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Next Generation Sequencing study on RNA viruses of *Vespa velutina* and *Apis mellifera* sharing the same foraging area

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Abstract

The predator Asian hornet (*Vespa velutina*) represents one of the major threats to honeybee survival. Viral spillover from bee to wasp has been supposed in several studies, this work aims to identify and study the virome of both insect species living simultaneously in the same foraging area. Transcriptomic analysis was performed on *V. velutina* and *Apis mellifera* samples and replicative form of detected viruses was carried out by strand-specific RT-PCR. Overall, 6 and 9 different viral types were reported in *V. velutina* and *A. mellifera*, respectively, and five of these viruses were recorded in both hosts. Varroa destructor virus-1 and Cripavirus NB-1/2011/HUN (now classified as Triato-like virus) were the most represented viruses detected in both hosts, also in replicative form. In this investigation, Triato-like virus, as well as Aphis gossypii virus and Nora virus, were detected for the first time in honeybees. Concerning *V. velutina*, we report for the first time the recently detected honeybee La Jolla virus. A general high homology rate between genomes of shared viruses between *V. velutina* and *A. mellifera* suggests the efficient transmission of the virus from bee to wasp. In conclusion, our findings highlight the presence of several known and newly reported RNA viruses infecting *A. mellifera* and *V. velutina*. This confirms the environment role as an important source of infection and indicates the possibility of spillover from prey to predator.

Keywords:

Virology; Next Generation Sequencing; honey bee viruses; *Vespidae*

1. Introduction

Several issues can lead to global bee declines, such as climate changes, use of pesticides, presence of pathogens and parasitic species, resulting in huge economic losses for beekeepers and a potential global crisis for the agrifood sector (Brown et al., 2016; Crenna, Jolliet, Collina, Sala, & Fantke, 2020). Moreover, a recent cause of honey bee reduction can be associated to the spread of invasive species, that is recognized as one of the major threat to biodiversity (Vitousek, Mooney, Lubchenco, & Melillo, 1997) and is generally caused by the intensification of human trades worldwide (Essl et al., 2015; Hulme, 2009; Pimentel, Zuniga, & Morrison, 2005). Among several invasive species, the honeybee predator *Vespa velutina* Lepeletier (Hymenoptera: Vespidae) is one of major concern because of its impact on the European domestic honeybee *Apis mellifera* Linnaeus (Hymenoptera: Apidae). The Asian hornet has been accidentally introduced from China

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into Europe, with the first detection in France in 2004 (Villemant, Haxaire, & Streito, 2006), and has rapidly spread to neighbouring countries, including Italy where the first yellow-legged hornet was trapped in 2012 in Loano (Liguria) (Demichelis, Manino, Minuto, Mariotti, & Porporato, 2014). Lately, hornet nests were found in 2013 in Liguria and Piedmont (Porporato, Manino, Laurino, & Demichelis, 2014); the sporadic presence of adults and nests has been described in other three Italian regions: Veneto, Lombardy and Tuscany (Bortolotti et al., 2016; www.stopvelutina.it). Recently, phylogenetic analysis on mitochondrial cytochrome *c oxidase subunit 1* of Italian *V. velutina* specimens has confirmed that the hornet has derived from the spreading southward of the *V. velutina* nigrithorax, initially established in France, which is recently also moving northward (Belgium, The Netherlands, Germany, United Kingdom) (Granato et al., 2019).

Although *V. velutina* can prey on various invertebrate species, its diet is mainly composed of honeybees workers, for which they show a marked dietary preference compared with other insects (Monceau et al., 2014; Cini et al., 2018). As a consequence, the finding of viruses common to the two species is highly probable and has been the subject of recent investigations. The presence of honeybee viruses in invasive honeybee predator species, mainly wasps (Vespoidea), has been confirmed by several authors (Forzan et al., 2017a; Garigliany et al., 2017; Mazzei et al., 2018, 2019). These results have shed light on the ability of such viruses to jump between superfamily taxa (Apoidea-Vespoidea) in view of a possible natural re-equilibrium prey/predator between honeybees and invasive hornets (Maurizio Mazzei et al., 2019), and on the role played by such viruses as potential biocontrol agents of honeybee predators (Forzan et al., 2017b; Dalmon et al., 2019). So far, in Italy, both *Vespa crabro* and *V. velutina* were found infected by a replicative form of Deformed Wing Virus (DWV) (Forzan, Sagona, et al., 2017; M. Mazzei et al., 2018). Moreover, in *V. velutina* sampled in South West of France the replicative form of Sacbrood virus (SBV), Moku virus and Israeli Acute Paralysis Virus (IAPV) were also detected (Garigliany et al., 2017; Mordecai et al., 2016; Yañez, Zheng, Hu, Neumann, & Dietemann, 2012). Yang et al. (2020) detected DWV and SBV in *V. velutina* sampled in France as well, while in such hornet species sampled in China, only IAPV was reported. Recently, both Kashmir bee Virus (KBV) and Black Queen Cell Virus (BQCV) were detected in *V. velutina* in Italy, although in an asymptomatic form (Maurizio Mazzei et al., 2019). In a recent study on *V. velutina* have been detected honeybee viruses, such as Acute bee paralysis virus (ABPV) and Bee Macula-like virus, and two new viruses: Acypi-like and Triato-like viruses (Dalmon et al., 2019).

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Next Generation Sequencing analysis (NGS) is a useful diagnostic tool that facilitates a high-throughput sequencing of the virome in a great variety of samples. NGS has been applied not only to discover host-specific honeybee viruses but also other viruses that could be considered as opportunistic, such as plants viruses (Granberg et al., 2013; Schoonvaere et al., 2016) or other invertebrate associated viruses, as Aphid Lethal Paralysis Virus (ALPV) (Liu, Vijayendran, Carrillo-Tripp, Allen Miller, & Bonning, 2014). Strictly related to honeybees, Galbraith et al. (2018) by the use of NGS applied to 12 bee species from 9 countries across the world, discovered 7 new viruses belonging to different viral families of which 5 having RNA and 2 having DNA genome. NGS studies performed on *V. velutina* allowed the detection of 19 known and unknown viruses (Dalmon et al. 2019). Therefore, metagenomic analysis applied to honeybees could represent a great tool to comprehend the dynamics of viral population, considering that some of those viruses are not only environmental contaminants but can actively infect honeybees (Li et al., 2014).

