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First detection of black queen cell virus, *Varroa destructor* macula-like virus, *Apis mellifera* filamentous virus and *Nosema ceranae* in Syrian honey bees *Apis mellifera syriaca*

Raied ABOU KUBAA[1,3], Giulia MOLINATTO[2], Bassem SOLAIMAN KHALED[4], Nouraldin DAHER-HJAIJ[5], Khaled HEINOUN[3], Maria SAPONARI[1]

[1]*CNR Istituto per la Protezione Sostenibile delle Piante (IPSP), UOS Bari, Italy*
[2]*DISAFA – Entomologia, Università di Torino, Grugliasco (TO), Italy*
[3]*Ministry of Agriculture and Agrarian Reform, Department of Plant Protection, Damascus, Syria*
[4]*Faculty of Agriculture, Plant Protection Division, Damascus University, Syria*
[5]*General Commission for Scientific Agricultural Research (GCSAR), Administration of Plant Protection Research, Damascus, Syria*

Abstract

The occurrence of honey bee viruses and *Nosema* spp. was investigated by PCR in five honey bee colonies suffering from depopulation, vestigial wings and dark colouring in Rural Damascus city (Syria). Deformed wing virus (DWV), detected in 95.8% of the samples, was the most prevalent virus, followed by *Varroa destructor* macula-like virus (VdMLV), black queen cell virus (BQCV) and *Apis mellifera* filamentous virus (AmFV) (70.8%, 29.2%, 16.7% of infection, respectively). *Nosema ceranae* was detected in two apiaries representing 8.3% of infection in the total tested samples. Simultaneous infections with two, three and four viruses together in the same sample were found. Phylogenetic analyses of the detected pathogens confirmed the high percentages of sequence identity at the nucleotide level with other isolates distributed worldwide. The present study reports the first detection and molecular characterization of VdMLV, BQCV, AmFV and *N. ceranae* in *A. mellifera* colonies with suspected infections in Syria.

Key words: honey bee, viruses, *Varroa destructor*, *Nosema ceranae*, Syria,

Introduction

The Syrian honey bee, *Apis mellifera syriaca* Skorikov (Hymenoptera Apidae), is native to the eastern Mediterranean region and one of the oldest honey bee races. It has the ability to produce honey in high quantities and shows an effective defence strategy against *Vespa orientalis* L. (Ruttner, 1988). Besides the aggressive attitude in defending the hive, typical traits of *A. m. syriaca* are bright yellow pigmentation, small size and nervous behaviour (Zakour *et al*., 2012). The Syrian honey bee is distributed in Syria, Lebanon, Iraq, Jordan, Palestine and the southern part of Turkey. This subspecies is well adapted to extreme hot and dry climates (Zaitoun *et al*., 2000) and has a superior ability to adapt egg laying to pollen availability and honey flow (Haddad and Fuchs, 2004) and considered as the honey bee of the mountain range bordering the eastern Mediterranean Sea (Ftayeh *et al*., 1994).

Beekeeping has a long tradition in Syria and it provided employment and income opportunities as well as economic security for farmers in rural areas. However, since 2011, most of the colonies have been destroyed or neglected, due to the difficulties in reaching the hives, in particular in the governorates of rural Damascus, Hama and Lattakia. Moreover, high queen losses have been reported after the introduction of European queens into the local Syrian colonies with the aim of replacing the indigenous population with more productive breeds. Besides, this practice turned out to be ineffective, as pure European colonies are not much more yielding than the native races under the local conditions and hybrids with Syrian bees are not gentle (Zakour *et al*., 2012).

In recent years, large-scale colony losses have been reported in different countries (Neumann and Carreck, 2010; Moritz and Erles, 2016). It has been proposed that parasites, like the mite *Varroa destructor*, and pathogens, including different RNA viruses and the microsporidia *Nosema ceranae*, could contribute to such losses, in addition to the toxic effects of pesticides and nutritional stress due to habitat depletion (Sanchez-Bayo and Goka., 2014; Doublet *et al*., 2015). Most viruses do not induce symptoms at low levels of infection but can reduce the longevity of bees to varying extents. Moreover, it was demonstrated that the exposure to neonicotinoid insecticides promotes viral replication by altering the honey bee immune system (Di Prisco *et al.*, 2013; Coulon *et al*., 2017).

