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Discovery and molecular characterisation of the first ambidensovirus in honey bees

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Discovery and molecular characterisation of the first ambidensovirus in honey bees

Abstract: Honey bees play a critical role in global food production as pollinators of numerous crops. Several stressors cause declines in populations of managed and wild bee species, such as habitat degradation, pesticide exposure and pathogens. Viruses act as key stressors and can infect a wide range of species. The majority of honey bee-infecting viruses are RNA viruses of the Picornavirales order. Although some ssDNA viruses are common in insects, such as densoviruses, they have not yet been found in honey bees. Densoviruses were however found in bumblebees and ants. Here, we show that densoviruses are indeed present in the transcriptome of the eastern honey bee (*Apis cerana*) from southern China. On the basis of non-structural and structural transcripts, we inferred the genome structure of the Apis densovirus. Phylogenetic analysis has shown that this novel Apis densovirus belongs to the *Scindoambidensovirus* genus in the Densovirinae subfamily. Apis densovirus possesses ambisense genome organisation and encodes three non-structural proteins and a split VP (capsid) protein. The availability of a nearly complete Apis densovirus genome may enable the analysis of its potential pathogenic impact on honey bees. Our findings can thus guide further research into the densoviruses in honey bees and bumblebees.

Key words: honey bees; densovirus; genome organisation; molecular characterisation

Odkritje in molekularna karakterizacija prvega ambidensovirusa pri čebelah

Izvleček: Čebele igrajo ključno vlogo v svetovni proizvodnji hrane kot opraševalci številnih poljščin. Številni stresorji povzročajo upad populacij gojenih in divjih vrst čebel, kot so degradacija habitata, izpostavljenost pesticidom in patogeni. Virusi delujejo kot glavni stresorji in lahko okužijo številne vrste. Večina virusov, ki okužijo čebele, so RNA virusi iz reda Picornavirales. Čeprav so nekateri ssDNA virusi pogosti pri žuželkah, na primer densovirusi, jih pri čebelah doslej še niso našli. Densovirusi pa so bili najdeni pri čmrljih in mravljah. Pokazali smo, da so densovirusi prisotni v transkriptomu azijskih čebel (*Apis cerana*) z južne Kitajske. Na osnovi nestrukturnih in strukturnih transkriptov smo ugotovili genomsko strukturo Apis densovirusa. Filogenetska analiza je pokazala, da novi Apis densovirus spada v rod *Scindoambidensovirus* v poddružini Densovirinae. Apis densovirus ima ambisense organizacijo genoma in kodira tri nestrukturne proteine in razcepljeni VP (kapsidni) protein. Dostopnost skoraj celotnega genoma Apis densovirusa bo omogočila analizo njihovega potencialno patogenega vpliva na čebele. Naše ugotovitve lahko privedejo do nadaljnjih raziskav densovirusov pri čebelah in čmrljih.

Ključne besede: čebele; densovirus; organizacija genoma; molekulska karakterizacija

1 INTRODUCTION

Honey bees (*Apis mellifera*) play a critical role in global food production as pollinators of numerous crops (Klein et al., 2007; Fürst et al., 2014). Several stressors cause declining populations of managed and wild bee species such as habitat degradation, pesticide exposure and pathogens (Goulson et al., 2015; Potts et al., 2010; Evans and Schwarz, 2011; McMenamin et al., 2016; McMenamin and Genersch, 2015). Viruses act as key stressors and can infect a wide range of species (Grozinger and Flenniken, 2019). Overt viral infections can result in a

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wide range of symptoms, including wing deformities, discoloration, hair loss, bloated abdomens, trembling, paralysis, and mortality (Chen and Siede, 2007). Honey bee populations have become increasingly susceptible to colony losses due to pathogenic viruses spread by parasitic Varroa mites (Martin et al., 2012).

The majority of honey bee-infecting viruses are RNA viruses of the Picornavirales order (Chen and Siede, 2007; Levitt et al., 2013; Brutscher et al., 2016; McMenamin and Flenniken, 2018; Beaurepaire et al., 2020). Common bee viruses include: the Dicistroviruses (Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV), and Black queen cell virus (BQCV)); the Iflaviruses (Deformed wing virus (DWV), Kakugo virus, *Varroa destructor* virus-1/DWV-B, Sacbrood virus (SBV), and Slow bee paralysis virus (SBPV)); and taxonomically unclassified viruses (Chronic bee paralysis virus (CBPV) and the Lake Sinai viruses (LSVs)) (reviewed in Chen and Siede, 2007 and Brutscher et al., 2016). Recently identified positive sense single-stranded RNA viruses (+ssRNA) viruses include Bee macula-like virus (BeeMLV) in the Tymoviridae family (Galbraith et al., 2018), *Apis mellifera* flavivirus and *Apis mellifera* nora virus 1 (Remnant et al., 2017). *Apis mellifera* rhabdovirus and bunyavirus were recently described (Remnant et al., 2017) and represent first bee-infecting negative sense single-stranded RNA viruses (-ssRNA).