The aims of the present study were the following: i) assess which viruses are present in honeybees and *V. velutina* individuals sharing the same foraging area; ii) determine the genomic homology rate between shared viruses to speculate possible pathogen transmission from *V. velutina* to *A. mellifera* and *vice versa*.

2. Material and Methods

2.1 Specimen collection

V. velutina and *A. mellifera* sampling was performed in Sanremo (Liguria region, Italy) in October 2018. Five *V. velutina* and 8 *A. mellifera* individuals were sampled in front of a single apiary during hornet predatory activity. Samples were the following: 2 *V. velutina* single-handedly; 3 *V. velutina* each one with a honeybee captured during their predatory activity; 5 *A. mellifera* individuals. After collection, samples were immediately frozen and stored at -80°C.

2.2 RNA extraction and metatranscriptomic analysis

RNA was extracted individually from the 5 *V. velutina* and 8 *A. mellifera* individual samples by RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Each insect was placed in a 2.0 ml microcentrifuge tube with RLT buffer and one steel bead, then disruption was performed by TissueLyser (Qiagen, Hilden, Germany). Brief and soft centrifugation was performed, then the supernatant was collected and used for RNA extraction. To

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point out any possible virus contamination among the hornet sampled together with the honeybee, and to get deeper information about single hornet or bee virome, we set up 5 specimens for NGS analysis: "V1" and "V2" for single *V. velutina* specimens; "VA" refers to a pool of the three *V. velutina* captured with *A. mellifera*; "AV" refers to a pool of the three *A. mellifera* collected with *V. velutina*; "A" a pool of five *A. mellifera*.

RNAs were pooled by mixing the same nucleic acid concentration of each insect after RNA quantification by Qubit RNA Assay Kits (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA 260/280 ratio quality was evaluated by spectrophotometer. Quality control (Agilent Bioanalyzer 2100), library preparation, and RNA sequencing on Illumina NovaSeq 6000 (2x150 paired-end reads) were carried out by IGA Technology Services (Udine, Italy).

2.4 Data processing

Adapter removal and low quality read filtering was carried out by IGA Technology Services (Udine, Italy). Galaxy software framework was employed for computational analysis (https://galaxyproject.org; https://galaxyproject.eu). Raw reads quality was evaluated on Galaxy project platform using FastQC v0.72 (Andrews, 2010). MultiQC v1.6 was employed to aggregate FastQC results and create a single report for forward and reverse reads (Ewels, Magnusson, Lundin, & Käller, 2016). A workflow was built to perform pre-processing and assembly of the raw sequencing data as diagrammed in Figure 1. High quality reads from two honeybees samples (AV, A) were aligned on *Apis mellifera* 04 Nov 2010 (Amel_4.5/apiMel4) reference genome with HISAT2 v2.1.0 (Kim, Langmead, & Salzberg, 2015). Output galaxy project files were then uploaded on Galaxy Europe platform and unmapped reads were then extracted from BAM file using Filter sequencing by mapping v0.0.6 (Cock et al., 2009). Such filtered reads and raw sequences from the hornet samples (V1, V2, VA) were employed in downstream analysis. The alignment of sequencing reads on a built-in index virome database consisting of invertebrate reference viruses with Bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012) was performed. Invertebrate viruses were retrieved from NCBI Virus (May 23rd, 2019; https://www.ncbi.nlm.nih.gov/genome/viruses/). The aligned reads matching the built-in index invertebrate dataset identified reference invertebrate viruses. For each mapped virus, all the available whole genomes belonging to the identified taxid were retrieved from NCBI. The dataset created for each taxid was used for mapping. Each alignment output was then assembled either through Trinity v2.8.4 (Grabherr et al., 2011) or Unipro UGENE (Okonechnikov, Golosova, &

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Fursov, 2012), and a consensus sequence generated. Retrieved consensus sequences were translated and aligned to the nr database of GenBank by BLASTx (Altschul et al., 1997) to confirm the virus sequences alignment.

Another analysis line of raw reads was performed: *A. mellifera* and *V. velutina* raw reads that did not map to the invertebrate virus database were aligned by Kraken2 v2.0.8 to fungi and plasmid databases (Wood & Salzberg, 2014). Unaligned reads were assembled by Trinity *de novo* assembly. The contigs obtained were translated and aligned by BLASTx using RNA viruses as database (taxid:2559587).

2.5 Viral genomes analysis: phylogeny, homology, recombinants detection

Mega-X software was employed to conduct evolutionary analyses and to generate a phylogenetic tree of the detected viruses (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). After viral sequences aligning and trimming by BioEdit (Hall, 1999), the tree was obtained by applying the Maximum Likelihood method and Tamura-Nei model algorithm (Tamura & Nei, 1993). The tree was built on the shared region of viruses found in all five samples and depending on the number of published sequences of related viruses in GenBank.

Recombinant viruses were analyzed and the recombination breakpoints were detected through the nucleotide sequence comparison plot generated with SimPlot (Lole et al., 1999).

To determine the homology rate among viruses, the genetic distance (p-distance of nucleotide acid) of the longest region shared by the five samples was calculated by MEGA-X. To determine the significance of the homology level, p-distance of the same region was conducted also on viral clone genomes deposited in GenBank.

2.6 Strand-specific RT-PCR

The replicative form of detected viruses was verified by strand-specific RT-PCRs (Maurizio Mazzei et al., 2014). Detection was performed for viruses that showed the highest alignment rates and which were either shared among samples or never detected in a host species under study. The assay was carried out using previously described primers for DWV (Forzan, Sagona, et al., 2017) or designing them on assembled sequences through Primer-BLAST (Ye et al., 2012). All primers are listed in Table 1. All cDNAs were amplified by PCR for the related viral target and amplicons were visualized on a 2% agarose gel.

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3. Results

3.1 RNA viruses detection

The alignment of sequencing reads on invertebrate virus genomes showed the presence of a variety of RNA viruses (Table 2). Coverage and position of consensus sequences of viruses detected in multiple samples were aligned against the reference whole genome (Figure 2).

