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The virome of an endangered stingless bee suffering from annual mortality in southern Brazil

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Abstract

Meliponiculture – the management of stingless bee colonies – is an expanding activity in Brazil with economic, social and environmental potential. However, unlike in apiculture, the pathogens that impact on meliponiculture remain largely unknown. In southern Brazil, every year at the end of the summer, managed colonies of the stingless bee *Melipona quadrifasciata* manifest a syndrome that eventually leads to collapse. Here we characterize the *M. quadrifasciata* virome using high-throughput sequencing, with the aim of identifying potentially pathogenic viruses, and test whether they are related to the syndrome outbreaks. Two paired viromes are explored, one from healthy bees and another from unhealthy ones. Each virome is built from metagenomes assembled from sequencing reads derived either from RNA or DNA. A total of 40621 reads map to viral contigs of the unhealthy bees' metagenomes, whereas only 11 reads map to contigs identified as viruses of healthy bees. The viruses showing the largest copy numbers in the virome of unhealthy bees belong to the family *Dicistroviridae* – common pathogenic honeybee viruses – as well as *Parvoviridae* and *Circoviridae,* which have never been reported as being pathogenic in insects. Our analyses indicate that they represent seven novel viruses associated with stingless bees. PCR-based detection of these viruses in individual bees (healthy or unhealthy) from three different localities revealed a statistically significant association between viral infection and symptom manifestation in one meliponary. We conclude that although viral infections may contribute to colony collapses in the annual syndrome in some meliponaries, viruses spread opportunistically during the outbreak, perhaps due to colony weakness.

INTRODUCTION

Stingless bee management in Brazil began with indigenous South Americans, who domesticated the non-aggressive native bees as a source for honey [1]. One of these bees, *Melipona quadrifasciata*, which is distributed from the state of Paraíba in the north of Brazil through to the southernmost state, Rio Grande do Sul [2], is the second most cultivated stingless bee in Brazil [3]. In spite of being widely cultivated, natural populations of stingless bees in the neotropics have reduced drastically in recent years due to different factors, ranging from habitat fragmentation to the introduction of exotic bees [4] that not only compete for resources, but also bring new pathogens to which native bees might be susceptible. In most of southern Brazil, wild populations of the subspecies *M. q. quadrifasciata* have been disappearing for 50 years. The species is currently mostly maintained through management

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Abbreviations: ABPV, *Acute bee paralisys virus*; BLAST, Basic Local Alignment Search Tool; BQCV, *Black queen cell virus*; BU, Barnard's Uunconditional test; CCD, Colony collapse disorder; CDS, Coding sequence; CMH, Cochran-Mantel-Haenszel; DWV, *Deformed wing virus*; IAPV, *Israeli acute paralysis virus*; KBV, *Kashmir bee virus*; MqC, *Melipona quadrifasciata cyclovirus*; MqC1, *Melipona quadrifasciata cyclovirus 1*; MqC2, *Melipona quadrifasciata cyclovirus 2*; MqD, *Melipona quadrifasciata densovirus*; MqV2, *Melipona quadrifasciata virus 2*; MqV1a, *Melipona quadrifasciata virus 1a*; MqV1b, *Melipona quadrifasciata virus 1a*; SBPV, *Slow bee paralysis virus*; VDV1, *Varroa destructor virus-1*.

MK190907 (MqC1), MK190906 (MqC2), MH340498 (MqD), MH340499 (MqV1a) and MH34500 (MqV1b), MK190905 (MqV2).

Two supplementary figures and four supplementary tables are available with the online version of this article.

in this region, and is therefore considered to be endangered [5, 6]. Managing stingless bees has been crucial for conservation, but on the other hand, practices that involve hive translocation may also spread pathogens [7]. Despite advances in beekeeping legislation and monitoring, there is no official bee surveillance in Brazil and, consequently, there are no regular records on bee mortality caused by pathogens. Nevertheless, it is a common knowledge among beekeepers from different localities in southern Brazil that every year at the end of the summer – normally during the first week of March – their *M. quadrifasciata* bees begin to manifest an unusual behaviour that may include tremors, crawling with everted proboscis and paralysis [8]. Although the disease symptoms are quite variable among stingless bees from different localities, during this period it is common to find large numbers of dead bees inside or in front of hives. Mortality, and the inability of bees to perform their regular activities, sometimes lead to colony collapse. Our previous studies on the *M. quadrifasciata* syndrome searched the associated microbiota for pathogenic bacteria or imbalances in bacterial community composition that could explain the symptoms [8]. However, no consistent differences in microbiota composition were found between healthy and unhealthy bees. While *Apis mellifera* shows a highly conserved core microbiota [9], *M. quadrifasciata*'s associated bacterial community is highly variable across colonies, independent of their health status. Furthermore, no pathogenic bacteria or other common bee pathogens such as *Nosema* and *Crithidia*, were detected in unhealthy bees.