Honey bees are infected by a small number of DNA viruses (Chen and Siede, 2007). Among double-stranded DNA viruses two honey bee-infecting viruses have been found. The *Apis mellifera* filamentous virus (AmFV) is from the Baculoviridae family and has been sequenced and characterized (Gauthier et al., 2015; Hartmann et al., 2015). The *Apis cerana* iridovirus from the Iridoviridae family has not yet been sequenced (Bailey et al., 1976; Bromenshenk et al., 2010; Tokarz et al., 2011). Very recently, a number of single-stranded DNA viruses (ssDNA) associated with *Apis mellifera* have been reported, belonging to circoviruses (Circoviridae) (Galbraith et al., 2018), genomoviruses (Genomoviridae) (Kraberger et al., 2019), CRESS DNA viruses (Cressdnaviricota) (Kraberger et al., 2019) and microviruses (Microviridae) that infect the honey bee bacterial community (Kraberger et al., 2019).

Although some ssDNA viruses are common in insects, such as densoviruses (Parvoviridae) (Cotmore et al., 2014; Pénzes et al., 2020), they have not yet been found in honey bees. Densoviruses were however found in bumblebees and ants (Schoonvaere et al., 2018; Valles et al., 2013). Here, we show that densoviruses are indeed present in the *Apis cerana* transcriptome from southern China. Genome organisation and phylogenetic analysis have shown that this novel Apis densovirus belongs to the *Scindoambidensovirus* genus in the Densovirinae subfamily. It is interesting that the Bombus and Apis densoviruses are not very similar and belong to different densoviral genera. Although the Bombus densovirus is also present endogenised in the *Bombus impatiens* genome, this was not the case for the Apis densovirus. The availability of a nearly complete Apis densovirus genome may enable the analysis of its potential pathogenic impact on honey bees. Our findings can thus guide further research into the densoviruses in honey bees.

2 MATERIALS AND METHODS

2.1 DISCOVERY OF THE APIS AMBIDENSOVIRUS IN PUBLIC TRANSCRIPTOMIC DATABASES

Sequence database searches were finished in July 2020. The protein queries were diverse densoviral NS1 and VP sequences. The database analysed was the Transcriptome Shotgun Assembly (TSA) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Comparisons were made using the TBLASTN program (Gertz et al., 2006), with the E-value cut-off set to 10^{-5} and default settings for other parameters. TBLASTN searching was restricted to different taxa (Protostomia, Hymenoptera, Apoidea and *Apis*). *Apis cerana* transcriptome (erroneously named *Apis mellifera carnica*) contains 52.177 contigs. Apis ambidensovirus sequences were compared to reference protein sequences of all parvoviruses. DNA sequences of the Apis ambidensovirus were translated with the Translate program (web.expasy.org/translate/).

2.2 ANALYSIS OF ENDOGENOUS VIRUS ELEMENTS

Endogenous copies of densoviruses were detected using the TBLASTN algorithm against hymenopteran genomes available in the Whole Genome Shotgun Database (WGS) and Sequence Read Archive (SRA) at the NCBI, using densoviral protein sequences as queries. The queries involved NS1, NS2, NS3 and VP protein sequences. Comparisons were made using the TBLASTN program (Gertz et al., 2006), with the E-value cut-off set to 10^{-5} and default settings for other parameters.

2.3 PREDICTION OF PROTEIN DOMAINS

In order to recognize potential protein domains in the protein sequences analysed, we used NCBI CDD database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi),

by applying a cut-off E-value of 0.01. All Apis and Bombus densovirus proteins were compared against SMART (smart.embl-heidelberg.de), InterPro (www.ebi.ac.uk/interpro/) and Pfam (pfam.xfam.org) protein domain databases at default parameters.

2.4 PHYLOGENETIC ANALYSIS

To infer the phylogenetic relationships among densoviruses, we used their NS1 protein sequences. Key representatives of the densoviral lineages were included in the phylogenetic analysis. 24 protein sequences of the NS1 were aligned using MAFFT (Katoh and Standley, 2013). Phylogenetic trees were reconstructed using the maximum likelihood (ML) method. For phylogenetic reconstruction, we used IQ-TREE with the in-built automated test to choose the best substitution model for each tree (Trifinopoulos et al., 2016). Branch support was computed for all trees using 100 replicates of parametric bootstrap, and 1000 replicates of the approximate likelihood ratio test and ultrafast bootstrap. The iTOL online tool (http://itol.embl.de/) was used for phylogenetic tree annotation (Letunic and Bork, 2016).