In *V. velutina* specimens (V1, V2, and VA), DWV-B genome was identified in all samples (Accession numbers: MT747986; MT747989; MT747988) and La Jolla virus only in V1 sample (Accession numbers: MT747979; MT747980). Both viruses are ascribable to the Iflavirus genus within the *Picornaviridae* family. DWV-B showed a high overall alignment rate especially in sample V1, for which a whole genome was obtained, while only a few sequences aligned to La Jolla virus. Two Dicistroviruses were also detected in sample V1, V2, and VA: ALPV and Cripavirus NB-1/2011/HUN. Cripavirus NB-1/2011/HUN and ALPV were more abundant in sample V2, since the total consensus sequence length was 8,754 and 3,877, respectively. In sample V2, one 150-bp read aligned to Culex Picorna/Iflavi-like virus belonging to the Picornavirales order. Despite the short sequence, the result can be supported by the BLASTn analysis that revealed the unique homology towards Culex Picorna-like virus 1 and Culex Iflavi-like virus 4 with an identity of 98% for both viruses and e-value ranging from e^{-10} to e^{-67}, no other significant matches were highlighted . The assembled viral genetic sequences were obtained through Unipro UGENE. The only exception was ALPV for which the longest consensus sequences were obtained by Trinity *de novo* assembly of ALPV aligned reads.

Concerning honeybees, both DWV-B (Accession numbers: MT747909; MT747987) and Cripavirus NB-1/2011/HUN were found with an abundant alignment rate, achieving full genome for DWV-B in both *A. mellifera* samples and Cripavirus NB-1/2011/HUN in sample A (Accession number: MT747985). ALPV and La Jolla virus were only detected in sample A, the first one in low abundance since were retrieved a consensus sequence length of 214 bp, while for la Jolla the total consensus sequence length was 2,915 bp but spread across 14 short contigs. Samples A and AV showed the dicistroviruses BQCV and Aphis gossypii virus (AGV) (Accession number: MT747981), respectively. BQCV was detected in very low quantity since only 2 consensus sequences with an 813 bp total-length were retrieved, while for AGV four consensus sequences were retrieved with 2,400 bp total-length. In sample A, a 360 bp sequence length of Thika virus and two 150-bp-reads sequences of Nora virus (Accession numbers: MT747983; MT747984) were detected. Nora virus BLASTn analysis of both 150 bp reads matched only to Nora virus sequences

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deposited in NCBI with an e-value ranging from e^{-10} to e^{-67}. In honeybee virome analysis, the longest consensus sequences of Cripavirus NB-1/2011/HUN and AGV were achieved by Trinity *de novo* assembly of mapped reads, while the other virus sequences were obtained through Unipro UGENE.

Thus, the two *Apis mellifera* samples (AV and A) resulted positive to four of the viruses also found in *V. velutina*. In detail, DWV-B and Cripavirus NB-1/2011/HUN were shared among honeybees and hornet samples; while La Jolla virus was detected only in A and V1 and ALPV in A and all hornets.

De novo assembly through Trinity allowed detecting a complete RdRp sequence ascribable to Hubei partiti-like virus 34 strain (Accession number: KX884207) in *V. velutina* (sample V1, not included in invertebrate virome database employed for initial screening. Then, Hubei partiti-like virus 34 mapping was carried out through Bowtie2 for all five samples. In *V. velutina* specimens (V1, V2, VA) the whole RdRp of such virus was retrieved, showing a high overall alignment rate. Hubei partiti-like virus 34 strain detected in sample V1 was deposited in GenBank (Accession number: MT747982). In honeybee, a short sequence (186 bp) of Hubei partiti-like virus 34 was retrieved only in sample A. *V. velutina* and *A. mellifera* detected viral consensus sequences are reported in Supplementary Table 1.

Phylogenetic analysis was performed on DWV-B RdRp (Figure 3). On the phylogenetic tree, DWV-Bs detected in this study clusterized in VDV-1 group. A different clusterization of DWV-B was obtained for V2 and AV in contrast to V1, VA, and A samples. A possible recombination site of DWV-B with DWV-A genome was identified among positions 1118- 1519 for sample V1 and 1135 – 1503 for sample AV (Figure 4).

3.2 Homology rate among bee and hornet viral regions

The longest genomic region shared by the detected viruses in all five samples was employed to determine viral homology rate. Only for DWV-B and Cripavirus NB-1/2011/HUN was it possible to identify a region shared by all the samples. The reference viruses employed to determine the significance of the homology rate are two VDV clones: HM067437 and HM067438 (Ryabov et al., 2014). P-distance of the latter is 0.220 (per thousand value), much higher than p-distance of the detected DWV-B in the present study (from 0.024 to 0.100). It was not possible to compare p-distance of Cripavirus NB-1/2011/HUN with other genomes deposited in GenBank since only one

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virus shows the analyzed region but also in this case p-distance values were rather low (from 0.000 to 0.086 per thousand).

3.3 Strand-specific RT-PCR

Strand-specific RT-PCR was conducted to determine the replicative form of detected viruses through metagenomics analysis. The replicative assay was conducted on Cripavirus NB-1/2011/HUN (in sample V1, A), ALPV (in sample V2), AGV (in sample AV), DWV-B (in sample V1, A) and Hubei partiti-like virus 34 (in sample V1). The negative-strand RNA was detected for all viruses obtaining amplicons of the expected sizes, except for Hubei partiti-like virus 34, for which it was not possible to obtain a definitive and reliable result (Figure 5).

4. Discussion

NGS analysis of transcriptome allowed to get a deeper knowledge about *V. velutina* and *A. mellifera* virome. The investigation conducted, successfully identified viruses shared among the two species and detect viruses never detected before in both hosts. Overall, 6 and 9 viruses were reported in *V. velutina* and *A. mellifera*, respectively.

Concerning DWV-B, it was detected with a high alignment rate in all samples (Accession numbers: MT747986; MT747989; MT747988; MT747909; MT747987), demonstrating the high diffusion of the virus worldwide (Wilfert et al., 2016). In recent studies conducted on *V. velutina*, DWV-A was found in adult hornets sampled in France (S. Yang et al., 2020), while in another study performed on hornets from the same country, DWV-B was found in infective form and DWV-C was found in low quantity in hornet gut as a contaminant or latent infectious agent (Dalmon et al., 2019). The capability to infect a wide range of pollinators was demonstrated by the presence of DWV-B replicative viral form. The existence of replicative virus and the high alignment rate may be related to symptomatic form (Dalmon et al., 2019; De Miranda & Genersch, 2010), especially referring to DWV-B, being a more virulent honey bee virus strain than DWV-A (McMahon et al., 2016). NGS analysis allowed to identify the whole DWV-B genome without significant differences in homology (from 0.024 to 0.100 per thousand) between samples, suggesting the efficient and previous transmission among *A. mellifera* and *V. velutina*. Considering the predatory activity of *V. velutina* on *A. mellifera* could be hypothesized a unique direction of transmission from prey to predator. Indeed, DWV spillover from honeybees to hornets was recently described (Dalmon et al., 2019; Forzan, Sagona, et al., 2017; B. Yang et al., 2017).

