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Research paper

Diverse single-stranded DNA viruses associated with honey bees (*Apis mellifera*)

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ABSTRACT

Honey bees (*Apis mellifera*) research has increased in light of their progressive global decline over the last decade and the important role they play in pollination. One expanding area of honey bee research is analysis of their microbial community including viruses. Several RNA viruses have been characterized but little is known about DNA viruses associated with bees. Here, using a metagenomics based approach, we reveal the presence of a broad range of novel single-stranded DNA viruses from the hemolymph and brain of nurse and forager (worker divisions of labour) bees belonging to two honey bees subspecies, Italian (*Apis mellifera linguistica*) and New World Carniolan (*Apis mellifera carnica*). Genomes of 100 diverse viruses were identified, designated into three groupings; genomoviruses (family *Genomoviridae*) ($n = 4$), unclassified replication associated protein encoding single-stranded DNA viruses ($n = 28$), and microviruses (family *Microviridae*; subfamily *Gokushovirinae*) ($n = 70$). Amongst the viruses identified, it appears that nurses harbour a higher diversity of these viruses comparative to the foragers. Between subspecies, the most striking outcome was the extremely high number of diverse microviruses identified in the Italian bees comparative to the New World Carniolan, likely indicating an association to the diversity of the bacterial community associated with these subspecies.

1. Introduction

Honey bees (*Apis mellifera*), as major polylectic pollinators, are crucial for the food chain pollinating not only a large percentage of the agriculturally important crops grown for human and animal consumption but also playing a central role in the pollination of a wide variety of wild angiosperms globally (Brown and Paxton, 2009; Hung et al., 2018; Southwick and Southwick, 1992). These eusocial insects form a complex society, comparable next only to that of humans, with a highly specialised division of labour, all working to benefit the hive. Each colony has a queen that is responsible for production of offspring, workers which perform a multitude of roles throughout their lifespan which is typically around 40 days for summer bees, and drones which are the only males and have the sole function of mating with a queen. Fundamentally dictated by age, two specific roles of worker bees are nursing and foraging. Nurse bees are the youngest in the colony and are in charge of caring for the larvae, cleaning the comb, and attending to the queen from the age of ~ 1 to 10 days old (Huang et al., 1994; Sakagami, 1953; Wagener-Hulme et al., 1999). Foragers are the oldest bees in the colony and they collect nectar, pollen and water to provide for the entire colony. Worker bees forage from about 21 days of age until death (Huang et al., 1994; Pankiw and Page, 2001; Wagener-Hulme et al., 1999).

Globally, several subspecies of honey bees are kept for honey production and pollination of major crops. In the United States of American the two most commonly kept are Italian (*Apis mellifera linguistica*) and New World Carniolan (*Apis mellifera carnica*).The ecology, behaviour and genetics of honey bees has been extensively studied and of late studies have focused on gaining insights into colony collapse disorder (CCD). CCD, characterized by the disappearance of adult bees, has most notably affected the commercial enterprises for pollination and honey production, as beekeepers continue to lose up to 40% of their functional population annually (Bruckner et al., 2018; Calderone, 2012; van Engelsdorp et al., 2008). Although likely caused by a combination of factors, several pathogens have been implicated as major contributors to CCD and more broadly to the overall hive productivity and health

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(Cornman et al., 2012.). As a result, efforts in pathogen research have increased over the last decade and a suite of viruses, predominantly single-stranded RNA viruses, have been identified in bees. Some of these viruses, such as deformed wing virus (DWV), are highly prevalent and can cause deformation, paralysis and death. Transmission routes differ with species, however, Varroa mite (*Varroa destructor*) play a crucial role in viral dynamics both within and between colonies (Chen et al., 2006; Mortensen et al., 2018; Ravoet et al., 2015). Little is known regarding DNA viruses in bees, with *Apis mellifera* filamentous virus currently being the sole identified DNA virus (Gauthier et al., 2015). Furthermore, despite an abundance of ssDNA viruses documented in a range of arthropods (Rosario et al., 2018), only one metagenomic based study identified contigs which share similarities to ssDNA viruses from honey bees sampled in the Middle East and North Africa (Haddad et al., 2018).

In the era of high throughput sequencing novel viruses discovery has continued to expand and with this has come an expansion of knowledge on DNA virus groups such as the circular replication-associated protein encoding single stranded (CRESS) DNA viruses (Rosario et al., 2012a). There are several established CRESS DNA virus families; *Bacillidnaviridae, Circoviridae, Geminiviridae, Genomoviridae, Microviridae, Nanoviridae* and *Smacoviridae,* (reviewed in Zhao et al. 2019). Several novel CRESS DNA viruses have also been reported in the last decade that have not been classified at a taxonomic level (Simmonds et al., 2017). These all encode a replication associated protein with conserved motifs important for initiation of replication (Rosario et al., 2012b). One of the most recently established families is *Genomoviridae* (Krupovic et al., 2016). Although members of this family have been isolated from a broad range of sample types and locations globally (Varsani and Krupovic, 2017), only one species in this family, *Sclerotinia sclerotiorum hypovirulence virus 1*, to date has a known host, the fungus *Sclerotinia sclerotiorum* (Yu et al., 2010). Members of the *Microviridae* family infect bacteria and are abundant in various environments including soil (Hopkins et al., 2014), the ocean (Labonte and Suttle, 2013) and guts of animals such as the marine invertebrate *Ciona robusta* and termites (Creasy et al., 2018; Tikhe and Husseneder, 2017). The family *Microviridae* is divided into two subfamilies, *Gokushovirinae* and *Bullavirinae* (International committee for virus taxonomy: 2018 release; https://talk.ictvonline.org/taxonomy/). Gokushoviruses are a rapidly expanding group with viruses that are known to infect parasitic bacteria – Bdellovirbio and Chlamidia, and the mollicute – Spiroplasma, and they typically range in size from 4 to 7 kb (based on those presently archived sequences in the public database GenBank). At least three recognised proteins are encoded by microviruses, i.e. major capsid, DNA pilot protein and a replication initiation protein (Cherwa and Fane, 2011).