3 RESULTS AND DISCUSSION

3.1 DISCOVERY OF THE ACTIVELY TRANSCRIBING DENSOVIRUS IN THE HONEY BEE TRANSCRIPTOME

Densoviruses are infecting diverse insect lineages (Cotmore et al., 2014; Penzes et al., 2020). Previous studies have found numerous endogenised densoviruses in insect genomes (Liu et al., 2011; Francois et al., 2016). Metatranscriptomic analyses of major invertebrate lineages have enabled the discovery of a very large number of novel RNA viruses (Shi et al., 2016). Recently, the metatranscriptomic analysis of diverse invertebrates has enabled the discovery of novel invertebrate DNA viruses (Porter et al., 2019). This methodology can identify actively transcribing DNA viruses in metatranscriptomic libraries. Here, we used this approach to find novel densoviruses in invertebrate transcriptomes at NCBI TSA database. We used both NS1 and VP proteins of diverse densoviruses as queries. A large number of novel densoviruses can be found in invertebrate transcriptomes; some are partial transcripts, while others represent separate NS and VP transcripts or nearly whole genomes. To our surprise, we found the first honey bee densovirus transcripts, with the size range between 1.9 and 2.6 Kb. These transcripts correspond either to the non-structural part of the densovirus genome (encoding NS proteins) or the structural part of the genome (encoding VP proteins). In the transcriptome of the eastern honey bee from China we found 8 VP transcripts (encoding a capsid protein) and 4 NS transcripts (Table 1). The size of the complete Apis densovirus VP protein is 760 amino acids, while the sizes of the NS3, NS2 and NS1 proteins are 177, 298 and at least 546 amino acids, respectively. Among NS transcripts only one encodes the complete set of NS3, NS2 and NS1 proteins (GALO01034698, 2215 bp long). NS1 protein is nearly complete, missing is only the C-terminal part (from 2 to 20 amino acids), depending on the most similar sequences that are quite divergent.

The most similar sequence to the NS1 protein of Apis densovirus is the ant *Solenopsis invicta* NS1; they are 49 %

Table 1: Apis densovirus transcripts

Transcript	NCBI accession number	Size of the transcript (in bp)	Presence of the intron
VP transcripts	GALO01020880	2454	no
	GALO01020879	2571	yes
	GALO01020878	2502	yes
	GALO01020884	2372	no
	GALO01020881	2489	yes
	GALO01020883	2420	yes
	GALO01020882	2425	no
	GALO01020885	2343	no
NS transcripts	GALO01034701	1921	no
	GALO01034700	1998	no
	GALO01034699	2138	no
	GALO01034698	2215	no

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aagcagaccatcgaaaaattaacaggagttttgtacccatctgttccaggtaagatatga
 K  Q  T  I  E  K  L  T  G  V  L  Y  P  S  V  P
ctagaaaattgaaacctccaccagacgaaagaccgaactatgaatttttaaatgagggcc

aaaaacgttatgcgtgggaacaatataaattggcacgtgttcgcagggattaccgatcg
                                              G  D  Y  R  S
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Figure 1: Apis densovirus possess a typical scindoambidensoviral intron in the VP1 gene. The VP1 intron is 117 bp long (*italic*). Splicing recognition sites are bold and underlined.

identical. Apis NS2 protein has best match in the *S. invicta* NS2 protein; they are 37 % identical. Apis NS3 protein is however unique and has no orthologs. Apis VP protein is more divergent and shows only 31 % amino acid identity with the *Planococcus citri* VP1 protein. We checked the conserved protein domains in the encoded Apis densovirus proteins and all of them are typical for densoviruses. In Apis VP protein, we can see the Parvo_coat_N domain (N-terminal region of the parvovirus VP1 coat proteins) and the large Denso_VP4 domain (capsid protein VP4 – four different translation initiation sites of the densovirus capsid protein mRNA give rise to four viral proteins, VP1 to VP4). Parvo_coat_N domain indeed encodes a special parvoviral phospholipase A_2 (PLA_2) that is necessary for their infectivity (Zadori et al., 2001). It is conserved in Apis VP protein and encodes at least 34 amino acids, with the conserved active site of the PLA_2 and Ca^{2+}-binding loop. In the NS1 protein, the DNA helicase protein that is required for the initiation of viral DNA replication is encoded in a protein domain named Parvo_NS1 superfamily. No conserved protein domains could be found in the NS2 and NS3 proteins.

In some of the Apis densovirus VP transcripts, we found an intron that is 117 bp long (Fig. 1). The presence of introns in a VP gene is typical for the *Scindoambidensovirus* genus. Members of the *Scindoambidensovirus* genus are characterized by a split VP-encoding ORF, which gives rise to the VP1 minor capsid protein *via* a spliced transcript as well as another major capsid protein (VP2) containing a unique N-terminal region, which has not been observed in any other parvovirus to date. The name "Scindo" refers to this split VP gene (Penzes et al., 2020, Tijssen et al., 2016). The Apis VP1 protein is 275 amino acids long, while the VP2 is 506 amino acids long. The presence of the split VP-encoding ORF in Apis densovirus indicates that it is most likely the new representative of the *Scindoambidensovirus* genus.