Up to date, little information exists on the sanitary status of honey bees in Syria, especially as far as virus infection is concerned. Although some viruses produce recognizable symptoms at sufficiently elevated titers, honey bee viruses generally persist naturally in the host population at low levels, without causing overt symptoms, using a variety of transmission routes (Gupta and Reybroeck., 2014). Out of more than 22 viruses known to infect the honey bee, deformed wing virus (DWV), chronic bee paralysis virus (CBPV), acute bee paralysis virus (ABPV) and sacbrood virus (SBV) were the only viruses reported from bees collected in Syria so far (Elbeaino *et al*., 2016; Barhoum *et al.*, 2017). The present study aims at estimating the occurrence of other

economically important honey bee viruses and *Nosema* spp. in some apiaries situated in Rural Damascus city, thus providing insights on possible causes of depopulation of bee colonies.

Materials and methods

In spring 2013, a total of 96 single bees showing crumpled and vestigial wings, decreased body size and some dark colouring were collected from five apiaries located in five different villages in Rural Damascus city in order to investigate whether those symptoms were associated with the presence of pathogens. Collected bees were kept in 70% EtOH in separate test tubes and stored at -20 °C prior to total nucleic acid (TNA) extraction. In the laboratory, heads were separated from the thorax and abdomen using a fresh scalpel for each individual to avoid cross-contamination of RNA/DNA between samples. Heads of two bees were pooled in a 2 ml microcentrifuge tube and conserved at −20 °C until further processing. The frozen samples (48) were crushed using a Mixer Mill MM300 (Retsch, Germany) and homogenized using 1 ml of CTAB buffer (2% Hexadecyl trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 20 mM EDTA and 1.4 M NaCl). After homogenization, samples were incubated at 70 °C and then chloroform-treated (1 ml). TNAs were then isolated by precipitating the supernatant with 0.7 volumes of cold 2-propanol and suspension was done in 100 µl of sterile water for each sample (protocol adapted from Murray and Thompson, 1980). RNA/DNA purity and concentration were quantified using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA).

For RNA viruses, reverse transcription was done using 8 µl of TNAs, mixed with 1 µl of hexamer random primers (Roche Diagnostics, Switzerland) (0.5 µg/µl) and 3.5 µl RNase-free water. The mix was denatured by heating at 70 °C for 5 min, followed by fast cooling, then mixed with 4 µl M-MLV (5×) first strand buffer, 2 µl DTT (0.1 M), 0.5 µl dNTPs (10 mM), 1 µl M-MLV RT enzyme (200 U/µl) (Invitrogen, Italy). The preparation was incubated at 39 °C for 60 min, 70 °C for 10 min and finally at 4 °C before use or stored at −20 °C. Samples were screened by conventional PCR for the DNA virus *Apis mellifera* filamentous virus (AmFV) and *Nosema*

Table 1. Primers used for the detection of honey bee viruses and *Nosema* spp. in the Syrian apiaries. *Oligonucleotides were designed with Primer3web version 4.1.0 (Untergasser *et al.*, 2012).