In an effort to advance our understanding of the honey bee virome, we undertook a metagenomics study on two commonly kept honey bee subspecies, the Italian and the New World Carniolan. This study specifically focused on DNA viruses and identified a broad range of novel viruses. This expands our current knowledge in bee virology and highlights the importance of further studies in order to determine the role of these viruses in honeybee health.

2. Materials and methods

2.1. Honey bee collection, dissection and isolation of viral DNA

Individual nurse and forager honeybees from single colonies of two different subspecies, New World Carniolan and Italian were sampled in June 2017 and August 2017, respectively, at the Arizona State University Bee Lab Annex in Mesa, Arizona, USA. Colonies were housed in typical 10-frame Langstroth hives cared for with standard honey beekeeping techniques. All colonies were housed in a common bee yard with access to the same foraging resources. The colonies appeared healthy, showing no signs of disease or mite infestation. Ten individuals from each working group and subspecies were randomly collected from individual colonies (one colony per subspecies), 40 individuals in total, and stored on dry ice. In the laboratory, these were sterilized and dissected to recover the brains and hemolymph. Each brain was dissected on dry ice using a dissecting microscope (VWR ocular microscope, USA), scalpel and forceps, and placed into individual 1.5 ml microcentrifuge tube. Hemolymph was extracted from the thorax from the incision where the head was removed using a 10 μl pipette, and placed into a separate 1.5 ml microcentrifuge tube. A total of 40 hemolymph and 40 brain samples were placed on dry ice for transport and stored at −80 °C until processed. The brain and hemolymph samples were homogenised in SM buffer [100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris–HCl (pH 7.4)] and the nucleic acid extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, USA). DNA for each sample was quantified using a Qubit 4 (ThermoFisher Scientific, USA) and circular DNA molecules were preferentially amplified by rolling circle amplification (RCA) using TempliPhi (GE Healthcare, USA). Samples were pooled into groups based on tissue type (hemolymph or brain), worker group (nurse of forager) and subspecies (Italian and New World Carniolan).

2.2. High throughput sequencing for identification of DNA viruses and determination of complete viral genomes

The RCA products were sequenced on an Illumina Hiseq4000 Sequencer (Illumina, USA) at Macrogen Inc. (Korea). The pair-end reads were de novo assembled using SPAdes v 3.12.0 assembly software (Bankevich et al., 2012) and the resulting contigs > 1000 nts were analysed with BLASTx (Altschul et al., 1990) against a viral protein database (the Viral RefSeq protein database downloaded from the GenBank). Contigs that encoded proteins that shared credible homology to known viral proteins were binned into two groups, those with similarities to members of the *Microviridae* family and those with similarities to other CRESS viruses. For the latter group, abutting primer pairs (Supplementary Table 1) were designed to recover the complete DNA viral genomes by PCR. PCR was undertaken with HiFi HotStart DNA polymerase (KAPA Biosystems, USA) and cycling conditions according to manufacturer's specifications. The amplified genomes were resolved on a 0.7% agarose gel using electrophoresis, the correct size amplicons we excised from the gel, purified using the Quick-spin PCR Product Purification Kit (iNtRON Biotechnology, Korea) and cloned into pJET1.2 cloning vector (ThermoFisher Scientific, USA). The recombinant plasmid was transformed into XL blue *E. coli* competent cells, plasmids from the transformants were purified using a DNA-spin Plasmid DNA Purification kit (iNtRON Biotechnology, Korea) and Sanger sequenced at Macrogen Inc. (Korea) by primer walking. Sanger sequencing contigs were assembled using Geneious software V11.0.3. All de novo assembled microviruses were verified by mapping raw reads with a coverage level of > 10× using BBMap (Bushnell, 2015).

2.3. Virus characterization and phylogeny

For those viral genomes which shared similarities to CRESS DNA viruses the *rep* gene sequences were extracted and a datasets was compiled with *reps* of CRESS DNA virus sequences available in GenBank (as of 10 Sept 2018). The same was done for the *major capsid protein (mcp)* gene (also referred to as the VP1 and F protein) of the microviruses. The nucleotide CRESS DNA virus *rep* and microvirus *mcp* gene sequences were translated prior to further analyses. The CRESS DNA virus Rep dataset was further broken down into family groupings, or most similar virus grouping. Since the datasets of Reps and MCPs are large, using BLASTp (with a minimum cut off e-value of 1×10^{-20}) against a locally curated dataset of Rep and MCP we identified the 10 nearest neighbours from the sequences of this study. The reduced datasets were aligned using MUSCLE (Edgar, 2004) and used to infer Maximum likelihood phylogenetic trees using PHYML (Guindon et al.,

Table 1
Sample information, outlining taxonomic groupings, genome size, GenBank accession number and isolation source for the genomoviruses, novel CRESS DNA viruses and microviruses recovered in this study.