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Phylogenetic analysis was performed on DWV-B RdRp, the only virus whose RdRp ORF was shared among samples. The phylogenetic tree shows a clear clusterization of detected DWV-B within VDV-1 group. It is necessary to consider that the VDV-1 has been recently renamed DWV-B (Mordecai et al., 2015), so the clusterization is consistent. The DWV-B cluster includes viruses originating from hosts different from those considered in this study, such as *Varroa destructor* (AY251269). The absence of a DWV-B clade among different hosts further underlines the high spread and capability of infection of DWV-B in the natural ecosystem, suggesting the interspecies transmission as effective as the intraspecies transmission (Dalmon et al., 2019; S. Yang et al., 2020). Detected DWV-B/DWV-A recombination features (Figure 4) are unique since have been identified for the first time in that specific genomic site. DWV-A and DWV-B recombination can naturally occur when the two genotypes are present at high levels during co-infection (McMahon et al., 2016; Moore et al., 2011; Wang et al., 2013). Furthermore, the discovery of chimeric DWV-A/DWV-B viruses has been linked to higher virulence in honeybee pupae (Ryabov et al., 2014). Indeed, the generation of recombinant genotype may be responsible for knock-on effects for long-term virulence evolution and host adaptation (Rambaut, Posada, Crandall, & Holmes, 2004).

Another virus detected in both species investigated is Cripavirus NB-1/2011/HUN (NC_025219). A high number of sequences were detected in *V. velutina* and for the first time in *A. mellifera* specimens, since so far it was only found in insectivorous bat faeces (Dacheux et al., 2014; Reuter, Pankovics, Gyöngyi, Delwart, & Boros, 2014). The whole genome retrieved from sample A was deposited in GenBank (Accession numbers: MT747985). The virus detected shows a high percentage of identity (99%) to another virus classified as Triato-like virus identified in 2014 from *V. velutina* specimens collected in the south-east of France (Dalmon et al., 2019). As stated by Reuter et al. (2014), the Cripavirus NB-1/2011/HUN was linked to Himetoby P virus (AB183472) by the sequence identity of their capsid protein and therefore classified as a taxonomic member of *Cripavirus* genus (from which the name Cripavirus NB-1/2011/HUN originated). Since 2015 Himetoby P virus has been classified as a member of *Triatovirus* genus (https://talk.ictvonline.org). Therefore, it seems necessary to provide for a nomenclature revision of the Cripavirus NB-1/2011/HUN species, as it could be wrongly attributed to *Cripavirus* genus. So, it seems more correct for future reference to classify the virus identified in our italian specimens as member of Triato-like viruses. Moreover, the presence of a replicative form of Triato-like virus in both hosts sampled in Italy indicates that the virus actively infects *V. velutina* and *A. mellifera*, confirming what was suggested by Dalmon et al. (2019). Since the high

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abundance of Triato-like virus in all samples, it would be intriguing to investigate its role on honeybee and *V. velutina* health. The low difference in homology rate (from 0.000 to 0.086 per thousand) suggests the efficient transmission of the virus from *A. mellifera* to *V. velutina*.

ALPV was detected in both *A. mellifera* and *V. velutina*. Only sample AV (honeybee) did not present sequences related to this virus. ALPV in *A. mellifera* was for the first time detected in Spain (Chen et al., 2004), and recently in France (Dalmon et al., 2019) confirming its presence in the European continent and such host. Recently ALPV has been detected in *V. velutina* in China (D. Yang et al., 2019). ALPV is globally distributed in honeybees and Asian hornets and its infectivity on these hosts is demonstrated by the presence of the replicative form of the genome in the tested samples. Considering the alignment rate, the highest values were attributed to *V. velutina* samples, in which was also detected the replicative form. This could suggest that the replication activity could be more efficient in hornets rather than in honeybees. However, according to Roberts et al. (2018), the low abundance of ALPV reads recorded in several studies does not allow a better understanding of its impact for *A. mellifera* and *V. velutina* health.

Although BQCV is a common honeybee virus it was only detected in very low abundance in sample A (honeybee). Nevertheless, BQCV was recently reported in a replicative form in *V. velutina* (Maurizio Mazzei et al., 2019). The presence in *Apis mellifera* only could be explained by a higher prevalence in *Apis* species compared to other social wasps (Manley, Boots, & Wilfert, 2015).

Two viruses associated with *Drosophila* spp. were also found in analyzed samples: La Jolla virus and Thika virus. La Jolla virus was only detected in two honeybee samples and, for the first time, in *V. velutina* (Accession numbers: MT747979; MT747980). This virus belongs to the *Iflaviridae* family and was recently detected in honeybee (Roberts et al., 2018). It is not possible to exclude an active role in *V. velutina* and *A. mellifera*, especially in the latter, in which a longer global consensus sequence length was retrieved. Thika virus, a taxonomically unclassified virus, was only detected in honeybee (sample A) and its presence was already ascertained in such host (Roberts et al., 2018). The low abundance of Thika virus reads suggests minimal importance for *A. mellifera* or an inability to replicate in honeybees.

Another virus conveyed by aphid and detected for the first time in honeybee is AGV (Accession number: MT747981). It is an unclassified Cripavirus and it was for the first time reported in *Aphis gossypii* sampled in Israel from 2015 to 2017 (GenBank: MH476200 to MH476204). *A. gossypii* is a polyphagous crop pest worldwide distributed and it plays an important role as plant virus vector

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(Gu et al., 2013). Its ability to replicate in *A. mellifera* has been observed and further studies are needed to verify if it plays a pathogenic role for honeybee, so considering honeydew produced by aphids a possible source of transmission.