Primer	Sequence (5'-3')	GenBank accession no.	Size (bp)	Reference
ABPV-F	TTATGTGTCCAGAGACTGTATCCA	AF150629	900	Benjeddou *et al*., 2001
ABPV-R	GCTCCTATTGCTCGGTTTTTCGGT			
CBPV-F	AGTTGTCATGGTTAACAGGATACGAG	AF461061	455	Ribiere *et al*., 2002
CBPV-R	TCTAATCTTAGCACGAAAGCCGAG			
BQCV-F	TGGTCAGCTCCCACTACCTTAAAC	AF183905	700	Singh *et al*., 2010
BQCV-R	GCAACAAGAAGAAACGTAAACCAC			
DWV-F	ATCAGCGCTTAGTGGAGGAA	NC-004830	702	Chen *et al*., 2004a
DWV-R	TCGACAATTTTCGGACATCA			
KBV-F	GATGAACGTCGACCTATTGA	NC-004807	415	Stoltz *et al*., 1995
KBV-R	TGTGGGTTGGCTATGAGTCA			
SBV-F	GCTGAGGTAGGATCTTTGCGT	AF092924	824	Chen *et al*., 2004b
SBV-R	TCATCATCTTCACCATCCGA			
IAPV-F	GGTCCAAACCTCGAAATCAA	NC009025	839	Palacios *et al*., 2008
IAPV-R	TTGGTCCGGATGTTAATGGT			
CWV-F	TCAAATGGACCAATGGACTTC	AF034543	358	This work*
CWV-R	GGTTGGCTATGAGTCATCATGT			
SBPV.F8156.F	GATTTGCGGAATCGTAATATTGTTTG	EU035616	868	de Miranda *et al*., 2010
SBPV.F8156.r	ACCAGTTAGTACACTCCTGGTAACTTCG			
VdMLV-F	ATCCCTTTTCAGTTCGCT	KT162924/5	438	de Miranda *et al*., 2015
VdMLV-R	AGAAGAGACTTCAAGGAC			
LSV-1F	TTATCTCGCGCCGCCACCTC	HQ871931/5	672	Runckel *et al*., 2011
LSV-1R	ATCGCCGCTGCAACGTGACC			
LSV-2F	CGGCCGGTCTAGCGTGGTTG	HQ888865	558	Runckel *et al*., 2011
LSV-2R	TGGCAAGCTGTGACGAATCCCT			
AmFV-BroN-F	CAGAGAATTCGGTTTTTGTGAGTG	KR819915	551	Gauthier *et al*., 2015
AmFV-BroN-R	CATGGTGGCCAAGTCTTGCT			
TS.AmFV28F	CGCATGTACCAACAACTCGTAC		332	
TS.AmFV28R	CACAGTTGGTGTAGCGCAGT			
Nos-F	TATGCCGACGATGTGATATG	U26534,GI857487	250	Fernandez *et al*., 2012
Nos-R	CACAGCATCCATTGAAAACG			
VTLV.3670	GAAGACTTCGACACCATGGC	KT162626	454	This work*
VTLV.4123	AGATGCGCACAAAGTTCAGG			

Table 2. GenBank accession numbers and geographical origins of the nucleotide sequences of BQCV, *N. ceranae*, VdMLV and AmFV used in the phylogenetic analyses.

Acc.No	Country
BQCV	
LN875569	Mongolia
KY363519	Sweden
GU903461	UK
EF517519	Poland
DQ364629	Uruguay
EU292211	Brazil
KY243932	Czech Republic
KX273081	Turkey
KX273100	Turkey
KY465685	Australia
KP730033	Japan
KT717337	Japan
KP730038	Thailand
KP730025	Thailand
JN185928	China
EU770973	Korea
HQ655461	USA
KX591576	Serbia
N. ceranae	
KC680637	Lebanon
KC680648	Thailand
KC680644	Morocco
KC680623	France
JX205151	Spain
FJ789795	Australia
EU025027	Argentina
EU045844	Austria
DQ673615	Switzerland
DQ374656	Germany
FJ481912	Korea
EU545141	Canada
DQ486027	Taiwan
KU937105	India
JN088169	Greece
KY022481	Saudi Arabia
KY022482	Saudi Arabia
VdMLV and/or BMLV	
AB859952	Belgium
KC880120	Belgium
AB859950	Belgium
AB859949	Belgium
AB859951	Belgium
KT956842	Belgium
JF289170	Jordan
KT162924	France
KT162925	USA
HQ916351	USA
AmFV	
KU047954	China, Henan
KT728709	China, Zhejiang
KR819915	Switzerland
JF304814	Switzerland
KJ685944	Belgium

spp. and by RT-PCR for 14 honey bee viruses: chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Kashmir bee virus (KBV), black queen cell virus (BQCV), sacbrood virus (SBV), acute bee paralysis virus (ABPV), cloudy wing virus (CWV), Israeli acute paralysis virus (IAPV), slow bee paralysis virus (SBPV), lake Sinai virus 1 (LSV1), lake Sinai virus 2 (LSV2), *V. destructor* macula-like virus (VdMLV), Varroa tymo-like virus (VTLV), aphid lethal paralysis virus (ALPV). The primers used in this study were synthesized by Macrogen (Korea) and are shown in table 1. The PCR reactions were carried out in a 25-µl mixture containing 12.5 µl of DreamTaq Green PCR Master Mix 2× (Thermo Fisher Scientific), 0.4 µl of 10 pmol/µl from each forward and reverse primer (Macrogen), 9.2 µl of nuclease-free water and 2.5 µl of cDNA or DNA of each sample. PCR products were separated by electrophoresis in 1.2% agarose gels and stained by GelRed (Biotium, USA), then purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced (Macrogen).