Family/subfamily/genus	Species grouping/Isolate name	Genome size (nts)	Accession number	Isolation source	
				Worker group/sample type	Bee subspecies
Genomoviridae/NA/Gemykibivirus	*Apis mellifera* genomovirus-1 BNH 2007	2208	MH973740	Nurse/Brain	*Apis mellifera carnica*
	Apis mellifera genomovirus-1 INH5 2007	2209	MH973737	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera genomovirus-1 IFH 184NG	2208	MH973739	Forager/Hemolymph	*Apis mellifera linguistica*
Genomoviridae/NA/Gemykrogvirus	Apis mellifera genomovirus-2 BNH 406	2218	MH973741	Nurse/Hemolymph	*Apis mellifera carnica*
CRESS DNA virus (Unassigned)	Apis mellifera DNA virus 1 BNH267	3033	MH973742	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 2 CFH6 2099	2323	MH973743	Forager/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 2 INH1208NG	2323	MH973744	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 3 INH5 2104	2347	MH973745	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 4 IFH156NG	2455	MH973746	Forager/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 4 INH5 59	2455	MH973747	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 5 BNH861	1901	MH973774	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 6 BCFH18929NG	1746	MH973749	Forager/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 6 BCNH17108NG	1746	MH973750	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 6 INH3 982	1746	MH973751	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 7 INH3 750	2719	MH973752	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 8 BCFH18425NG	1780	MH973753	Forager/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 8 BCNH16643NG	1780	MH973754	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 8 IFH251NG	1780	MH973755	Forager/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 8 INH3 98	1780	MH973757	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 9 BCFH13578NG	2200	MH973758	Forager/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 9 IFH186NG	2200	MH973759	Forager/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 9 INH3 67	2201	MH973761	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 10 INH3 2469	1893	MH973764	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA virus 11 CNH 1691	3263	MH973765	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 12 CFH6 2802	1769	MH973766	Forager/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 12 CNH1 2802	1769	MH973767	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 13 BNH4529	1876	MH973768	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 14 BFH508NG	1909	MH973769	Forager/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 14 BNH302	1907	MH973770	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 15 BNH198	1715	MH973771	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA virus 16 BFB18385	2006	MH973772	Forager/Brain	*Apis mellifera carnica*
	Apis mellifera DNA virus 16 BNH4370NG	2006	MH973773	Nurse/Hemolymph	*Apis mellifera carnica*
Circular DNA molecules (Unassigned)	Apis mellifera DNA molecule 1 INH3 2498	1586	MH973775	Nurse/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA molecule 2 CNH20931NG	1516	MH973776	Nurse/Hemolymph	*Apis mellifera carnica*
	Apis mellifera DNA molecule 2 IFH297 NG	1516	MH973777	Forager/Hemolymph	*Apis mellifera linguistica*
	Apis mellifera DNA molecule 2 INH2 1118	1516	MH973778	Nurse/Hemolymph	*Apis mellifera linguistica*
Microviridae/Gokushovirinae	Apis mellifera microvirus-1 BeeINH SP 292	4279	MH992217	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-2 BeeINH SP 246	4436	MH992206	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-3 BeeINH SP 275	4331	MH992215	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-4 BeeINH SP 208	4576	MH992192	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-5 BeeINH SP 330	4117	MH992223	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-6 BeeCNH SP 3574	4195	MH992164	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-7 BeeINH SP 273	4332	MH992214	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-8 BeeINH SP 309	4211	MH992222	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-9 BeeINH SP 302	4259	MH992220	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-10 BeeCNH SP 3231	4387	MH992163	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-11 BeeNHemo SP 392	4135	MH992171	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-12 BeeINH SP 298	4269	MH992219	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-13 BeeFHemo SP 62	4538	MH992166	Forager/Hemolymph	Apis mellifera carnica

(*continued on next page*)

Table 1 (*continued*)

Family/subfamily/genus	Species grouping/Isolate name	Genome size (nts)	Accession number	Isolation source	
				Worker group/sample type	Bee subspecies
	Apis mellifera microvirus-14 BeeINH SP 289	4286	MH992216	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-15 BeeINH SP 253	4412	MH992208	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-16 BeeINH SP 224	4519	MH992199	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-17 BeeINH SP 228	4502	MH992201	Nurse/Hemolymph	Apis mellifera liguistica
	Apis mellifera microvirus-18 BeeINH SP 306	4231	MH992221	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-19 BeeIFH SP 71	4506	MH992180	Forager/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-20 BeeINH SP 206	4582	MH992190	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-21 BeeINH SP 231	4485	MH992202	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-22 BeeCNH SP 2675	4770	MH992157	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-22 BeeIFH SP 61	4770	MH992174	Forager/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-23 BeeINH SP 214	4559	MH992195	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-24 BeeINH SP 257	4394	MH992209	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-25 BeeINH SP 259	4392	MH992210	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-26 BeeINH SP 266	4355	MH992211	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-27 BeeCNH SP 2766	4691	MH992158	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-28 BeeINH SP 207	4578	MH992191	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-29 BeeINH SP 236	4469	MH992204	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-30 BeeFHemo SP 55	4703	MH992165	Forager/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-31 BeeNHemo SP 324	4345	MH992170	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-32 BeeNHemo SP 302	4410	MH992169	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-33 BeeINH SP 293	4278	MH992218	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-34 BeeINH SP 217	4548	MH992197	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-35 BeeINH SP 177	4719	MH992185	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-36 BeeINH SP 268	4346	MH992213	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-37 BeeINH SP 211	4566	MH992194	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-38 BeeINH SP 209	4569	MH992193	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-39 BeeINH SP 174	4737	MH992184	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-40 BeeINH SP 151	4893	MH992181	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-41 BeeCNH SP 3010	4529	MH992162	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-41 BeeIFB SP 218	4529	MH992172	Forager/Brain	Apis mellifera linguistica
	Apis mellifera microvirus-41 BeeIFH SP 70	4529	MH992179	Forager/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-41 BeeINH SP 221	4529	MH992198	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-42 BeeCNH SP 2929	4584	MH992161	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-43 BeeINH SP 198	4608	MH992188	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-44 BeeCNH SP 2907	4600	MH992160	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-44 BeeIFH SP 66	4600	MH992177	Forager/Hemolymph	Apis mellifera linguistica
		4600	MH992189	Nurse/Hemolymph	

(*continued on next page*)

Table 1 (*continued*)