Several viruses have already been identified in *A. mellifera* in Brazil [10], mainly in samples from areas where abnormal population declines have been observed [11]. These have included *Iflaviridae* such as the *Deformed wing virus* (DWV), and *Dicistroviridae* such as the *Black queen cell virus* (BQCV), *Acute bee paralysis virus* (ABPV) and *Israeli acute paralysis virus* (IAPV) [11, 12]. Characteristic symptoms of DWV infections are the death of pupae, or adult bees with deformed wings, sometimes associated with shortened and bloated abdomens, and miscolouring [13]. However, DWV may persist in covert infections with no apparent symptoms [14, 15], in spite of having strongly deleterious effects on honeybee foraging and survival [16]. Similarly, dicistroviruses may also persist with no obvious symptoms at the individual or colony level [17]. Nevertheless, it has been shown that an increase in BQCV titre kills developing queen larvae, whose necrotic remains result in a pupal cell with a darkened colour [18]. Other viruses of this family, such as ABPV and IAPV, are known to cause paralysis [19, 20]. They are extremely virulent when injected into pupae or adults, causing disease in the honeybee with fewer than 100 viral particles [21]. Honeybees experimentally infected with IAPV develop symptoms such as crawling, disorientation and trembling wings, which progress to paralysis and death within or outside the hive [22, 23]. Furthermore, viruses with RNA genomes such as the *Iflaviridae* and *Dicistroviridae*, which show higher mutation rates, quickly evolve into many variants, allowing them to switch between different bee hosts [24–26]. An example of viral host switching between bee species is the detection of honeybee virus ABPV in managed colonies of *M. scutellaris* suffering from an unexpected increase in worker bee mortality in Brazil [27].

The main routes of virus transmission between bees are vectors such as mites or pollen resource sharing [28]. The most studied vector in honeybees is *Varroa destructor*, which transmits different lineages of DWV, *Varroa destructor virus*-1 (VDV1) and IAPV [29–31]. These and many other viruses are also transmitted within and between species via floral sources [24, 32]. Bee species sharing habitats and resources tend to have similar associated micro-organisms. A study on viral distribution via pollen sources showed that in apiaries with colonies affected by colony collapse disorder (CCD), IAPV virus was associated with *A. mellifera* as well with non-*Apis* hymenopteran pollinators [32]. At the same time, in nearby apiaries not affected by CCD, the virus was present in neither *A. mellifera* nor non-*Apis* hymenopterans. The persistence of BQCV, DWV, ABPV and *Slow bee paralysis virus* (SBPV) in wild bumblebee populations in Europe, often at putatively low levels, may also result from indirect interactions with local *A. mellifera* through the shared use of floral resources [24].

Recent studies using high-throughput sequencing technologies expanded the list of known honeybee viruses, previously restricted to the Picornavirales [33]. Among the recently identified viruses are those from the family *Rhabdoviridae* with −ssRNA genomes , ssDNA viruses belonging to the families *Circoviridae* and *Parvoviridae*, and dsDNA viruses from the family *Nudiviridae* [34–36]. These viruses were identified in honeybees displaying variable health conditions. Hence, the viromes of bees have revealed that, in addition to the complex network of host-associated bacteria [9], a very diverse range of viruses are probably acting on and interacting in the bacterial network. Different bee viruses are frequently detected in healthy and unhealthy hosts [24, 34, 35, 37]. Chronic viral infections may become harmful by increasing their loads in genetically predisposed individuals, depending on the environmental conditions [37–39], or may become symbiotic [40].

Given that the symptoms observed in *M. quadrifasciata* affected by the annual syndrome resemble those of honeybee viral diseases such as IAPV and ABPV, and given that stingless bees probably host yet unidentified viruses, some of which may have been spread by honeybees due to beekeeping practices, we focus our investigation on viruses that might be implicated in the disease. Using high-throughput sequencing to characterize the virome of *M. quadrifasciata,* we identify seven novel stingless bee viruses.

RESULTS

Viromes of healthy and unhealthy *M. quadrifasciata*

In order to find viruses associated with the *M. quadrifasciata* syndrome, worker bees from a single colony showing disease symptoms such as tremor and paralysis (unhealthy bees), as well as workers from another colony where none of the individuals showed such symptoms (healthy bees), were

sampled from the same meliponary (place where stingless bees are reared). Each bee pool was macerated and underwent subsequent centrifugation steps, including an ultracentrifugation with a sucrose cushion for virus enrichment. The nucleic acids were then subjected to high-throughput sequencing, yielding the following number of paired-end reads: 640 953 (DNA sample from unhealthy bees=UDNA), 535 631 (DNA sample from healthy bees=HDNA), 582 603 (RNA sample from unhealthy bees=URNA) and 834 753 (RNA sample from healthy bees=HRNA). After assembly, viral contigs larger then 200 bp were classified based on the best BLASTX hit against the nr database, resulting in a total of 989 viral sequences. From these, only five were recovered from healthy bees (three from DNA and two from RNA), whereas all remaining 984 viral sequences were assembled from unhealthy bees.

From the total of 989 viral sequences (Table S1, available in the online version of this article), 901 were identified as bacteriophages and 17 were identified as viruses of unclassified or unknown hosts, and for the purpose of the present study, we focused on the remaining 71 sequences, which were identified as eukaryotic viruses (Fig. 1). The HRNA sample was left out of Fig. 1, since both viral contigs assembled from the RNA of healthy bees were identified as bacteriophages. Thus, the virome of healthy bees contained only three sequences from eukaryotic viruses that belonged to two different families. The virome of unhealthy bees, on the other hand, contained 38 sequences from viruses belonging to 11 different families, as well as 30 contigs showing similarity to unclassified viruses previously found in eukaryotes (Fig. 1). Using sequencing depth as a proxy for abundance, i.e. the number of copies of viruses within each virome, the most representative viruses in unhealthy bees (50–140 X depth) belonged to the families *Parvoviridae* (ssDNA genome) and *Circoviridae* (ssDNA genome). Viruses identified as *Dicistroviridae* (+ssRNA genome) that included potential honeybee pathogens showed lower sequencing depth, ranging from 8 to 15 X.