Two short sequences of Nora virus were detected in sample A (*A. mellifera*) (Accession numbers: MT747983; MT747984). Despite the short sequences identified (150 bp lenght), the possibility of artifacts of NGS it is unlikely due to the reliability of BLASTn analysis. Indeed, the two sequences only aligned to other Nora virus deposited in NCBI with an e-value ranging from e^{-10} to e^{-67}, much lower than e^{-5}, the typical threshold for a good e-value. Nora virus is an unclassified ssRNA positive-strand virus and it is for the first time detected in such host, even if it has been frequently associated with *Drosophila* species. Nora virus was also detected in a recent study in *V. velutina* (Dalmon et al., 2019). This opens the scenario to a possible wasp-bee transmission, although it is necessary to establish the route of infection, probably indirect, and effects of the virus on the two hosts' health.

Culex Picorna/Iflavi-like virus was detected in sample V2 and it has been only detected in *Culex* sp. in previous studies (Sadeghi et al., 2018). Even if only two 150 bp sequences were retrieved, it is possible to assume that they are uniquely ascribable to Culex Picorna/Iflavi-like virus because following BLASTn analysis the only matches with e-value lower than e^{-5} were Picorna and Iflavi-like viruses isolated from hosts belonging to Culicidae family. It is unlikely that this virus replicates in *V. velutina* given the short sequence obtained, and therefore could be considered contamination rather than an infection event.

The last virus detected in high quantity in *V. velutina* samples was Hubei partiti-like virus 34 (Accession number: MT747982), sharing high homology of the genome and translated protein similarity to the reference genome (Accession number: KX884207). This virus belongs to *Partitiviridae* family and was only found in Chinese land snail (Shi et al., 2016). The detected viruses share also from 63.21 to 64.06 of translated protein similarity with Vespa velutina partiti-like virus 1, detected in *V. velutina* sampled in the south-east of France (Dalmon et al., 2019). Given the relative geographical proximity with the samples analyzed in the present study, it is plausible that the presence of this virus could be found in Asian hornet in southern Europe. Although it was not possible to identify the replicative form of Hubei partiti-like virus 34, the high alignment rate recorded can be related to an active viral replication in *V. velutina*, although its impact on hornet health has not yet been investigated. In *A. mellifera* the virus was absent (sample

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AV) or present in very low quantity (sample A), supposedly as environmental or indirect contamination from *V. velutina*.

5. Conclusion

In conclusion, our findings evidence the presence of several viruses infecting both *A. mellifera* and *V. velutina*, confirming the role of the environment as an important source of infection. Interestingly, NGS approach allowed us to retrieve genomic sequences belonging to viruses never reported before in both insects analyzed in this study. Newly associated viruses to *A. mellifera* were Triato-like virus, AGV, and Nora virus, while for *V. velutina* La Jolla virus was for the first time reported. The general high homology rate between viral genomes identified in this study indicates the possibility of multiple pathogen transfer from the environment to the hosts or spillover among the two insect species. In some cases the presence of replicating virus has been demonstrated, suggesting an active role in transmission for different species and new possible pathogenetic implications. Moreover, the transmission route from *A. mellifera* to *V. velutina* was confirmed by the evidence of intense viral replication in Asian hornet of DWV-B. Another evidence could be observed for the Triato-like virus that showed high replicative activity in *A. mellifera* and also in *V. velutina*. In this case, it is not clear whether the *A. mellifera* could play an active role in viral transmission, or both species resulted infected by other environmental sources. Since the presence of replicative strand in *V. velutina* was clearly detected, it is unlikely that it derives from dead ingested bees. Indeed, if the RNA of the replicative virus had been present only in the gastrointestinal tract of the wasp, this would not have been easily detected by molecular assays. Furthermore, the wasps were captured in October, the period of the year in which *V. velutina* exerts the major predation pressure and after bee capturing it removes all parts of the bee's body except the thorax, which is carried to the nest (Perrard et al., 2009; Arca et al., 2014; Monceau et al., 2018). Those data need to be deeply clarified to better understand the role of both insects to define a possible natural re-equilibrium prey/predator. Finally, NGS has been confirmed as an effective diagnostic tool to identify new pathogens, for highlight epidemiological knowledge especially for alien or newly introduced species in a defined environment. Such information could be extremely useful in planning early actions to contrast pathogens spread.

Author contributions

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F.M., M.F, A.F. and M.M conceived and designed the experiments; L.B, A.F provided the samples; F.M., M.F and M.M. performed the experiments; F.M, M.F., M.I.P., M.S.R-F., A.F. and M.M analyzed the data; F.M and M.M. wrote the paper; F.M., M.F., L.B., M.I.P., M.S.R-F., A.F. and M.M. checked and finalized the manuscript; A.F. and M.M, funding acquisition. All authors read and approved the final manuscript.

Conflict of interest statement

Authors have no competing interests to declare.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Ethical approval

No ethical approval is required as this article is based on invertebrates samples.