Family/subfamily/genus	Species grouping/Isolate name	Genome size (nts)	Accession number	Isolation source	
				Worker group/sample type	Bee subspecies
	Apis mellifera microvirus-44 BeeINH SP 200				Apis mellifera linguistica
	Apis mellifera microvirus-45 BeeIFH SP 57	4859	MH992173	Forager/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-46 BeeINH SP 247	4431	MH992207	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-47 BeeINH SP 169	4756	MH992183	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-48 BeeIFH SP 63	4633	MH992176	Forager/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-48 BeeINH SP 193	4633	MH992187	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-49 BeeINH SP 242	4448	MH992205	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-50 BeeINH SP 180	4708	MH992186	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-51 BeeCNB SP 10118	4469	MH992156	Forager/Brain	Apis mellifera carnica
	Apis mellifera microvirus-52 BeeINH SP 225	4507	MH992200	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-53 BeeIFH SP 67	4587	MH992178	Forager/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-54 BeeINH SP 153	4882	MH992182	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-55 BeeCNH SP 2813	4661	MH992159	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-55 BeeIFH SP 62	4661	MH992175	Forager/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-56 BeeCFH SP 3639	4557	MH992155	Forager/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-56 BeeINH SP 216	4557	MH992196	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-57 BeeINH SP 267	4348	MH992212	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-58 BeeINH SP 235	4470	MH992203	Nurse/Hemolymph	Apis mellifera linguistica
	Apis mellifera microvirus-59 BeeCFH SP 3351	4728	MH992154	Forager/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-60 BeeNHemo SP 226	4702	MH992168	Nurse/Hemolymph	Apis mellifera carnica
	Apis mellifera microvirus-61 BeeNHemo SP 71	6391	MH992167	Nurse/Hemolymph	Apis mellifera carnica

2010) with best-fit models (rtRev+I + G + F for genomovirus Reps; LG + I + G for CRESS DNA virus Reps; LG + I + G for gokushovirus MCPs) determined using ProtTest (Darriba et al., 2011). For all phylogenetic trees branch with < 0.8% SH-aLRT support were collapsed in TreeGraph2 (Stover and Muller, 2010). The geminiviruses Rep sequences were used to root the genomovirus phylogenetic tree, the CRESS DNA virus Rep tree was midpoint rooted and MCP sequences of bullaviruses were used to root the gokushovirus phylogenetic tree.

2.4. Statistical analyses

A general linear mixed model with binomial error distribution and logit transformation was used to assess whether subspecies or the worker group differed in their viral prevalence. We used presence/absence of each virus group (Table 1) as our binomial response variable, and subspecies and worker group as predictor variables. We treated the virus groupings as a random effect, because of likely inherent differences between infectiveness of the viruses (Juarez et al., 2013). We used a backward model selection approach where we started with the interaction between task group and subspecies, and if it was not significant, we dropped that term, and then evaluated the individual factors. All statistics were implemented in Program R (R Development Core Team, Vienna, Austria) version 3.3.3.

3. Results and discussion

This study explores the viral diversity of ssDNA viruses in honey bees. From two honey bee subspecies (Italian and New World Carniolan) we collectively describe four genomovirus genomes, 28 eukaryote-infecting CRESS DNA viruses which currently do not fall into any taxonomically classified families, and 70 prokaryote-infecting CRESS DNA viruses (i.e. microviruses in the subfamily *Gokushovirinae*) and five novel circular DNA molecules (Table 1).

3.1. Eukaryote-infecting CRESS DNA viruses

3.1.1. Genomoviruses

The *Apis mellifera* genomoviruses ($n = 4$) recovered in this study display classical features of this group with genomes ranging from 2208 to 2218 nts in size and two bidirectional (ORF) encoding a *rep* and a *capsid protein (cp)*. These genome sequences fall phylogenetically into two genera. Three genomes MH973740 [BNH 2007], MH973737 (INH5 2007) and MH973739 (IFH184 NG) cluster with the gemykibiviruses and one MH973741 (BNH406) with the gemykrogviruses. The three gemykibiviruses isolates were identified in the hemolymph of both Italian foragers and nurses and in New World Carniolan nurses (Fig. 1). The novel *Apis mellifera* associated gemykibiviruses have a “TAATGT TAT” nonanucleotide motif and their Reps and CPs share 95–98% and