Characterization of novel *M. quadrifasciata* viruses

Seven eukaryotic viral sequences with significant length and depth were selected as candidates for further investigation: *Melipona quadrifasciata cyclovirus* (MqC), *Melipona quadrifasciata cyclovirus* 1 (MqC1) and *Melipona quadrifasciata cyclovirus* 2 (MqC2) from the family *Circoviridae*; *Melipona quadrifasciata densovirus* (MqD) from the family *Parvoviridae*; *Melipona quadrifasciata virus* 1a and 1b (MqV1a and MqV1b) from the family *Dicistroviridae*; and *Melipona quadrifasciata virus* 2 (MqV2), which is unclassified (Table S1). To evaluate whether these sequences represent already known or novel viruses, predicted proteins derived from their coding sequences (CDSs) were used for phylogenetic analyses. None of them showed sufficient similarity to other known viruses to be regarded as the same species.

The most abundant virus found in unhealthy stingless bees belonged to the family *Parvoviridae* (MqD; Table S1). The MqD sequence (4214 nt) probably represents a complete genome, encoding the non-structural (534 aa) and structural (655 aa) proteins, containing conserved domains Parvo_NS1 (cl24009, e-value $4.57e^{-10}$) and Denso_VP4 (cl03545, e-value $4.83e^{-15}$), respectively. Phylogenetic inference based on their concatenated amino acid sequences groups MqD with other *Parvoviridae* found mainly in insects, within the genus *Densovirus* (Fig. S1), such as the recently described *Bombus cryptarum densovirus* [34].

Two *Circoviridae* sequences from unhealthy bees also contained recognizable CDSs: MqC1 and MqC2 (Table S1). MqC1 (1587 nt) is probably a complete genome as well, from which the non-structural protein (228 aa), with a P-loop_NTPase domain (cl21455, e-value $7.94e^{-18}$), and the structural protein (224 aa) are predicted. MqC2 (673 nt) is a partial virus genome bearing a partial non-structural protein (224 aa) with the P-loop_NTPase domain (e-value $3.34e^{-18}$). These two viruses cluster with viruses from the family *Circoviridae* within the genus *Cyclovirus* (Fig. 2), a sister clade of the genus *Circovirus*, that were found recently in *A. mellifera* (Circo-like 1 and Circo-like 2) [35].

Two other partial viral genomes show similarity to *Dicistroviridae* viruses: MqV1a (5913 nt) and MqV1b (1236 nt) (Table S1). A single protein is predicted from each, i.e. the structural protein (1888 aa), with RNA_dep_RNAP (cd01699, e-value $9.44e^{-83}$) and RNA_helicase (pfam00910, e-value $1.24e^{-31}$) domains, is encoded by MqV1a, whereas the partial non-structural protein (412 aa), with RHV_like (cd00205, e-value $5.94e^{-23}$) and Dicistro_VP4 (cl13011, e-value $4.36e^{-08}$) domains, is predicted from MqV1b. Both proteins group these viruses within *Dicistroviridae* known to infect bees and other arthropods (Fig. 3), such as ABPV and IAPV. However, the amino acid identities between MqV1a or MqV1b proteins and known ABPV and IAPV proteins are within the order of 28–15% and 29–16 %, respectively.

MqV2 (3730 nt) shows an unclassified virus as best hit (Table S1), and encodes a non-structural protein (479 aa), which contains RT_like (cl02808, e-value $8.87e^{-11}$) and RdRP_3 (cl24119, e-value $2.48e^{-05}$) domains. MqV2 is related to other unclassified RNA viruses that are found to be associated with a diversity of hosts, from plants to animals such as birds, molluscs and bees (Fig. S2) [41].

Virus detection in stingless bees and honeybees

Having characterized seven novel eukaryotic viruses found in high abundance in unhealthy stingless bees, we tested whether their presence is associated with the manifestation of disease symptoms (Table S2). We reasoned that if a virus is directly implicated in the annual syndrome, it should be detected more often in unhealthy than in healthy bees of different meliponaries. Virus detection by PCR was performed by testing individual worker bees to determine if the disease symptoms were present in three different meliponiaries, using primers designed to amplify fragments of about 300 bp from each virus genome (Table 1). Table 2 summarizes the results of Cochran–Mantel–Haenszel (CMH) tests, which were used to verify the association between the detection of each candidate virus with the manifestation of symptoms, controlled