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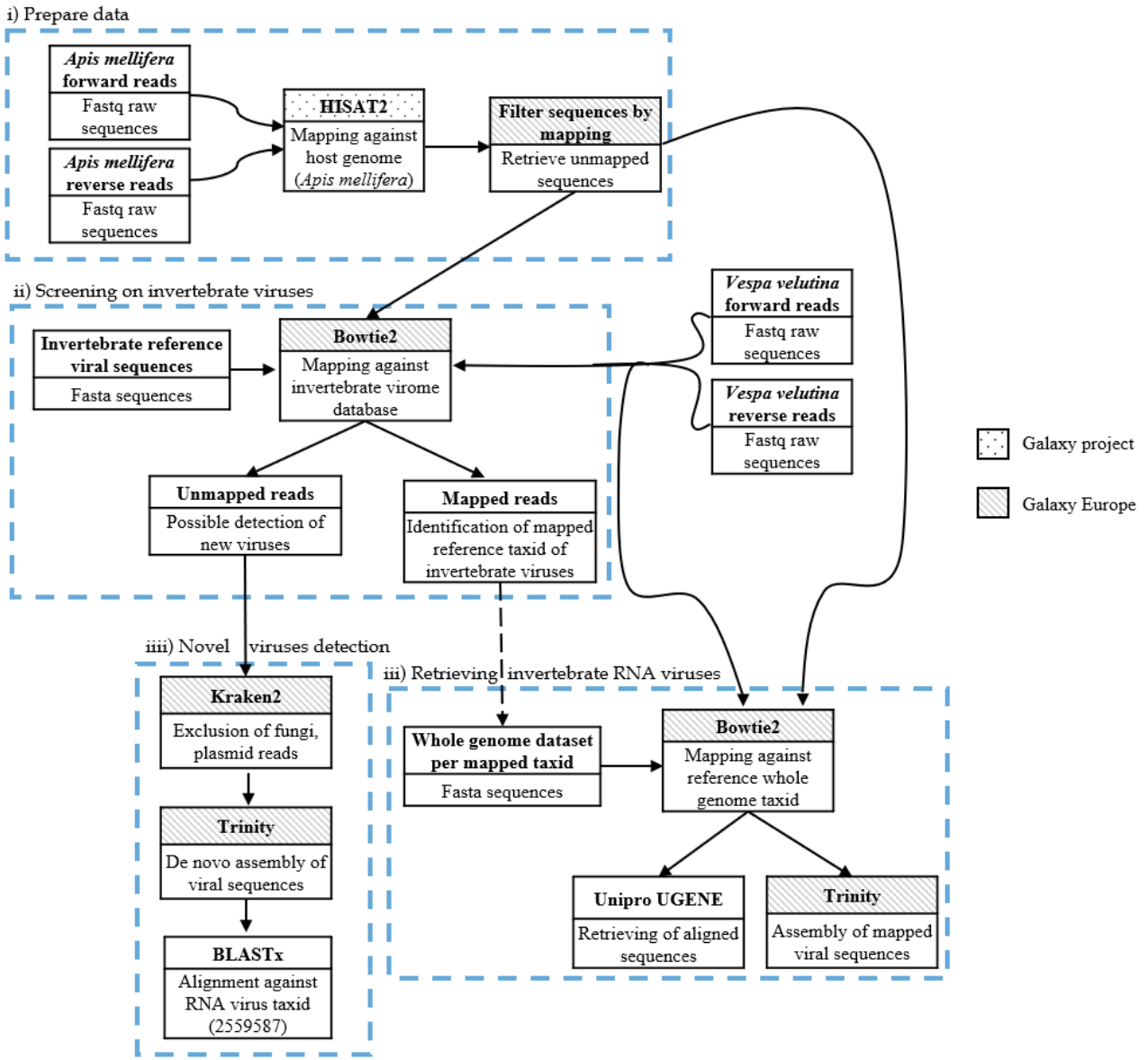

Figure 1. Customized Galaxy workflow used in the current study. Sub-workflows (dashed line) indicate the stages of data analysis. "Dot shading" indicates processing in Galaxy project (https://usegalaxy.org/); "oblique line shading" indicates processing in galaxy Europe (https://usegalaxy.eu/).

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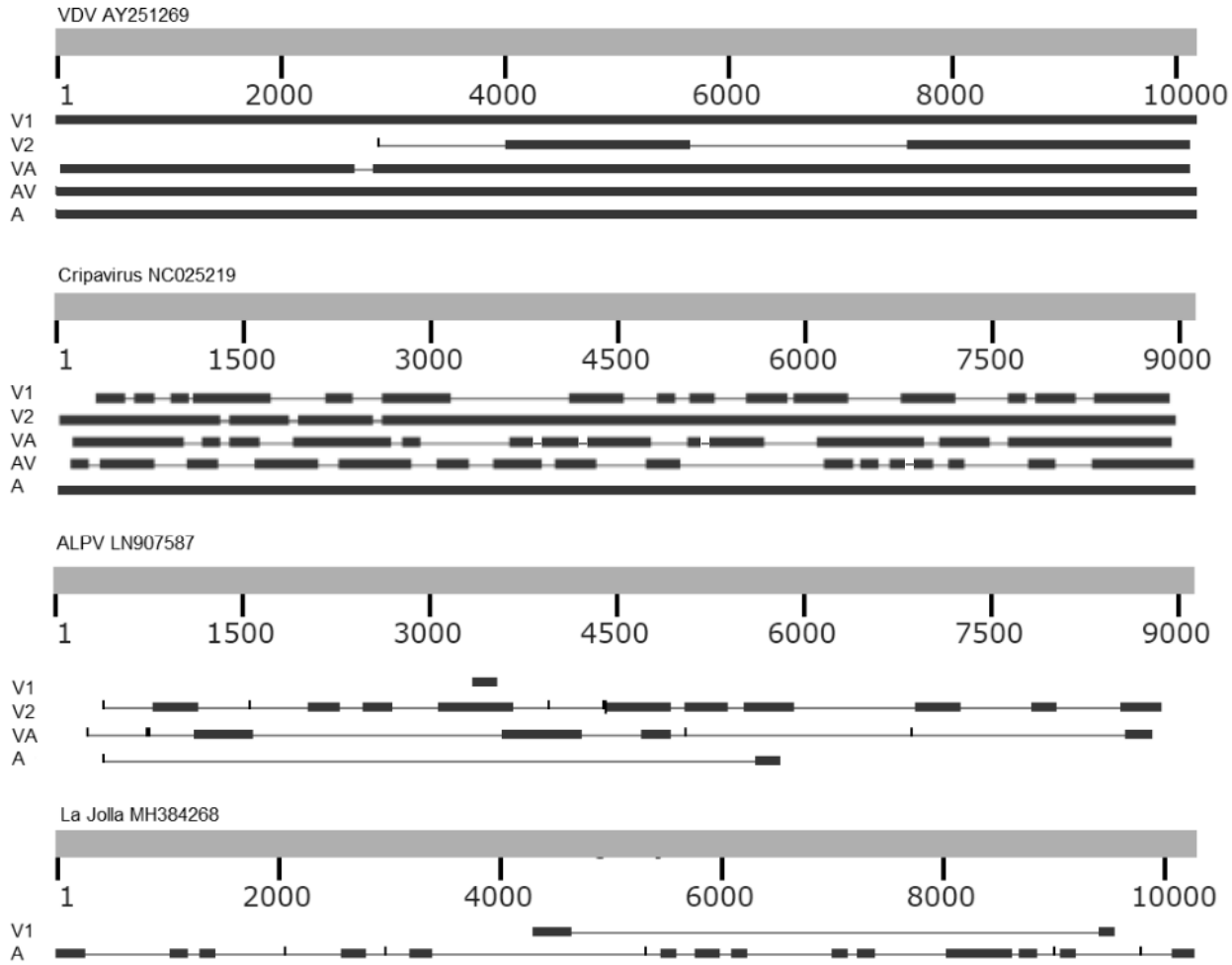

Fig. 2. Coverage and position of consensus sequences aligned against the reference whole genome. Accession number of the reference genome is indicated on the box referred to each detected virus. Thick lines represent the consensus sequences obtained for viral genomes detected.