3.2 APIS DENSOVIRUS IS A MEMBER OF THE *SCINDOAMBIDENSOVIRUS* GENUS

To infer the phylogenetic affinity of Apis densovirus and relationships among densoviruses, we used their

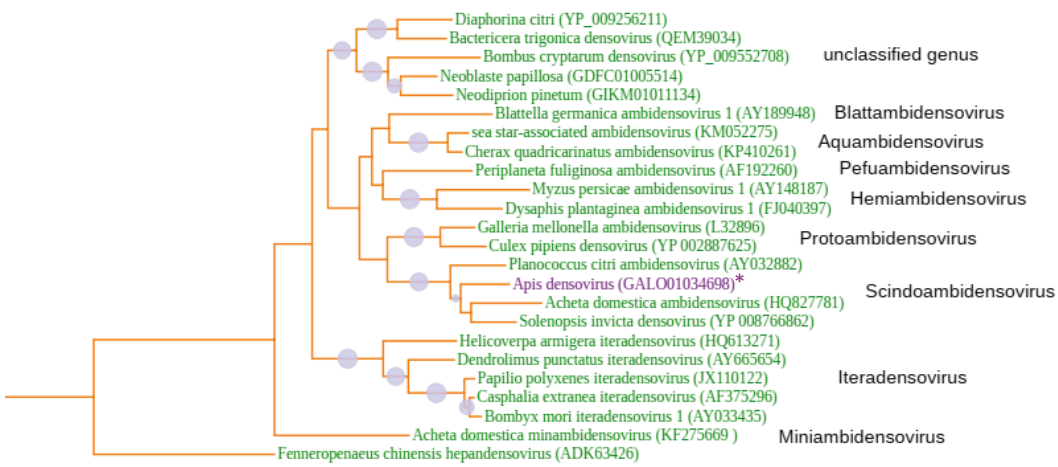

Figure 2: Maximum likelihood phylogeny of the densoviruses. The tree was inferred by IQTree program under a LG + F + I + G4 model from the NS1 proteins. Only bootstrap values larger than 80 % are shown as circles. The hepandensovirus was used to root this tree. Apis densovirus is shown in cyan color with asterisk.

NS1 protein sequences. Representatives of eight classified and one unclassified Densovirinae genera were included in the phylogenetic analysis. Best-fit model according to Bayesian information criterion was LG + F + I + G4. Tree was rooted with the hepandensovirus. Maximum likelihood phylogenetic analysis confirmed that Apis ambidensovirus is a new member of the *Scindoambidensovirus* genus (Fig. 2). On the other side, the bumblebee (*Bombus impatiens*) densovirus is a representative of a novel unclassified Densovirinae genus.

3.3 INFERRED GENOME ORGANISATION OF THE APIS AMBIDENSOVIRUS

Ambidensoviruses share a genomic characteristic: all of them exhibit antisense genome organisation. They maintain the division of the genome into separate non-structural (NS3 to NS1) and structural (VP capsid) gene cassettes; these cassettes are inverted with respect to one another. In ambisense densovirus genomes, the non-structural proteins (NS3 to NS1) are expressed from an ORF in the left half of the genome. The capsid proteins are translated from an ORF on the right hand side of the genome, but from an RNA generated in the opposite orientation (Mietzsch et al., 2019). Although we lack direct evidence for the Apis densovirus genome, we can simply infer its genome sequence from the available NS and VP transcripts. While the obtained genome sequence is not complete, it contains nearly 90 % sequence of the Apis densovirus. Missing are only terminal inverted repeats with promoters and the 50–100 bp in the center of the genome. The assembled partial Apis densovirus genome is currently 4786 bp long, while the expected complete genome size could be up to 5300 bp long (Fig. 3).

3.4 APIS DENSOVIRUS IS NOT ENDOGENISED

Previous studies of densoviruses in invertebrate genomes have found numerous endogenised densoviruses, some of them possessing complete genomes (Liu et al., 2011; Francois et al., 2016). We searched the available Apis genomes at the NCBI WGS and NCBI SRA databases for the presence of endogenised densoviruses. No endogenised densovirus sequences can be found in the available Apis genomes. The search for endogenised densoviruses in hymenopteran genomes showed that besides their presence in ant genomes they are also present in the bumblebee (*Bombus impatiens*) genome (AEQM02016195, 3848 bp long). This Bombus densovirus encodes intact NS1, NS2 and VP proteins (Fig. 4). It is most similar to the *Bombus cryptarum* and *Diaphorina citri* densoviruses but has very low level of similarity to the Apis densovirus (Fig. 2).