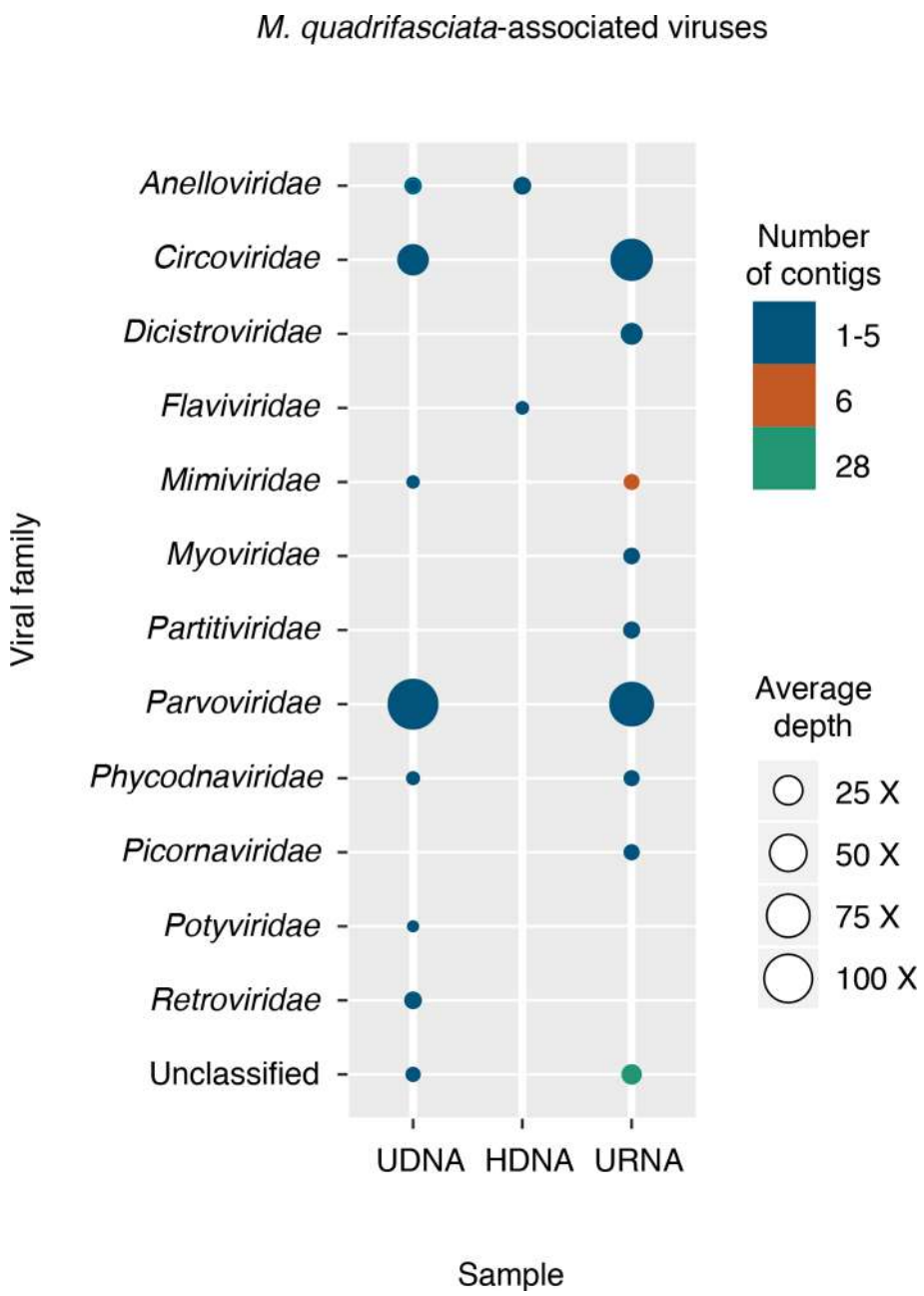

Fig. 1. Taxonomic distribution of *M. quadrifasciata* viral contigs in metagenomes of healthy (HDNA) and unhealthy bees (UDNA and URNA). Only contigs larger than 200 bp and showing best hits on eukaryotic viruses are considered in this plot. Sequences of viral families showing larger sequencing depth (larger circles) were chosen as candidates in subsequent analyses.

by locality, and the results of Barnard's unconditional (BU) tests applied to individual localities. All viruses were detected in both healthy and unhealthy bees, and only MqV1a was detected in bees from all meliponaries. None of the viruses were found to be significantly more associated with the syndrome manifestation in all localities in view of the CMH test. However, MqC, MqD and MqV1a viruses were more often associated with unhealthy bees in the Boqueirão do Leão (BL) meliponary, where *M. quadrifasciata* was sampled to build the viromes. Interestingly, the three circoviruses (MqC, MqC1 and MqC2) and the unclassified virus MqV2 were also detected in *A. mellifera* colonies from one of the sampling localities [Bom Princípio (BP)] (see Table 2).

DISCUSSION

Bee populations have been declining for some years, leading to global concern over the plight of plant pollinators [42, 43].