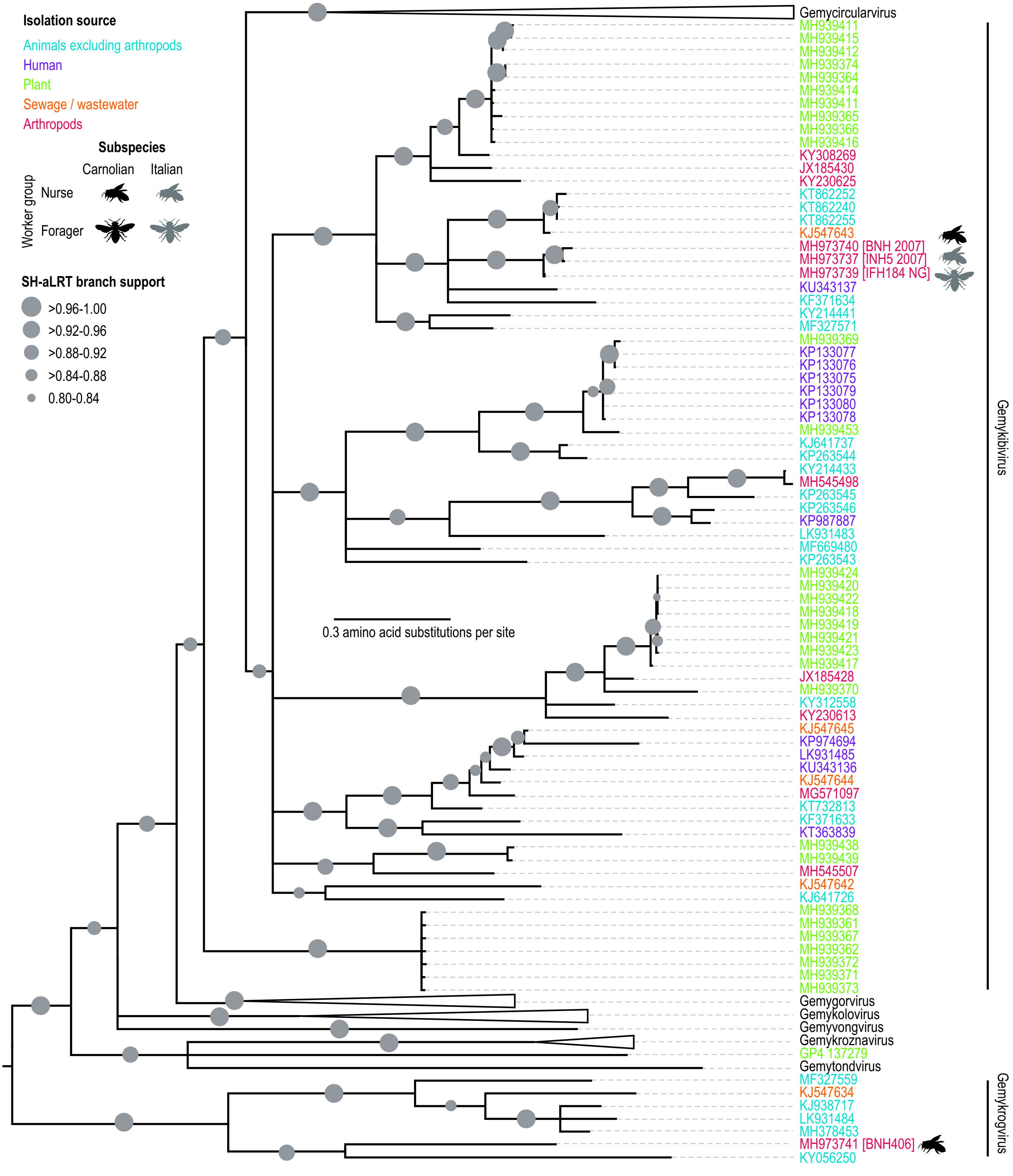
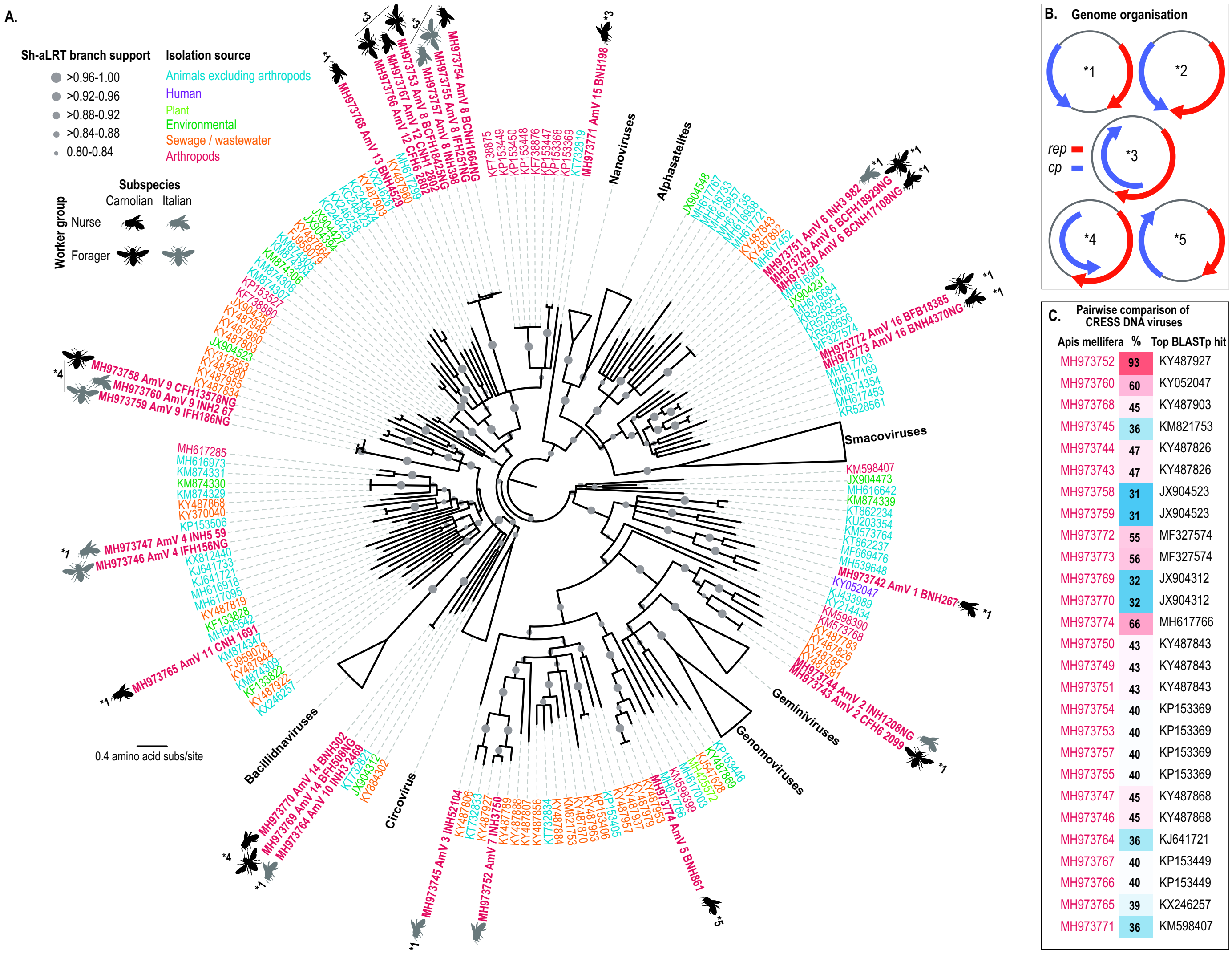


Fig. 1. Replication-associated protein maximum likelihood phylogenetic tree of the genomoviruses. Isolation source for genomoviruses is indicated by coloured isolate name; those recovered in this study are highlighted in bold and *Apis mellifera* sub species and worker group indicated by symbol and black or grey colouring, respectively.