3.5 POTENTIAL IMPACT OF THE APIS DENSOVIRUS ON HONEY BEES

The relationship between densoviruses and their arthropod hosts ranges from mutualism (Xu et al., 2014) to severe pathology (Szelei et al., 2011), which is especially problematic in large insect rearing facilities (Tijssen et al., 2016; Schoonvaere et al., 2018). Densoviruses are highly pathogenic for insects at larval stages, which are infected through the ingestion of contaminated food

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NS3

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RKGTVFIHVSTVRSACRVSRAYKAICGNCAKPVQLKVYDAKGIKLDPIHF
CWNDSYTVTGNVYPPDGRPRIRKRYYR*

NS2

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NS1

MESYMGFASDSGDRGNSVGSTSDMGGPSGENIGKITPERGFTGHFPVRKI
KDLFPEQFKILEDKLQKVVGHYIRRIEEQDFGKTKRYISDVILLQGQEHR
DRCIRHLRQESGSYMGKLFIWVVETDHLHIVHDCPWSNGSCRCRILDVPF
IRRHVQKSVRRTKYISELDRTDYEGILLYFIVSKWESEREIWIGRRIQRL
PDQDEIVQWQDLSRTASELLARETEGGGHIGPEGSSYNDPRGSDIYEEFD
GTSKKRRAIDDGPRRAKETKWTKILSKIQAVLTEFMPIPAVHVRDLLVGI
PEYEYLHDPNIDKYYTNACSYYVNSISNFNFIDFYNLYNNRTPIFYANNL
NPFSYYHTREDSFQYLNRLLTYQLGGDTDIVREFLFNMKEWFNRKGWTGN
PKINAIAVIGPPNSGKNYFFDAVASIAYNVGHIGRVNNKTNNFALQECYS
KRFIVGNEISMEEGAKEDFKKLCEGTALNIRVKYQGDKIYKKTPVLLISN
SMLDICSDPAFKGIRLVTFTWNVAPFLRDSTLKPYPLAIFDLYNMYG

VP1

MASEAVGWDRSIIDKFLKNRTTQEEEHRLLDYNEVDNEHFGIEETAFNGE
PDYNYTSSGIYNSTSDISVGEGNVSRQRESIGSTNERNPNADGLRRRGGT
RGSLRISPSEGAGASESVASGVGASVGAVSSASAVGAGSSLAAPTLLASA
AIGTAAVGIGGYLTEKITNRRGYTLPGSDYVGPGNSIPIEAAKNPVDQIA
RDHDLKYQEIQEKYEKGQIDKSSFVAEVKEADREAATRFAEESGIHAAIG
KVGLDVKQTIEKLTGVLYPSVPGKI*

VP2

MRAKNVMRGNNINWHVFAGDYRSIILFQKLKNHKARTIRLLRKLIRITST
IILCHQSENNKLRLIVHLIHLVLPKAKKRKLTGTGQEQGNSNDVASDNSS
LQRLPSPLVSIHSHIRYYRKVHRILTYGLAYRAISFKLNNTDNSRIGYIL
STPLCEIPWDRMFLYINQGEFNVLPNGSTVNKIKCEIRTRNVRIAFPTNS
TDNNLATLNQNKSTVHAVGLNLSLSTMPIKYTSFQANQPMIPTSMDKVDD
SDYLNIHYNMYGMNYDITRVPRHQCGIPQVLPIYLGMVFAPFENQTDKTN
VGWECLQEKVVENLAEDAMSRELISVEYEPLEGLCKTPITPKWYGIPQAA
NKDKTSTVTVNYGPSEQSPQAKIITMNVNSEPHSYANSELKTDQNGYFGL
TQKIERSQEIWRGIYPHTHPRAQPSLHVGVQPTVALSTKTLVLDDSNNSF
TDTQGYFDVIAEMEINTQYPVYRPHIENCITGEGDFYVMRAATDESVPTF
SGLYQV*

Figure 3: The nucleotide sequence of the Apis ambidensovirus genome with encoded NS and VP proteins. Partial Apis densovirus genome was reconstructed from the two transcripts (GALO01034698 and GALO01020879). This genome is 4786 bp long, the gap in the middle of the genome is from 6 to 60 bp long (or 2 to 20 amino acids long); missing are ITRs.