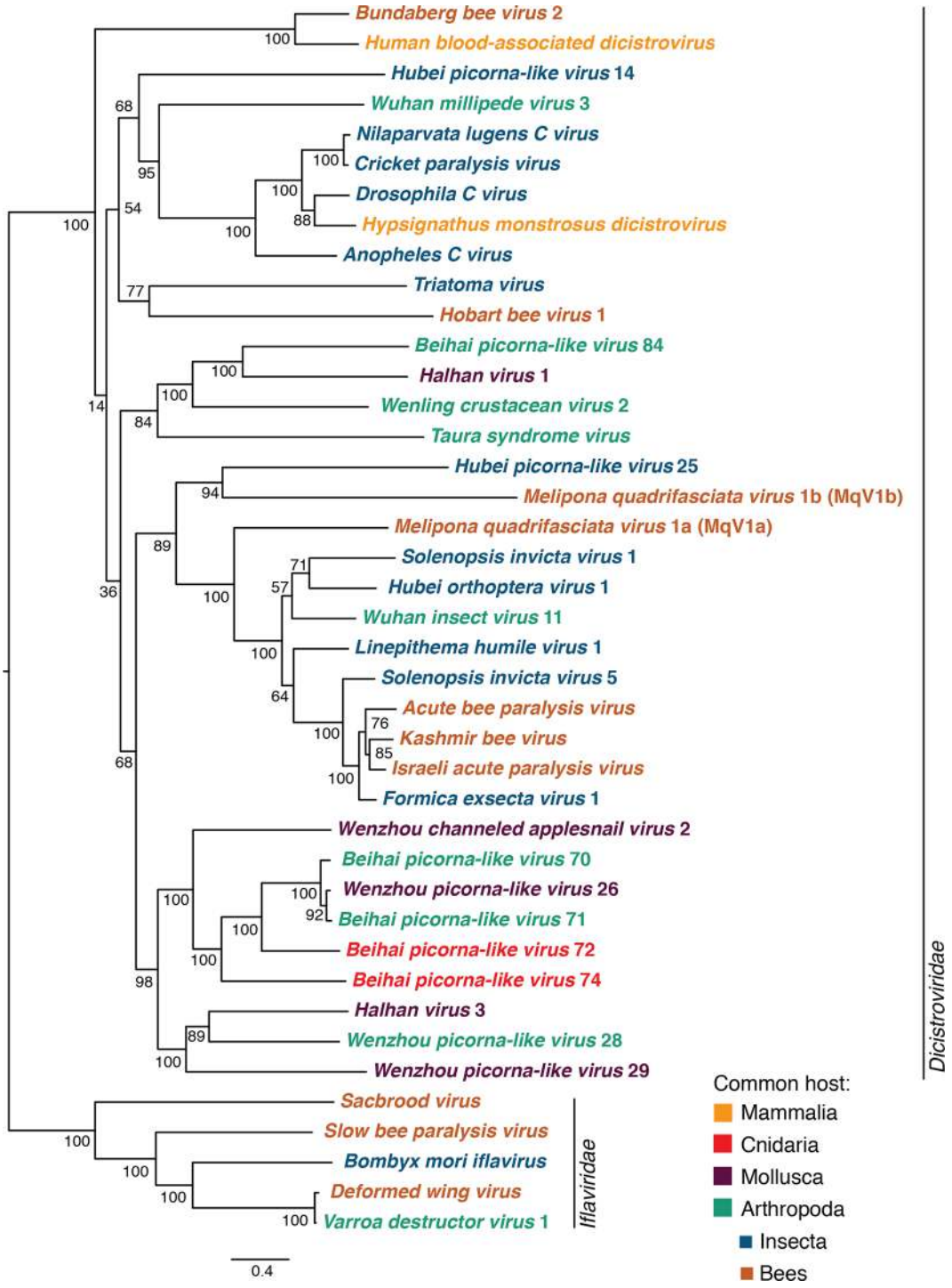

Fig. 2. Phylogenetic inference of MqC1 and MqC2 viruses (*Circoviridae*). The maximum-likelihood tree was inferred with 100 bootstrap resamples based on an alignment of 502 aa. The colours of the species names represent the viruses' common hosts (see lower right).

Viruses that are prevalent in weak colonies of honeybees are probably directly implicated in the population decline of different bee species [44–46]. For example, honeybee colonies affected by CCD, a syndrome characterized by the sudden disappearance of honeybees [47], often show higher prevalence of IAPV, ABPV or *Kashmir bee virus* (KBV) [48, 49]. The apparent spread of CCD in the USA suggests that viruses, among numerous other factors such as pesticides, stress, climate and habitat change, and other natural enemies, could cause honeybee populations to drop so much

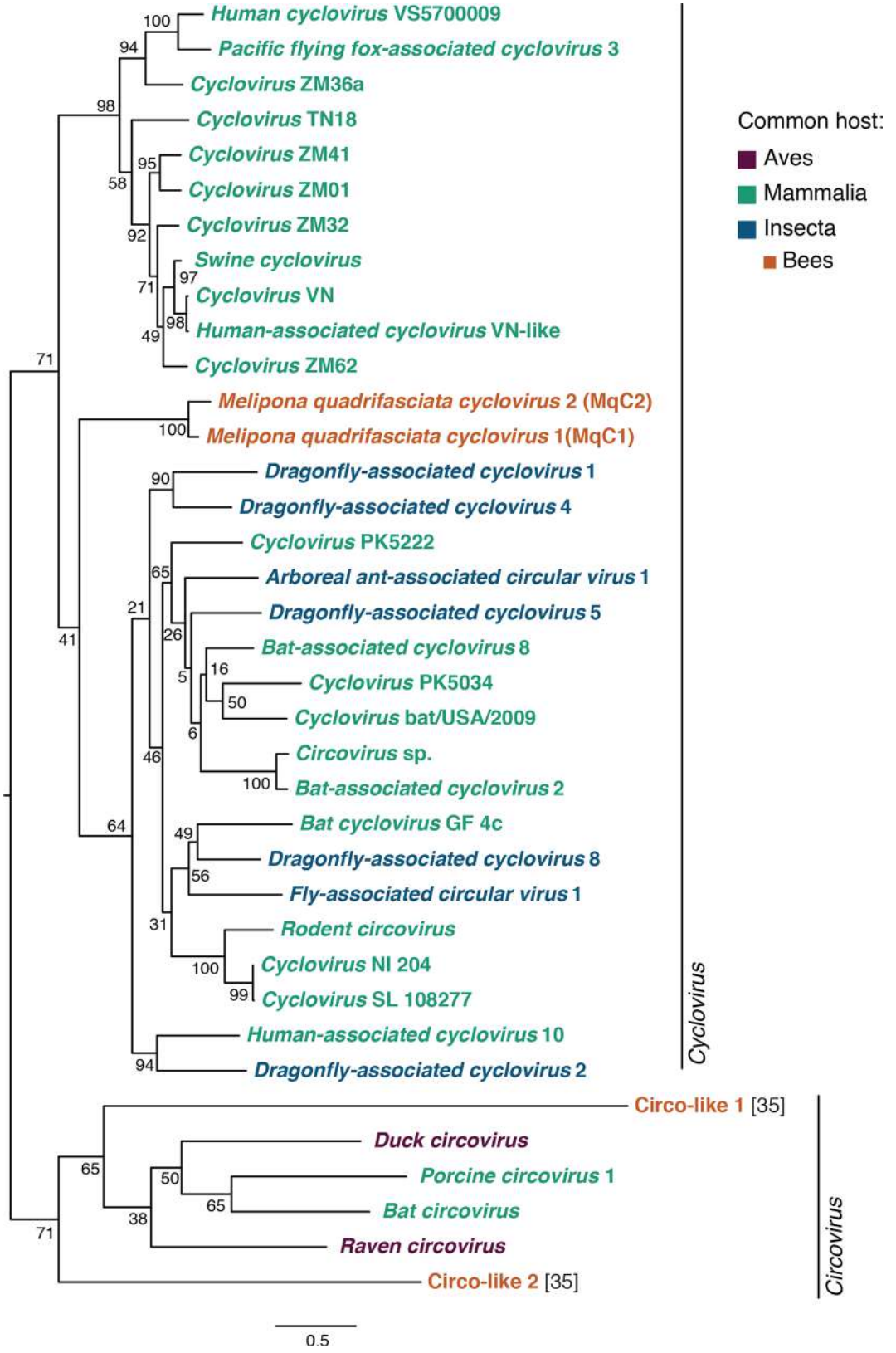

Fig. 3. Phylogenetic inference of MqV1a and MqV1b viruses (*Dicistroviridae*). The maximum-likelihood tree was inferred with 100 bootstrap resamples based on an alignment of 2748 aa. The colours of the species names represent the viruses' common hosts (see top right).