Bioinformatics tools used for sequence analyses and for local alignments at the nucleotide level included NCBI-BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), BioEdit (Hall, 1999) and MEGA6 (Tamura *et al.*, 2013) software. The sequences used to understand the phylogenetic relationships between the detected honey bee viruses and *N. ceranae* in Syria and those distributed in other countries all over the world were obtained from NCBI database (table 2).

Results

Overall, in this study, 97.9% of samples were infected with one or more viruses. DWV, BQCV, VdMLV, AmFV and *N. ceranae* were found in more than one sample, whereas other viruses, namely CBPV, KBV, SBV, ABPV, CWV, IAPV, SBPV, LSV1, LSV2, VTLV and ALPV, could not be detected. The most prevalent virus was DWV, which was found in all surveyed apiaries infecting 95.8% of the samples, followed by VdMLV (70.8%), BQCV (29.2%) and AmFV (16.7%). *N. ceranae* was detected only in four samples representing 8.3% of infection (table 3).

Simultaneous multiple infections were detected in apiaries as shown in table 4. Single infections with DWV were rare, as this latter was generally found in multiple infections with one to three other viruses. *N. ceranae* was found in virus-infected honey bees.

One representative isolate for each detected pathogen was sequenced to determine its putative phylogeny. Obtained sequences have been deposited in the EMBL, GenBank sequence databases under accession numbers: LT844588 (BQCV), LT855556 (VdMLV), LT844586 and LT855555 for BroN and thymidylate synthase genes, respectively (AmFV), and LT841357 (*N. ceranae*). BLASTn analyses showed that the DWV isolate identified in this study was identical to the previous reported sequence from Syria, DWV-TAR56 (Acc. Number: LN851544), showing 99% of identity at the nucleotide level. As for the new identified viruses from

Table 3. Percentages of infection of honey bee viruses and *N. ceranae* detected in five apiaries in Rural Damascus city.

	No. Bees	No. samples	No. infections									
			DWV	%	BQCV	%	AmFV	%	VdMLV	%	*N. ceranae*	%
Apiary 1	20	10	10	100	2	20	6	60	6	60	2	20
Apiary 2	20	10	10	100	2	20	2	20	10	100	2	20
Apiary 3	20	10	10	100	4	40	0	0	10	100	0	0
Apiary 4	20	10	8	80	2	20	0	0	2	20	0	0
Apiary 5	16	8	8	100	4	50	0	0	6	75	0	0
Total	96	48	46		14		8		34		4	
% infection			95.8		29.2		16.7		70.8		8.3	

Table 4. Frequencies of simultaneous virus and *N. ceranae* infections in honey bee samples.

Detected viruses	apiary1	apiary2	apiary3	apiary4	apiary5	%
0	-	-	-	2	-	4.2
DWV	2	-	-	4	-	12.5
DWV, VdMLV	2	6	6	2	4	41.7
DWV, BQCV	-	-	-	2	2	8.3
DWV, AmFV, *N. ceranae*	2	-	-	-	-	4.2
DWV, VdMLV, BQCV	-	2	4	-	2	16.7
DWV, VdMLV, AmFv	2	-	-	-	-	4.2
DWV, VdMLV, BQCV, AmFV	2	-	-	-	-	4.2
DWV, VdMLV, AmFV, *N. ceranae*	-	2	-	-	-	4.2
DWV, VdMLV, BQCV, AmFV, *N. ceranae*	-	-	-	-	-	0.0

Syrian *A. mellifera*, VdMLV clustered in the phylogenetic tree together with the Belgian VdMLV isolate (Acc. Number: AB859952) showing 97% of sequence identity (figure 1A), BQCV clustered with the Turkish BQCV isolate (Acc. Number: KX273100) showing 99% of sequence identity (figure 1B), and AmFV was located in a separated branch, although showing 97% of sequence identity with the Chinese AmFV isolate (Acc. Number: KU047954) (figure 1C). Finally, the Syrian *N. ceranae* isolate showed high sequence identity (98%) with most available isolates in the GenBank and clustered in the same group with isolates identified in Saudi Arabia (Acc. Numbers: KY022481-2) and Iran (Acc. Number: JF431546) (figure 1D).