ATAAACACGATACTAGAATATCTCAGACATACCTCAATGGGTGATATCGGCGAATTGTTTTGTCGCGAGG
ATATACCAGAAGAGTTCGAGACATTTGTGGATCAAGTAATAGCTGGTGAGTGCGAAAATGAATCAAATTT
TGGATATATTGCCGAAAACCGATCTAACATTGGAGAAGCTTCATGTTCTGGAAATGTGCCTATGGAGATT
AGCGAAAGAGTATATGGATTCTCAGAACAAGGATCAGCTGCTTATATGGTTCCACCGGGTGAAAGCATTA
CAGCAACACAGAGAGATGCTGCTGAAAGACGAAAATTGCTCAGGCAAATATTCTTGGAAAGATTTGAGAG
CCAGCATAGACGCAACTCAGTCTGCCATCAAATTTTTAGTAAAAGAAGTATCCCAGGAGCTAATGCTGAA
ATTAAAAGAGTGGTACAAGGAAATTTTCGAGGAACAGCTTATTTGGTATGCGATCACGGAGACCACTATC
ACATTGTGCACGACTGCCATAGATCAGGGCAAAGATGTCGCTGTCATCGACTCGACGAAACCAGGAACGT
CTTCGGTCGAGCAGTGTCTGAACGAGTTGTTAGAGACAACATCTTCGACATCGAACATTGGATCAATCTC
GCAGAGTATTTCCAAAAAGACGAACGGCACCTTATCTACATGGAAGTCTGCGGGAGAGAAAGGACTGAAT
GTGTTCAAAATAGAAAAGTATTCGTTCAAGGAAGTGTCCAAGCTAGACAAGACGAAATGGTGGATGACAC
CGTCAGCAGTGAGAGCCCTATTCGTGACTTCCTCTCCTTTGGATCCTGTGGCGATACATGCAGACCAAGT
ACTGCTGCTGGGGACGAAGAGGTTGACCAAGCTGCCCGGAGTTCAGAAGGAGGAAAAACAACTAATGTCG
AAAAATACATACGAAAGTTTCTCACGTCTCCAATAACACATTTACTATCTACATCATATTGGATTAATTC
GAAGTACTATATGATCAATACACAATCTAATTGGTTTCAATGTGTTATGCGTAAGATATCTTTTTCATTT
AATCGTATGACTATATACGAATTATTTCAATACGCTAAACCACTGGATATGGATAAGCTGCTATATAGTA
GTCCTACGGAAATGATATTCGATTATTACTATGATATACGCACAAGTGTATACATTTTGGATGAGCTACT
ACGATTCCAGATGCAAGATGAAGATGAAATTGTATCATTCTTGGAAGTTCTATTAGCTGTCTTGGATAAA
TCAATACCGAAGAAGAATTGCTTACATATACAAGGACCACCATGTTCAGGGAAGAACCTATTCTTCGACT
GTGTGACATCGTTTTGTATTAATTGCGGACACTTGGGCAATTTCAACAAGTATAATTCTTTTCCGATGAT
GGATTGTATAGATAAACGTGTTATCATGTGGAATGAGCCTATCTTAGAAGTATCAGCACTCGAAACATTA
AAAATGGTATTCGGTGGAGATACTTGTCCTGTTAAAGTTAAATATCTCGGAGATAAGTTATTGCTTCGTA
CTCCGATTATCTGTTTATCGAATAATCAGCCATTTCCACAGGATGACGCATTTACCTGTCGTATGTTTAC
TTACCAATGGCAACAATGTAATGATTTAAAGAAAGTATCAAAAAAACCTCATCCTTTAGCTTTTCCTTAC
CTATTAATAAAATATAAGATATGGGAAGATGTAGAATTGAATGAGAAAGAAAAAGAATATTTATATTAAT
AAACATTTATTCAGCATAATATATGTGTACATTTAATTTACAGTATCCTGACCCATATTACTTAGTCTAT
CAAATTTATTTCCTAAATTACGACGAACAGGTCCCATCCTCTCATCATCCCTATATGCCATCTTATATTG
AGCTGCTCTTGGTGGATTTGGTGGAGTGAAGTCCTCTGTTTGTGTTGATCTTGTAGCAGGTGGTTCAGGT
GCTGGCATAGCAACCAATCCAGATAGTTGACAATATCCATTGTATGCTCTATTTCCAACCCATATAGTAG
TATCTGATGGAAATTGTACTGTGCCATTCTCCGAATAGTATGATCCATGATGTAGTTCAACATCTAATTC
AGCTTCAACTGCCCAATATGCAGATGTATTCTGGAATGTAGTACTATCCGATCCTGGAGTCATAGCTGGT
ACTGCCTGAATTCCTATATGTATTTGTGGTTGTGCTTTAAATGGGAATCCTCCATTCTGGAAATTATGTC
CACCTTTCTCCAAAAATTGTTCTACATTTGTTACGGGAGTAGAATTGTAATCATTTATTTTATCGCTTAT
ATCTGTAGTATGTGCTGCACCTAATGTTGTACTATGATCTCCATTCTTTATTACTTCTAATGCAAATGCT
CTAGTACGTGGACCACCATAATGTACATATCTTGTATGAACATTAGGAGGATTATAATAGGGTGCACTAA
TAGGAGCATGTTTACACTTATACGTATAATTAACTACAGGTTTTCCTATTGCTGTGTTTATTAGAAATTG
ATCTACAAATTGATCTTTACGCATCTGTCCGTGATTGTTAGTGAACACTGTTGCACCAGTCGTAGGTCCT
GGACTAACAAATGCTGCATATCCCACTGTATGTCTTGTGACTAGATTAGTATGTGAATCATCCGCTCTAT
AGTATTTGTTAATTATATCGCTAGCATTAATCAATGACATATTATTCGGTTTCATATTATCCATTGTTCC
ATATGTACAATTTACAATTTCTGTATTTACATTCAGTCCTACAGATACTAATCCTATAGGTACAAATTCG
TTAGTAGTATGTCCTGATGTTGTCCCTCCAAATTCAAACGCAGTACGAATACCGAGAGGTGTTACTTTAA
CACGACATTCTTTAGCCCATGCATTATTTGGTAATTCTCTAAATTCGGTAGGTGATAAATAAAATCCAAC
TAAATCTGTAGGTATTAAAGCTAATGGTGTTGTATAATAATCATCGCTGTTATTATTTATTCTCTTTGTA
CATATTCCATAGCTGAAGAATATTCTATTCTTACGGAATGTAACAATAGATGGTTTAGGTGCAAGAGGTC
TAGGTATAGTAACAATGTGTCCTACAGTGTGACTACCACTACTACGTCCACTAGCACCTGATGGACCAGA
TGCTGCTAACACCTGATTATCATTCACACCATTTATACTTGGTACATCGAACTCCATGCCTTCTATATCG
TTAAACAATCCCAATAGACTATTATCTGATCCCTGACTACTGTCTGGTTGTTGTATTTGTTCTTCTTGTT
GTTCCTGTGGTACGTATTTACTTGTTCCTGGCTGATTTCTCTTTAATTCGTCCCAGGCGTCCTTTGAATG
GGCTTGTTGTATCTCTCGAAAGGATAATCCTGTCTCTCTACTAGTATCTGCTAATTGTCTCTGTATTTGT
GCGTACCTCTTTCTTTGTTCCAACTGTGCTGGTGTTGGTTGTCTTCTCTTCATATTAGGATATAACACTC
CTGTCAAGGATTCTACACCATACTTAGCTGCTAATCCTGCAGCACCCAAATAACCATGTGGTGATTGTAA
TTCCCAAAAATGTTTTATGGCGTCTCGATCTGCCTCTCTTATATCTTCCTCAGTCTTTGCACGATCATAC
AAATTATCATGTATTCTCGCTATTTCATCGTCTTCATCCACTGGTTCACCGTTCTTCAATTTATTCCCTG
GCCCTAAATAGCGATGGCCGTACCAATTCATACTGACTTATATCATCTCATCGCATGTCTTATATAGTCT
GAATAGATCGTATGGTAGGGTGCTCCGAGAATCACAGGTAAACTATTATGATCTATTCCATCCTTACT