Table 1. Primer sequences designed for virus detection

Name	Sequence	Amplicon length (bp)
MqC F	5′ ACTTCTGTGCCGTTGGTAGT 3′	300
MqC R	5′ TCTCTCAAATTGCTTGCGCC 3′	
MqC1 F	5′ GCCACTTCTGTGCCGGATAT 3	304
MqC1 R	5′ TCCTTCAAAGGTGCCAGCTC 3	
MqC2 F	5′ ACTACTGGGAGCACGGAGAT 3	318
MqC2 R	5′ ATCCGTCCCACCATTCTCCT 3	
MqD F	5′ TCAAGCAGCGATTCTGGAGA 3′	300
MqD R	5′ CCACACAGGCGCTACTATTT 3′	
MqV1a F	5′ CTTCTTTGCCATCGACAAGCT 3′	300
MqV1a R	5′ TCAGCCGCAAACTTCTTCGA 3′	
MqV1b F	5′ TTAGTCTAAACGGGCCCACT 3′	300
MqV1b R	5′ TGGACATGCCCAACACTACA 3	
MqV2 F	5′ GCGCTGTATTGCATCTGGTG 3′	304
MqV2 R	5′ CGACGCGCATTTTCCTCAAT 3′	

that agricultural pollination will become unsustainable [50]. In spite of being less important with regard to crop pollination than honeybees, stingless bees and other native bees are fundamental to sustain nesting and alternative flower resources for crop pollinators, which are typically unavailable within intensively managed crop fields [51]. Moreover, the disappearance of *M. quadrifasciata* wild nests in southern Brazil is worrying because the management practices that are used to maintain bee populations in this region are not well regulated, and the trade in stingless bee colonies across the country could potentially spread viruses and other pathogens.

The seven most abundant eukaryotic viruses found in the virome of unhealthy *M. quadrifasciata* are novel viruses. Two partial dicistrovirus genomes were obtained: MqV1a and MqV1b. Although each contains one of the two CDSs of a typical dicistrovirus, our attempts to bridge them by PCR failed (data not shown), suggesting that they may indeed represent related, but different, viruses. Both show low identity to other dicistroviruses causing similar pathological symptoms in honeybees (IAPV and ABPV). Their high divergence from honeybee dicistroviruses and the lack of detection of MqV1a and b sequences in honeybees indicate that the presence of dicistroviruses in *M. quadrifasciata* does not represent a horizontal transmission event from *A. mellifera* to stingless bees, as previously suggested for ABPV infections in *M. scutellaris* [27]. Rather, our phylogenetic analyses indicate that MqV1a and b belong to an evolutionary branch that diverged from the honeybee IAPV long before other dicistroviruses found in unrelated hosts such as ants and crustaceans. Similarly, a recent study on the prevalence of DWV in *Melipona subnitida* and *A. mellifera* colonies from a single island in Brazil showed that DWV strain C, which is typically found in the stingless bee, is virtually absent in the honeybee [52]. Such dissimilar scenarios concerning the simultaneous presence of dicistroviruses in stingless bees and honeybees in Brazil probably represent different stages of the evolutionary divergence process that follows a host-switching event.

Interestingly, the most abundant *M. quadrifasciata* viruses in our study have ssDNA genomes: MqD (genus *Densovirus*, family *Parvoviridae*), MqC, MqC1 and MqC2 (genus *Cyclovirus*, family *Circoviridae*). These viral families have not been

Table 2. Candidate virus detection in *M. quadrifasciata* and *A. mellifera*. The number of *M. quadrifasciata* individuals that were positive for each virus is shown in relation to the total number of tested bees. Differences in the proportion of positive samples between healthy (H) and unhealthy (U) bees were tested for each meliponary (BL, Boqueirão do Leão; BP, Bom Princípio; and RL, Rolante) using Barnard's unconditional (BU) test and for the total number of sampled bees controlled by locality using the Cochran–Mantel–Haenszel (CMH) test. Pools of bees from three colonies of *A. mellifera* from BP were tested for the same viruses

	BL			BP			RL			CMH	*A. mellifera*		
	H	U	p£	H	U	p£	H	U	p£	p	1	2	3
MqC	8/19	40/57	0.029	0/6	0/3	NT*	1/6	0/3	0.579	0.094	+	+	+
MqC1	13/19	49/57	0.107	2/6	2/3	0.492	0/6	0/3	NT*	0.106	+	–	+
MqC2	5/19	27/57	0.122	0/6	0/3	NT*	0/6	0/3	NT*	NT**	+	+	–
MqD	6/19	6/57	0.031	0/6	0/3	NT*	0/6	0/3	NT*	NT**	–	–	–
MqV1a	0/6	4/6	0.021	8/38	7/27	0.668	0/6	2/37	0.776	0.192	–	–	–
MqV1b	1/6	3/6	0.303	3/38	5/27	0.249	0/6	0/37	NT*	0.165	–	–	–
MqV2	0/6	2/6	0.197	1/38	0/27	0.569	0/6	0/37	NT*	0.919	+	–	+

£, BU test.