99–100% amino acid (aa) identity amongst themselves, respectively. Their Reps and CPs share 74–77% and 56–58% aa identity, respectively, to those of their related gemykibivirus - Human associated gemycircularvirus (KU343137) (Halary et al., 2016) (Fig. 1 and Supplementary Data 1).

The single gemykrogivirus sequence was identified in hemolymph of Carniolan nurses (Fig. 1). *Apis mellifera* gemykrogivirus has a relatively less conserved “CATATTTAT” nonanucleotide motif and shares



C. Pairwise comparison of CRESS DNA viruses

Apis mellifera	%	Top BLASTp hit
MH973752	93	KY487927
MH973760	60	KY052047
MH973768	45	KY487903
MH973745	36	KM821753
MH973744	47	KY487826
MH973743	47	KY487826
MH973758	31	JX904523
MH973759	31	JX904523
MH973772	55	MF327574
MH973773	56	MF327574
MH973769	32	JX904312
MH973770	32	JX904312
MH973774	66	MH617766
MH973750	43	KY487843
MH973749	43	KY487843
MH973751	43	KY487843
MH973754	40	KP153369
MH973753	40	KP153369
MH973757	40	KP153369
MH973755	40	KP153369
MH973747	45	KY487868
MH973746	45	KY487868
MH973764	36	KJ641721
MH973767	40	KP153449
MH973766	40	KP153449
MH973765	39	KX246257
MH973771	36	KM598407

Fig. 2. A) Replication-associated protein maximum likelihood phylogenetic tree of the novel CRESS DNA viruses. Isolation source is indicated by coloured isolate name; those recovered in this study are highlighted in bold and *Apis mellifera* subspecies and worker group indicated by symbol and black or grey colouring, respectively. Genome organisation type 1–5 is denoted by the * next to each *Apis mellifera* CRESS DNA virus grouping and B) shows depiction of *rep* and *cp* orientation and organisation in genome. C) Blastp Rep amino acid sequence comparison of *Apis mellifera* CRESS DNA virus and percentage similarity to nearest neighbour and GenBank accession of nearest neighbour.

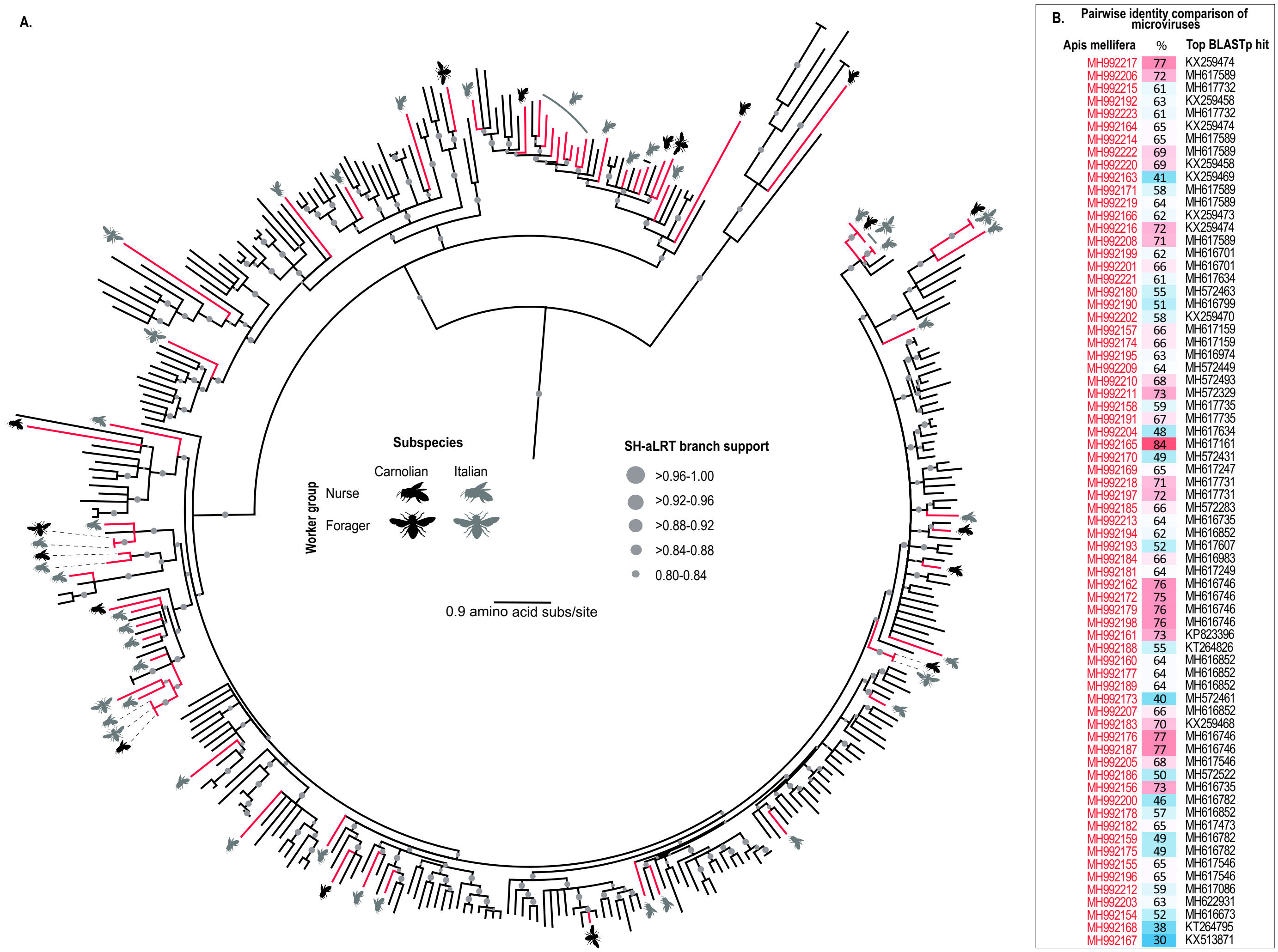
highest Rep pairwise identity of 51% with that of Chicken stool associated gemycircularvirus (KY056250) (Lima et al., 2017) and the CP identity of 43% with that of Giant panda associated gemycircularvirus (MF327559) (Zhang et al., 2017) (Fig. 1 and Supplementary Data 1).