Fig 4 continues on the next page

NS1 (70% aa id with *Bombus cryptarum* densovirus)
INTILEYLRHTSMGDIGELFCREDIPEEFETFVDQVIAGECENESNFGYI
AENRSNIGEASCSGNVPMEISERVYGFSEQGSAAYMVPPGESITATQRDA
AERRKLLRQIFLERFESQHRRNSVCHQIFSKRSIPGANAEIKRVVQGNFR
GTAYLVCDHGDHYHIVHDCHRSGQRCRCHRLDETRNVFGRAVSERVVRDN
IFDIEHWINLAEYFQKDERHLIYMEVCGRERTECVQNRKVFVQGSVQARQ
DEMVDDTVSSESPIRDFLSFGSCGDTCRPSTAAGDEEVDQAARSSEGGKT
TNVEKYIRKFLTSPITHLLSTSYWINSKYYMINTQSNWFQCVMRKISFSF
NRMTIYELFQYAKPLDMDKLLYSSPTEMIFDYYYDIRTSVYILDELLRFQ
MQDEDEIVSFLEVLLAVLDKSIPKKNCLHIQGPPCSGKNLFFDCVTSFCI
NCGHLGNFNKYNSFPMMDCIDKRVIMWNEPILEVSALETLKMVFGGDTCP
VKVKYLGDKLLLRTPIICLSNNQPFPQDDAFTCRMFTYQWQQCNDLKKVS
KKPHPLAFPYLLIKYKIWEDVELNEKEKEYLY*

NS2 (76% aa id with *Bombus cryptarum* densovirus)
MNQILDILPKTDLTLEKLHVLEMCLWRLAKEYMDSQNKDQLLIWFHRVKA
LQQHREMLLKDENCSGKYSWKDLRASIDATQSAIKFLVKEVSQELMLKLK
EWYKEIFEEQLIWYAITETTITLCTTAIDQGKDVAVIDSTKPGTSSVEQC
LNELLETTSSTSNIGSISQSISKKTNGTLSTWKSAGEKGLNVFKIEKYSF
KEVSKLDKTKWWMTPSAVRALFVTSSPLDPVAIHADQVLLLGTKRLTKLP
GVQKEEKQLMSKNTYESFSRLQ*