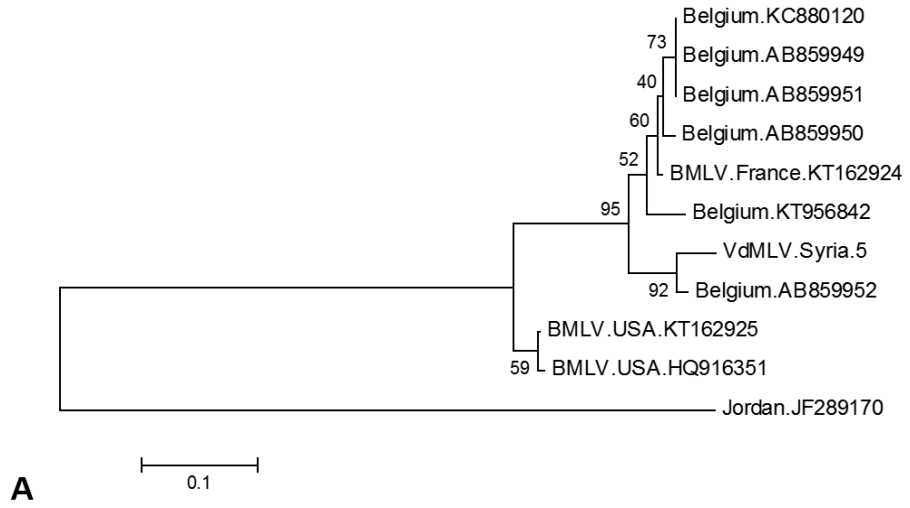
Discussion and conclusions

Due to the increasing interest in honey bee pathogens, we collected symptomatic samples from five different apiaries located in Rural Damascus city in Syria. These samples were used to investigate on the presence of several honey bee viruses and the microsporidia *N. ceranae* by PCR. With the present work, we could provide new information about the health status of some symptomatic honey bees in Syria and we could identify at the genetic level some possible stressors involved in colony losses. Although our survey was limited and cannot reflect the overall situation in the country, this study represents the first molecular detection of BQCV, VdMLV, AmFV, and *N. ceranae* in *A. mellifera* in Syria.

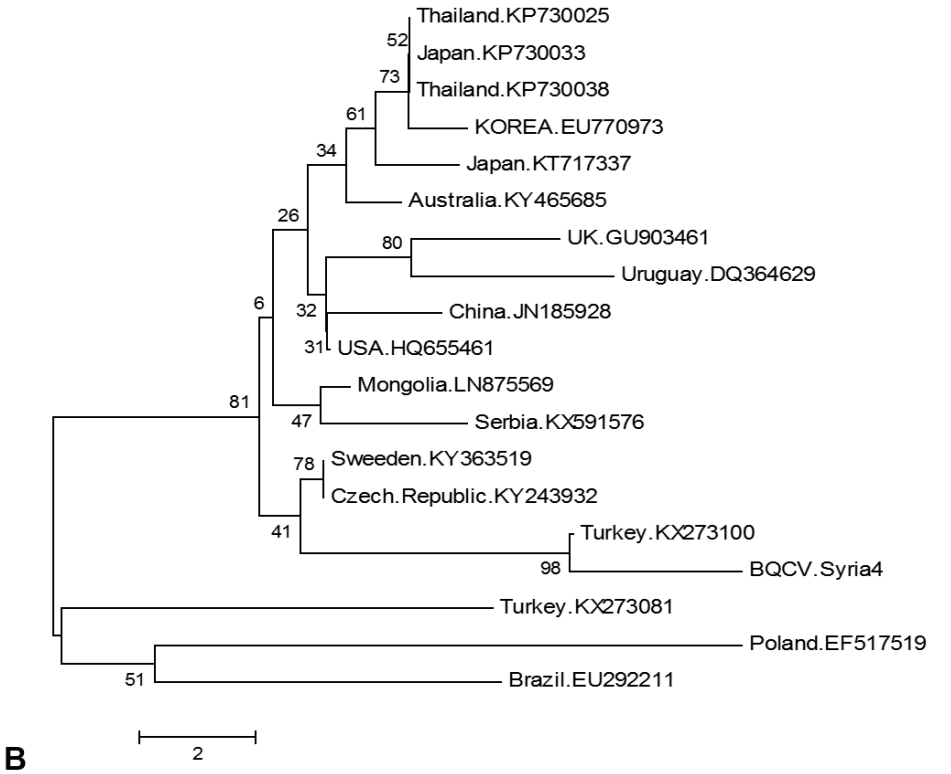
BQCV and DWV are the two most prevalent viruses in European honey bees around the world and have been associated with colony declines (Zhang *et al.*, 2012). The detection of BQCV and DWV in adult bees from all the visited apiaries shows that these viruses are present within the colonies of *A. m. syriaca* throughout the year as reported by Tentcheva *et al*. (2004) for European colonies. DWV was first reported from Syria in our previous work (Elbeaino *et al*., 2016) showing 25.5% infection rate in samples collected in September-November 2014. In this survey, all visited apiaries shared a higher rate of infection with DWV, which was detected in 95.8% of tested samples.