3.1.2. Unclassified CRESS DNA viruses

In addition to the novel Genomoviruses, 28 divergent CRESS DNA viruses (MH973742 -MH973774) were identified clustering into 16 CRESS DNA virus groupings (MH973742 -MH973774), referred to here as *Apis mellifera* virus (AmV) 1 – 16 (Table 1). The genomes were clustered into groups based on 95% similarity threshold at a genome level. The genomes of these CRESS DNA viruses range in size 1715 to 3263 nts (Table 1) and all encode a Rep and a putative CP (which all have an arginine rich N terminus). The Reps of these 16 groups of CRESS DNA viruses are the only protein that can be broadly compared with other CRESS DNA viruses. Thus the top 10 neared neighbour Rep sequences determined using BLASTp for each novel CRESS DNA virus recovered in this study were used for the phylogenetic analysis together with representatives from each well-established CRESS DNA virus family (Fig. 2). The Rep phylogeny illustrates the level of diversity amongst the novel *Apis mellifera* CRESS viruses with sequences broadly dispersed throughout the phylogenetic tree (Fig. 2A). The highest Rep sequence BLASTp hit to each of the Rep is noted in Fig. 2B, these range from 93% (AmV 7, MH973752 to KY487927) to 31% (AmV 16, MH973769–70 to JX904523) similarity. Interestingly, AmV 7 (MH973752) shares 82% full genome pairwise identity with a virus isolated from wastewater (KY487927) collected in Florida, USA (Pearson et al., 2016). Waste treatment plants are often open ponds prior to being expelled as wastewater and therefore this closely related wastewater virus may have originated from a honey bee or related arthropod which got trapped and perished in the pond.

The genome organisation of the CRESS DNA viruses varies amongst the different groups (see Fig. 2C and the corresponding * next to the sequence names). The most common genome organisation is type 1 in Fig. 2C which is bidirectional with the *rep* encoded in the virion sense and the *cp* encoded in the complementary sense and has two intergenic regions. Nonetheless, the CRESS DNA virus genomes identified here are either bidirectional or unidirectional and have at least one intergenic region (Fig. 2C).

Four of the novel AmV CRESS DNA virus groups (AmV 2, 6, 8 and 9) were identified in both Italian and New World Carniolan bees, and in both nurses and foragers. All the other AmVs were all only detected in a



B. Pairwise identity comparison of microviruses

Apis mellifera	%	Top BLASTp hit
MH992217	77	KX259474
MH992206	72	MH617589
MH992215	61	MH617732
MH992192	63	KX259458
MH992223	61	MH617732
MH992164	65	KX259474
MH992214	65	MH617589
MH992222	69	MH617589
MH992220	69	KX259458
MH992163	41	KX259469
MH992171	58	MH617589
MH992219	64	MH617589
MH992166	62	KX259473
MH992216	72	KX259474
MH992208	71	MH617589
MH992199	62	MH616701
MH992201	66	MH616701
MH992221	61	MH617634
MH992180	55	MH572463
MH992190	51	MH616799
MH992202	58	KX259470
MH992157	66	MH617159
MH992174	66	MH617159
MH992195	63	MH616974
MH992209	64	MH572449
MH992210	68	MH572493
MH992211	73	MH572329
MH992158	59	MH617735
MH992191	67	MH617735
MH992204	48	MH617634
MH992165	84	MH617161
MH992170	49	MH572431
MH992169	65	MH617247
MH992218	71	MH617731
MH992197	72	MH617731
MH992185	66	MH572283
MH992213	64	MH616735
MH992194	62	MH616852
MH992193	52	MH617607
MH992184	66	MH616983
MH992181	64	MH617249
MH992162	76	MH616746
MH992172	75	MH616746
MH992179	76	MH616746
MH992198	76	MH616746
MH992161	73	KP823396
MH992188	55	KT264826
MH992160	64	MH616852
MH992177	64	MH616852
MH992189	64	MH616852
MH992173	40	MH572461
MH992207	66	MH616852
MH992183	70	KX259468
MH992176	77	MH616746
MH992187	77	MH616746
MH992205	68	MH617546
MH992186	50	MH572522
MH992156	73	MH616735
MH992200	46	MH616782
MH992178	57	MH616852
MH992182	65	MH617473
MH992159	49	MH616782
MH992175	49	MH616782
MH992155	65	MH617546
MH992196	65	MH617546
MH992212	59	MH617086
MH992203	63	MH622931
MH992154	52	MH616673
MH992168	38	KT264795
MH992167	30	KX513871

Fig. 3. A) Major capsid protein maximum likelihood phylogenetic tree of the microviruses from the *Gokushovirinae* subfamily. Those from this study have branches highlighted in red, and *Apis mellifera* sub species and worker group indicated by symbol and black or grey colouring, respectively. B) Blastp MCP amino acid sequence comparison of *Apis mellifera* microviruses, percentage similarity to nearest neighbour and GenBank accession of nearest neighbour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

single subspecies (Fig. 2A), four (AmV 4, 12, 14, 16) of which were however recovered from both working groups. The hemolymph was the predominant source of all the viruses with the exception of AmV 16 which was identified in both the brain and the hemolymph (Fig. 2A). The New World Carniolan subspecies harboured the highest number of AmV groups, 12 groups, compared to the Italian with eight groups. Regardless of subspecies all 16 AmV groups were identified in the nurses whereas only eight where identified in the foragers, phylogenetically these are broadly distributed across various clades indicating nurses may harbour higher levels of diversity than foragers.