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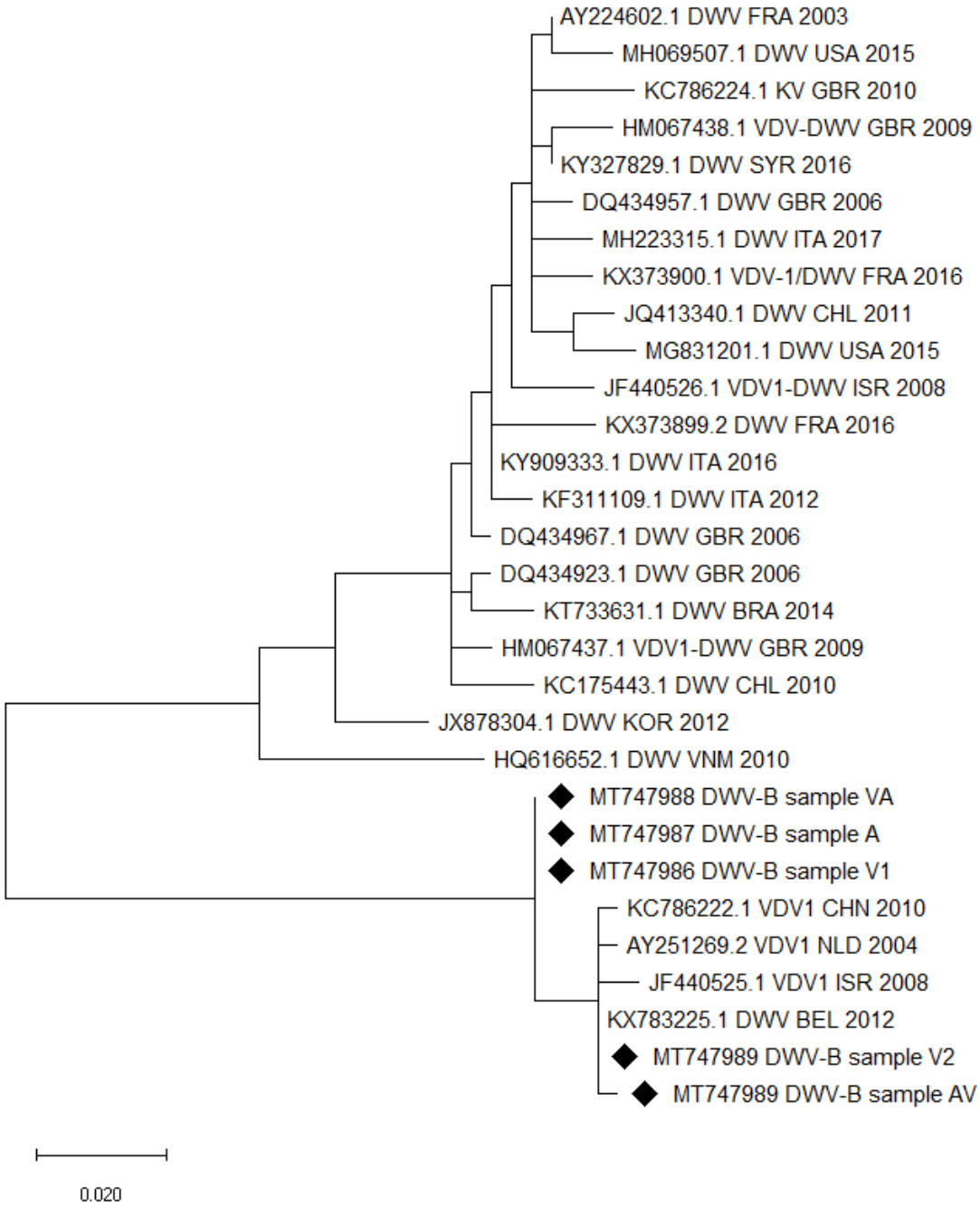

Fig. 3. Molecular Phylogenetic analysis for RdRp ORF of Deformed Wing Virus (DWV) and Varroa Destructor Virus (VDV) by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The branch lengths of the tree measured the number of substitutions per site. The analysis involved 30 nucleotide sequences. There were a total of 315 positions in the final dataset. Accession number, country, and year of available GenBank DWV and VDV sequences are shown. The DWV-B sequences obtained from Italian *Vespa velutina* and *Apis mellifera* from this study are marked with a rhombus. (FRA: France, USA: United States of America, GBR: United Kingdom, SYR: Syria, ITA: Italy, CHL: Chile, ISR: Israel, BRA: Brazil, KOR: South Korea, VNM: Vietnam, CHN: China, NLD: Netherlands, BEL: Belgium).