*, not tested; virus not detected.

**, not tested; virus detected in a single locality.

found to be associated with other bee species until recently. Using high-throughput sequencing technologies, viruses belonging to the *Nudiviridae* (dsDNA) and *Parvoviridae* (ssDNA) were found in *Bombus* sp. [34], and *Circoviridae* viruses (ssDNA) were identified in *A. mellifera* [35], with no apparent pathological consequences. Densoviruses are known to establish both mutualistic and pathogenic relationships with their arthropod hosts [53–55]. Viruses from the genus *Cyclovirus* are not known to cause pathology in insects, but have been detected in the cerebrospinal fluid of human patients with neurological conditions [56, 57], and in secretions from patients with respiratory disease [58]. Here, the three cycloviruses were detected in honeybees sharing their habitat with *M. quadrifasciata*. It is known that the pollen resources of honeybees and *Melipona* spp. overlap in different regions of Brazil [59–61], and by foraging on the same flowers they may potentially exchange viruses. Inter-species transmission of bee viruses such as DWV, IAPV, SBPV and BQCV through pollen sources is already well documented [32, 62]. It is not possible to confirm that the *M. quadrifasciata* cycloviruses are in fact replicating within the bee body, but the high loads of DNA viruses such as MqD (136 X), MqC (107 X) and MqC1 (83 X) in the metagenome derived from a RNA sample at least suggests that they are active.

Even though no direct association was found between the manifestation of the *M. quadrifasciata* annual syndrome and the detection of the seven candidate viruses in adult stingless bees from all localities, we do not rule out the possibility that these viruses indeed play a role in the outbreaks. Our viromes were built using pools of bees from BL, where they showed the most intense disease symptoms, and three viruses (MqC, MqD and MqV1a) were found significantly more often in unhealthy bees. This indicates that viruses apparently replicate opportunistically on a local basis, and that, given the regular timing of the outbreaks on different meliponaries, the common denominator might be a higher susceptibility of bees at the end of the summer. In the case of honeybee CCD, no single factor explains the manifestation of the disorder; rather, it seems that a combination of several factors may act synergistically [50]. Known examples of synergistic interactions of honeybee viruses with other factors include *Varroa* mites that destabilize DWV–host dynamics via suppression of the honeybee NF-kB immune response [63] and pesticides that reduce honeybee resistance to viral infections [64]. Bee nutrition, which varies across landscapes and seasons, is also known to affect resistance to viruses and other pathogens [65].

In conclusion, there is no obvious answer to the question of whether or not viruses are implicated in the *M. quadrifasciata* annual syndrome. In spite of having shown a higher diversity and abundance of viruses in the virome of unhealthy bees in one of the studied meliponaries, our study suggests that viruses are not the direct cause of the syndrome, but may influence adult bee symptoms in certain localities. It is worth mentioning that eusocial bees differ from other animals in having an additional higher-level immunity referred to as social immunity [66, 67]. Because social immunity is negatively correlated with host density [68], it is possible that the annual syndrome actually represents a mechanism of density control in *M. quadrifasciata* populations at the end of the summer. Nevertheless, our study calls attention to the presence of a diversity of as yet unknown bee viruses in stingless bees, and their ability to spread across species boundaries. Given their pathogenic potential, and global concern about the decline of bee populations, viruses must be taken into account when planning management practices and conservation strategies.

METHODS

Worker bee sampling

M. quadrifasciata worker bees were collected from four different meliponaries in southern Brazil: 76 bees were taken from BL (29°18′5.47″S/52°25′57.96″W), 43 bees were taken from Rolante (RL) (29°38′19.78″S/50°26′49.77″W) and 65 bees were taken from BP (29°31′2.30″S/51°17′29.00″W). Sampling was performed in February, March and September (i.e. before, during and after the syndrome outbreak, respectively) in different years. Worker bees with symptoms such as tremors and paralysis (unhealthy) and symptomless bees (healthy) were collected using an entomological pooter, stored temporarily in sample pots and brought alive or in RNAlater (Thermo Fisher Scientific, USA) to the laboratory where they were stored individually at −80 °C until nucleic acid extraction. Healthy individuals were only collected in colonies where none of the bees presented symptoms. During the 2017 syndrome outbreak at BL, an additional 25 healthy and 25 unhealthy worker bees were collected from a colony affected by the syndrome and one not affected by it, respectively, pooled and stored in 5 ml of RNAlater (Thermo Fisher Scientific, USA) at 4 °C until nucleic acid extraction. Additionally, three pools of ~100 *A. mellifera* workers were sampled in September 2018 from different colonies of the BP location and stored as described.

Nucleic acid extraction for metagenome sequencing

Nucleic acids (RNA and DNA) were extracted from samples enriched for viral particles following the protocol of de Sales Lima and collaborators [69]. Briefly, two pools of 25 worker bees (healthy and unhealthy) were individually ground with sterile sand in 3 ml of phosphate-buffered saline (PBS). The homogenate was filtered in a cell strainer to remove bee parts (i.e. wings, legs and cuticles) and then centrifuged at 3000 r.p.m. for 15 min at 4 °C. The supernatant was collected with a syringe and filtered through a 0.45 µm filter (Sigma-Aldrich, USA) to remove bacteria-sized particles. The filtrate was transferred to a centrifuge tube in an SW-28 Ti rotor (Beckman Coulter, USA) already containing a sucrose cushion (25 % sucrose in Tris-Cl 10 mM, EDTA 1 mM) and ultracentrifuged at 27 000 r.p.m. for 4 h at 4 °C. The pellet, enriched for viral particles, was eluted in 200 µl DEPC-treated water and split into two aliquots of 100 µl each, one for DNA extraction with the universal phenol-chloroform

protocol [70] and another for RNA extraction with TRIzol (Ambion, USA), following the recommendations of the manufacturers. The yield and quality of nucleic acid purification were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and a Qubit fluorometer (Invitrogen, USA). Aliquots of the purified RNA were used for cDNA synthesis with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, USA). The cDNA second strand was synthesized using Klenow fragment DNA polymerase (New England Biolabs, USA). Fragment libraries were further prepared with 1 ng of purified DNA or double-stranded cDNA using the Nextera XT DNA Library Preparation kit (Illumina, USA) and paired-end sequenced (2×150 nt) using the MiSeq Reagent kit v2 (Illumina, USA) on a MiSeq instrument (Illumina, USA).

