RNA molecular identification of investigated viruses in real-time RT-PCR
**Table 1.** Primers and probes (TaqMan Probe®) used for RNA molecular identification of investigated viruses in real-time RT-PCR

| Primer/Probe name | Sequence | Primer | Virus | Primer/Probe authors |
|-------------------|----------|--------|-------|----------------------|
| DWV958F           | 5'-AAATTCTCTCACAGTCCAAG-3' | Forward | Deformed Wing Virus | Chantawannakul et al., 2006 |
| DWV9711R          | 5'CAACAGGTAATTTTCTTTTAG-3' | Reverse | |
| DWV9627T          | 5'-CATGCTCGAGGATTGGGCGGTGCTG-3' | Probe | |
| APV95F            | 5'-TCCTATATCGACGACGAAAGACAA-3' | Forward | Acute Bee Paralysis Virus | Chantawannakul et al., 2006 |
| APV159R           | 5'-GGGCTTTAATTCCCATCCAATTIGA-3' | Reverse | |
| APV121T           | 5'-TTTTCCCGGACTTGAC-3' | Probe | |
| SBV311F           | 5'-AAGTTGGAGGCGGGYATTG-3' | Forward | Sacbrood Virus | Chantawannakul et al., 2006 |
| SBV380R           | 5'-CAATGTCTCTTACDAAGYAAGATTG-3' | Reverse | |
| SBV331T           | 5'-CGGAATGGAAAGAT-3' | Probe | |
| CBPV1F            | 5'-CGCAAGTACGCTTGATAAAGAAC-3' | Forward | Chronic Bee Paralyses Virus | Blanchard et al., 2007 |
| CBPV2R            | 5'-ACTACTGAAACCTCGCTTTCG-3' | Reverse | |
| CBPVT             | 5'-TCAAGAAGGAGACCACCGCAAGTTG-3' | Probe | |
**Table 2 (on next page)**

Prevalences of single and simultaneous virus infections in honey bee samples from Serbia. DWV - deformed wing virus; CBPV - chronic bee paralysis virus; ABPV - acute bee paralysis virus; SBV - sacbrood virus
Table 2. Prevalences of single and simultaneous virus infections in honey bee samples from Serbia. DWV - deformed wing virus; CBPV - chronic bee paralysis virus; ABPV - acute bee paralysis virus; SBV - sacbrood virus

| No. of viruses in simultaneous infection | Type of infection            | No. of samples | %   |
|----------------------------------------|-----------------------------|----------------|-----|
| 0                                      | /                           | 19             | 12.67 |
| 1                                      | DWV                         | 32             | 21.33 |
|                                         | ABPV                        | 6              | 4.00  |
|                                         | SBV                         | 4              | 2.67  |
|                                         | CBPV                        | 1              | 0.67  |
| 2                                      | DWV, ABPV                   | 44             | 29.33 |
|                                         | DWV, CBPV                   | 6              | 4.00  |
|                                         | DWV, SBV                    | 11             | 7.33  |
|                                         | ABPV, SBV                   | 11             | 7.33  |
| 3                                      | DWV, SBV, ABPV              | 12             | 8.00  |
|                                         | DWV, ABPV, CBPV             | 3              | 2.00  |
| 4                                      | DWV, ABPV, SBV, CBPV        | 1              | 0.67  |