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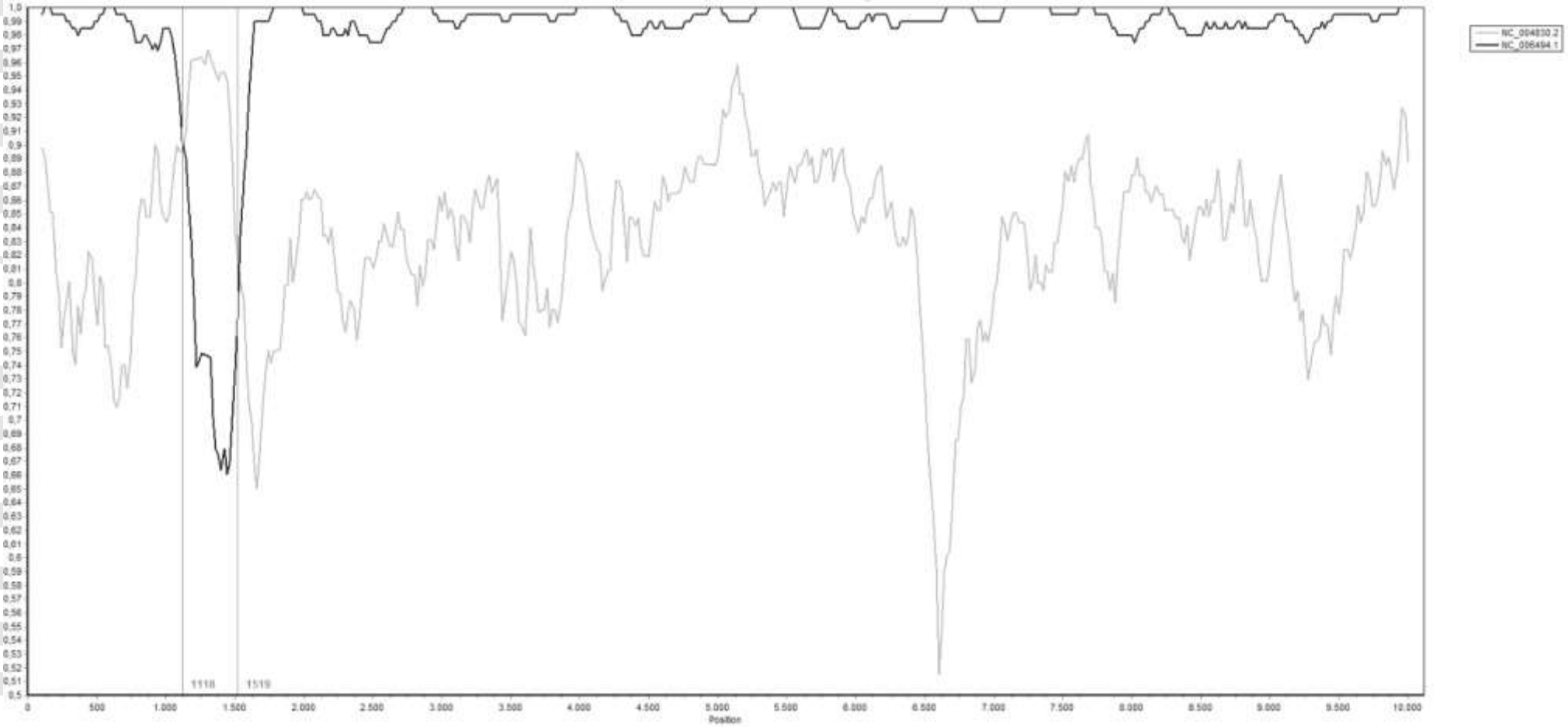
(a)



(b)

Fig. 4. Genome recombination of DWV-B detected in *Vespa velutina* (sample V1- box a) and *Apis mellifera* (sample AV – box b) with DWV-A. Sequences similar to DWV-B (NC_006494.1_VDV) and DWV-A (NC_004830.2_DWV) are in black and grey, respectively. Nucleotide sequence comparison plots were generated with SimPlot (Lole et al., 1999). Genome position of recombination is indicated by a vertical line.

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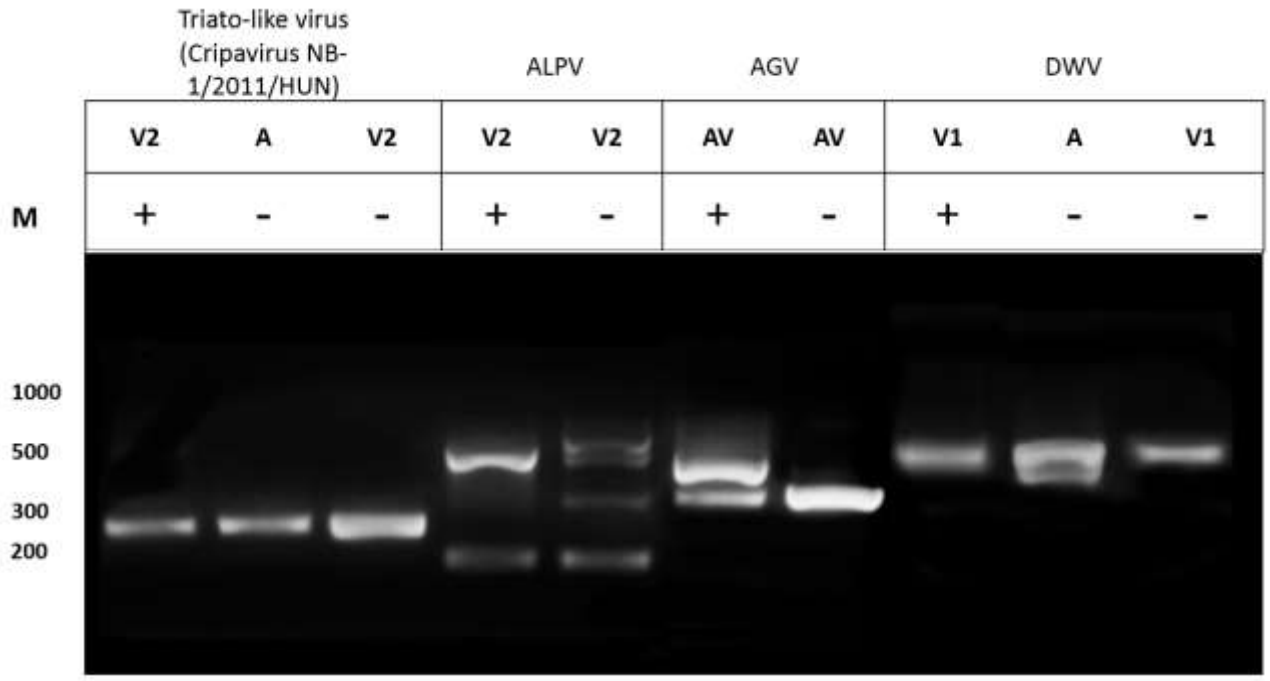

Fig. 5. Evidence of replicating and genomic viruses in *Vespa velutina* (V1, V2) and *Apis mellifera* (AV, A) samples. Gel electrophoresis of strand specific RT-PCR. Replicative strand (-) and genomic strand (+). Amplicon length: Triato-like virus (Cripavirus NB-1/2011/HUN) (294 bp); Aphid Lethal Paralysis Virus - ALPV (157 bp); Aphis gossypii virus - AGV (389 bp); Deformed Wing Virus – DWV (504 bp).

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