In addition, VdMLV and AmFV were detected in 70.8% and 16.7% of the tested samples, respectively. Little information is still available on the potential impact, transmission and spread of these viruses. According to recent studies, *V. destructor* is considered to be a primary host for VdMLV, rather than the honey bee (de Miranda *et al*., 2015). Thus, the presence and distribution of VdMLV in varroa mites from the infected apiaries should be investigated. One of the possible ways of spread of this virus is by shared contact with contaminated flowers (Ravoet *et al*., 2014), which has also been reported as a transmission pathway for other RNA viruses (Singh *et al.*, 2010). As for the DNA virus AmFV, this is the first report for the whole Middle East. Our results further confirm that viral infections of honey bee, which threaten the future of apiculture, are worldwide spread.

The economically most important honey bee species *A. mellifera* was formerly considered to be infected by one microsporidian, *Nosema apis*. After the first finding of *N. ceranae*, a pathogen of *A. ceranae*, in colonies of *A. mellifera* in Europe (Higes *et al*., 2006), Klee *et al*. (2007) demonstrated its importance as an emergent pathogen of *A. mellifera* and proved also that *N. ceranae*



A



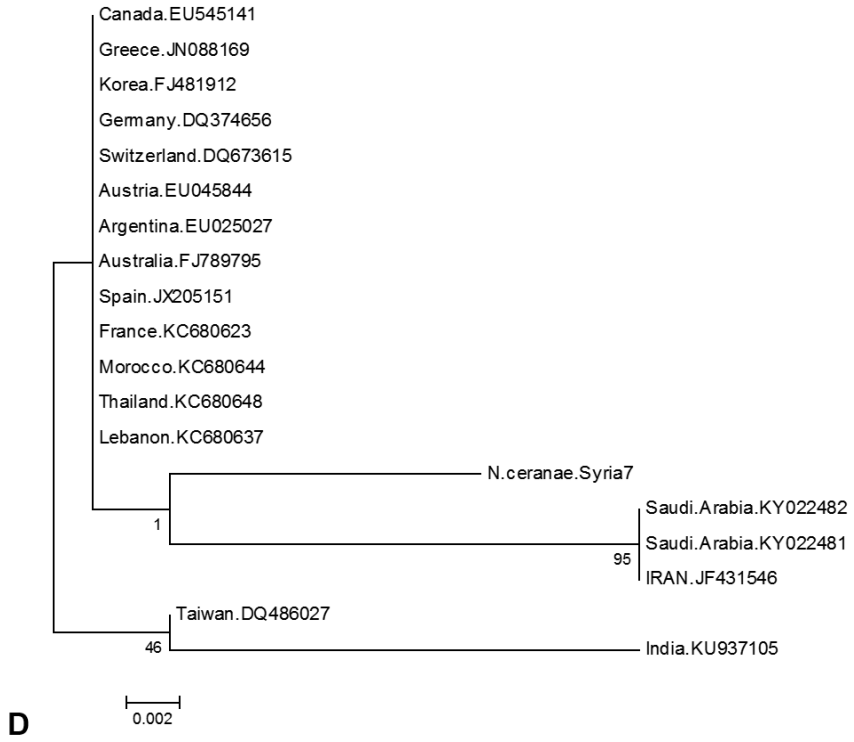
B

C

(continued)