De novo assembly and contig filtering

Paired-end reads from healthy and unhealthy bee DNA and RNA fragment libraries were debarcoded and used for *de novo* assembly. Datasets derived from DNA samples were assembled with SPAdes v3.10.1 [71], whereas RNA-derived reads were assembled with Trinity v2.2.0 [72]. Within SPAdes, reads were assembled with k-mer lengths 21, 33 and 55, without a filtering step before assembly. Within Trinity, a read quality trimming step (*--trimmomatic*) was added with the default settings [73]. Contaminations were removed by building subsets (bins) of the NCBI nucleotide (nt) database with the *blastdb_aliastool* command of BLAST [74]. Contigs with significant hits (e-value cutoff $1e^{-12}$) to bins including sequences from bacteria (taxid: 2), fungi (taxid: 4751) or plants (taxid: 33090) were filtered out. Another bin was used to filter out hits against the *M. quadrifasciata* genome (LIRP00000000.1) using the same parameters.

Virome sequence analysis and candidate virus selection

For taxonomic characterization, the remaining contigs were matched with BLASTX to a viral protein database created from the virus RefSeq database (taxid: 10239). This strategy provided consistent results independently of the BLAST algorithm (BLASTX or BLASTP) or database (nr or RefSeq) used in the analyses (Table S3). Only contigs matching the viral database were retained (e-value cutoff $1e^{-5}$). False positives were then filtered out by comparing the candidate viral dataset with the total protein database (nr) from the NCBI using BLASTX, and removing contigs showing best hits to non-viral sequences. Taxonomic information for retained contigs larger than 200 bp was retrieved with taxdb. Contig depth was estimated with the depth command from Samtools v1.3.1 [75] by mapping the trimmed reads onto the virome with Bowtie 2 v2.3.1 [76], and used as a proxy for abundance. The taxonomic and sequencing depth data were used as inputs for bubble plots drawn with the ggplot2 package [77] in R. Contigs that had a sequencing depth larger than 8 X, were longer than 500 bp and showed best BLASTX hits on eukaryotic viruses were chosen as candidates for further investigation. Coding sequence prediction and annotation were performed with the prokaryotic genome annotator Prokka v1.12 [78] using the command options *--kingdom* (viruses) and *--metagenome*. To validate annotations with protein-conserved domains, predicted coding sequences were submitted to NCBI's conserved domain database (CDD) with the web application Batch CD-Search (www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi; e-value cutoff $1e^{-3}$).

Phylogenetic inferences

The predicted coding sequences of six candidate viruses (all candidate viruses, except MqC, which does not have predicted protein) were used to search for similar viral sequences in GenBank (e-value cutoff $1e^{-5}$), avoiding species redundancy. These sequences, plus relevant reference sequences (see Table S4), were used for phylogenetic inferences. Sequences were aligned using MAFFT as implemented in Geneious R11 [79], removing columns with more than 60 % gaps. For each alignment, the best-fitted model of amino acid substitution was selected using ProtTest v3.4.2 [80] under corrected Akaike information criteria (AIC). Maximum-likelihood phylogenies with 100 bootstrap resamples of the alignment datasets were generated with RaxML v8.2.9 [81] and then edited and visualized with FigTree v.1.4.2 [82].

Nucleic acid extraction for PCR and RT-PCR

For PCR-based detection of viruses, the extraction of DNA, RNA or simultaneous DNA/RNA from individual bees was performed with the DNeasy Blood and Tissue kit (Qiagen, Germany), TRIzol (Ambion, USA) and the QIAamp Cador Pathogen Mini kit (Qiagen, Germany), respectively, according to the manufacturers' protocols. The nucleic acid extractions tested for the presence of RNA had been treated previously with TURBO DNase (Thermo Fisher Scientific, USA) and were then used for cDNA synthesis with the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, USA).

Virus detection by PCR and RT-PCR

Primers for virus detection were designed based on the seven candidate viral contig sequences (Table 1). An aliquot of 100–300 ng of nucleic acids purified from each of the tested samples was used as a template for PCR or RT-PCR, according to the sample type, mixed with 1 U of Platinum *Taq* DNA polymerase (Invitrogen, USA), 1× PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.2 µM of each forward and reverse primer in a final reaction volume of 50 µl. For MqV1a, the temperature cycling protocol used was 94 °C for 5 min, 60 °C for 1 min and 72 °C for 2 min, followed by 20 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a touchdown of 0.5 °C per cycle, and another 20 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min, and a final extension step of 72 °C for 10 min. For the remaining six viruses a similar touchdown procedure was used, with primer annealing temperatures

starting at 54 °C and ending at 44 °C. The amplicons were analysed by conventional agarose gel electrophoresis. To test for the association of virus detection in individual bees with the manifestation of disease symptoms we conducted CMH test with Compare2 v.3.85 from WinPep [83] and two-way Barnard's unconditional tests with the Barnard package [84] in R.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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