VP1 (59% aa id with *Bombus cryptarum* densovirus)
MNWYGHRYLGPGNKLKNGEPVDEDDEIARIHDNLYDRAKTEEDIREADRD
AIKHFWELQSPHGYLGAAGLAAKYGVESLTGVLYPNMKRRQPTPAQLEQR
KRYAQIQRQLADTSRETGLSFREIQQAHSKDAWDELKRNQPGTSKYVPQE
QQEEQIQQPDSSQGSDNSLLGLFNDIEGMEFDVPSINGVNDNQVLAASGP
SGASGRSSGSHTVGHIVTIPRPLAPKPSIVTFRKNRIFFSYGICTKRINN
NSDDYYTTPLALIPTDLVGFYLSPTEFRELPNNAWAKECRVKVTPLGIRT
AFEFGGTTSGHTTNEFVPIGLVSVGLNVNTEIVNCTYGTMDNMKPNNMSL
INASDIINKYYRADDSHTNLVTRHTVGYAAFVSPGPTTGATVFTNNHGQM
RKDQFVDQFLINTAIGKPVVNYTYKCKHAPISAPYYNPPNVHTRYVHYGG
PRTRAFALEVIKNGDHSTTLGAAHTTDISDKINDYNSTPVTNVEQFLEKG
GHNFQNGGFPFKAQPQIHIGIQAVPAMTPGSDSTTFQNTSAYWAVEAELD
VELHHGSYYSENGTVQFPSDTTIWVGNRAYNGYCQLSGLVAMPAPEPPAT
RSTQTEDFTPPNPPRAAQYKMAYRDDERMGPVRRNLGNKFDRLSNMGQDT
VN*

Figure 4: The nucleotide sequence of the endogenised *Bombus impatiens* densovirus genome with encoded NS and VP proteins. Partial ambisense *Bombus impatiens* densovirus genome was obtained from the NCBI WGS database (AEQM02016195). This genome is 3848 bp long, missing are ITRs.

(Tijssen et al., 2016). In honey bees, brood diseases are common and are caused by fungi, bacteria and viruses (Brutscher et al., 2016). Until now, among the honey bee-infecting viruses, only the Sacbrood virus was connected with the honey bee brood disease (Brutscher et al., 2016). The question to be resolved is whether densoviruses infecting pollinators (honey bees and bumblebees) exert any detectable pathological effects on them.

3.6 INFORMATION ABOUT THE ORIGIN OF THE EASTERN HONEY BEE TRANSCRIPTOME

The honey bee transcriptome in which we found the first Apis densovirus was erroneously classified as *Apis mellifera carnica* transcriptome (SRR922440, 52.177 contigs) and is still available under such name. It was produced from the whole heads of the workers by the researchers from the Yangzhou University, Jiangsan Province, China (Ji et al., 2014). The analysis of the SRR922440 transcriptome showed that 86 % of contigs have strong signals to the *Apis cerana* and only 1.2 % to the *Apis mellifera*. China, the largest producer of honey, introduced *A. mellifera* (diverse subspecies, including *carnica)* besides the native *A. cerana*. Since the erroneous classification of transcriptomes is misleading, we also investigated the origin of the SRR922440 transcriptome with complete mitochondrial genomes of *A. cerana* and *A. mellifera carnica*. This analysis demonstrated that the SRR922440 transcriptome originates from the *Apis cerana*, since numerous sequences show 99 % identity with the mitochondrial genome of *A. cerana*. The authors of this transcriptome (Ji et al., 2014)

provided some information about the origin of the *A. cerana* colonies: M and C colonies were unrelated local strains in the same *A. cerana* population and were bred in the Guandong Entomological Institute, Guangzhou (also known in the West as the Canton), southern China. All Apis densovirus transcripts were obtained from the C colony that was Varroa resistant. No information about the health status of the bees was provided in the original reference (Ji et al., 2014). We checked if these bees were infected with some RNA viruses, discovering only a full-length Sacbrood virus in this transcriptome (in the sequence GALO01042235). It should be noted that the additional *A. cerana* brain transcriptome is available at the NCBI TSA database (SRR361851), but shows the absence of the Apis densovirus. This transcriptome was produced by the researchers from the Fujian University: eastern honey bees originated from the Honey bee Research Institute, Jiangxi Agricultural University, Nanchang, Jiangxi province, east China. The availability of diverse *A. cerana* transcriptomes therefore shows limited presence of Apis densovirus in southern China.

4 CONCLUSIONS

Viruses, especially RNA viruses, pose a major threat to the survival of honey bees. Combined with additional stressors, the consequences for honey bees and agriculture can be extremely severe. Here, we present the first densovirus in honey bees, which can pose a potential threat to them. Densoviruses are often associated with high mortality and great economic losses in commercially important infected insects, such as farmed crickets and silk moths. Given that densoviruses have also been detected in bumblebees, their potential pathogenicity could pose a serious threat to diverse pollinators (honey bees and bumblebees) and consequently to agriculture. The availability of the nucleotide sequence for the honey bee and and bumblebee ambidensovirus genomes, its transcripts, and all coding proteins provides a good starting point for more detailed studies of the pathogenicity of densoviruses in honey bees and bumblebees. Research on the effects of infection on the survival of honey bee colonies is also needed, as larvae are the most common victims of densoviruses in the majority of infected insects. We detected the densovirus only in eastern honey bees (*Apis cerana*) from southern China. Of course, this does not mean that this virus is not more widespread or that it lacks the potential to rapidly spread around the world. Dead larvae should be tested for the presence of the Apis densovirus. Research on densoviruses in diverse pollinators and their impact on the survival of honey bees and bumblebees is therefore urgently needed.

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