3.2. Prokaryote-infecting CRESS DNA viruses

3.2.1. Microviruses

Microviruses are abundantly present in honey bees we sampled and likely infect bacteria associated with the bees. The 70 genomes identified falling into 61 *Apis mellifera* microvirus groups in the *Gokushovirinae* subfamily. The subfamily *Gokushovirinae* represents a group of highly diverse viruses with > 1000 full genome sequences available in the public database, as with the CRESS viruses we compiled a dataset of the MCP which is the most conserved protein in the family *Microviridae*. A phylogenetic tree of the MCP was constructed with 10 nearest neighbour's bases on BLASTp analysis (Fig. 3 and Sup Fig. 1) and demonstrates the diversity of the microviruses identified in the bees. Distributed throughout the microvirus MCP phylogeny, the *Apis mellifera* microviruses are highly diverse (Fig. 3) with their MCPs sharing 84% (MH617161 from fish tissue) to 30% (KX513871 from human gut) amino acid sequence identity (Fig. 3B).

The microvirus analyses between honey bee subspecies phylogenetically indicates the biodiversity of microviruses is higher in the Italian comparative to New World Carniolan (Fig. 3A), of the 70 microviruses identified, 51 were from Italian compared to 19 from New World Carniolan bees. Similar to the trend seen in the worker groups for the CRESS DNA viruses, for the microviruses we note that the hemolymph of the nurses harbours more viruses ($n = 56$) compared to the hemolymph of the foragers ($n = 12$) and the brain of foragers ($n = 2$) (Fig. 3A).

3.3. Circular DNA molecules

Five novel circular DNA molecules encoding a CP and an unknown protein were identified and recovered. Four are 100% identical and referred to as *Apis mellifera* circular DNA molecule (AmCM) 2 (1516 nts) and the fifth is referred to as AmCM 1 (1586 nts). BLASTp comparison of the CP of AmCM 1 and 2 reveals that it is most closely related to CP of Dragonfly cyclicusvirus (JX185418) sharing 29% identity and 34%

with "CRESS virus sp." from minnow tissue (MH617741), respectively. AmCM 1 was only identified in the hemolymph of Italian nurse bees whereas AmCM 2 was identified in the hemolymph of Italian nurses and foragers and New World Carniolan nurse hemolymph. These AmCM 1 and 2, based on their CP sequence similarities to CPs of CRESS DNA viruses are likely subgenomic molecules or possibly components of multicomponent CRESS DNA viruses.

4. ssDNA virus prevalence

We undertook statistical analyses to investigate prevalence of ssDNA viruses across variables of two *Apis mellifera* subspecies (Italian and New World Carniolan), and two worker groups (nurse and forager) within each of these subspecies (Supplementary Fig. 2 and Supplementary Tables 2 and 3) and the interactions of the coupled as variable predictors. Our evaluation showed although subspecies and worker group interactions did not show significant differences for the CRESS DNA viruses, nurses alone did show higher prevalence than foragers (Supplementary Fig. 2A and Supplementary Table 2). The Italian nurses harboured significantly more microviruses and New World Carniolan foragers the least (Supplementary Fig. 2B and Supplementary Table 3).

5. Concluding remarks

Our study reveals a broad diversity of previously uncharacterised viruses; four genomoviruses, 28 unclassified CRESS DNA viruses, and 70 microviruses in the *Gokushovirinae* subfamily. Interestingly a trend in virus diversity and prevalence in worker groups is apparent. Overall, for all virus groups described in this study, nurses harboured a much higher abundance and diversity of viruses than the foragers (Figs. 1, 2, 3, Supplementary Fig. 2, and Supplementary Tables 2 and 3). Although we performed all of these experiments under sterile conditions and dissected individuals instead of working with whole bees we cannot definitively state that these viruses are directly associated with the bees. Nonetheless, this is a noteworthy trend and regardless of whether all or some are infecting the bees, the commonality of viruses between subspecies indicates these viruses are associated with the bee and their environment. The identification of a larger number and diversity of viruses in the nurses compared to foragers begs the question – Why? It makes perfect sense that ideally a hive, and thus by proxy its residents, maintain a greater sterility compared to the foragers. However, could it be that having a diverse complement of viruses may confer some benefit to the nurses in the way of "immunization"? This is certainly an area of research that needs more inquiry.

An obvious trend is a higher diversity and prevalence of eukaryote-infecting CRESS DNA viruses in the New World Carniolan bees was identified compared to the Italian bee, whereas it is the opposite for the microviruses. Based on how little we currently know about the hosts of the unclassified eukaryote-infecting CRESS DNA viruses, majority of which have been identified though metagenomic studies, we cannot speculate on the hosts of these viruses identified in this study other than that they are to likely infect eukaryotes. The microviruses, however, are known to infect bacteria and be associated with the bacterial flora of plants and animals. Extrapolating from this it may be that the microvirus diversity is linked somewhat to the host bacterial diversity i.e. the bacterial diversity of the Italian bees is likely greater than that of the New World Carniolan bees. Although no study to date has investigated this specifically in subspecies, the gut flora community profile in *Apis mellifera* does differ between countries and even on a local scale between colonies (Evans and Schwarz, 2011; Moran et al., 2012). A comparative analysis of Italian and New World Carniolan bee microbiomes coupled with microvirus diversity may help address this observation. Overall this study adds to current knowledge on DNA viruses associated with bees and paves the way for further studies to investigate the role these may play in disease, environment and overall health of the bee, both positive and negative.

Accession numbers

MH973742 - MH973779; MH992154 - MH992223.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.03.024.

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