Figure 1. Neighbor-Joining phylogenetic tree based on nucleotide sequence alignment of *V. destructor* macula like virus (**A**), Black queen cell virus (**B**), *A. mellifera* Filamentous virus (**C**) and *N. ceranae* (**D**) isolates obtained from infected honey bees in Syria and their homologues available in the GenBank. The numbers at each node represent bootstrap values as the result of 1000 replicates.

(Figure 1 continued)



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most likely jumped host from *A. ceranae* to *A. mellifera*. This microsporidian is nowadays contributing to the worldwide colony losses (Smith, 2012). Previous studies showed a positive correlation between BQCV epidemiology and *Nosema* infection (Bailey and Ball, 1991; Tentcheva *et al*., 2004). Conversely, in our study, simultaneous detection of *N. ceranae* and BQCV was not observed and the prevalence of *N. ceranae* was rather low. However, since *N. ceranae* infection appears to be more common in warmer climates (*N. ceranae* spores are capable of surviving high temperature) and in specific geographical areas (Fenoy *et al*., 2009), the origin of the imported bees should be taken into account, especially in the case of bordering countries of Syria where *Nosema* infection and some honey bee viruses, which have not detected yet in Syria, were reported previously, such as Iraq (Alzubaidy and Ali., 1994), Jordan (Haddad, 2014; Al-Abbadi *et al.*, 2014), Egypt (Abd-El-Samie *et al.*, 2017), Saudi Arabia (Abdel-Baki *et al.*, 2016; Ansari *et al.*, 2017; El-Shemy *et al*., 2012), Turkey (Whitaker *et al.*, 2011, Oğuz *et al.*, 2017) and Iran (Nabian *et al*., 2011; Aroee *et al*., 2016; Ghorani *et al*., 2017).

Samples infected with multiple viruses were common in our study. Indeed, more than 83% of the examined samples were infected with more than one virus. The most common pair of viruses in double infection was VdMLV and DWV, representing 41.7% of infection, and these were the two most common viruses found in bees overall. A recent study demonstrated that significant positive correlations between AmFV and DWV and between AmFV and BQCV were detected in spring and summer (Hartmann *et al.*, 2015). Although this was not so clear in our case, a part of our samples was simultaneously infected with AmFV and DWV together with/without the presence of BQCV. The effects of multiple virus infection on the immune system of bees, as well as the possible recombination between viruses, allowing for the production of new virus variants (Chen *et al*., 2004c) are still unknown.

Although most honey bee RNA viruses have been correlated with colony declines, the impact of viral diseases is still not completely appreciated, as they are widespread and often cause unapparent, multiple infections in seemingly healthy honey bee colonies. In Syria, the lack of information about viral bee diseases requires further studies to describe the dynamics of virus outbreaks, which may shed light on colonies losses of the Syrian honey bees.

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Authors' addresses: Raied ABOU KUBAA (corresponding author: raied.aboukubaa@ipsp.cnr.it), Maria SAPONARI, CNR Istituto per la Protezione Sostenibile delle Piante (IPSP), UOS Bari, via Amendola 165/A, 70126 Bari, Italy; Giulia MOLINATTO, DISAFA - Entomologia, Università di Torino, largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy; Khaled HEINOUN, Ministry of Agriculture and Agrarian Reform, Department of Plant Protection, Damascus, Syria; Bassem SOLAIMAN KHALED, Damascus University, Faculty of Agriculture, Plant Protection Division, Damascus, Syria; Nouraldin DAHER-HJAIJ, General Commission for Scientific Agricultural Research (GCSAR), Administration of Plant Protection Research, Damascus, Syria.

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