Comparative Virome Profiling and Discovery of Novel Viruses in Managed and Wild Bees using RNA-seq

Zixiao Zhao[1]; M. Catherine Farrell[2]; Rameshwor Pudasaini[1]; Jayla Marvin[2] ; Sandra M. Rehan[3]; Hongmei Li-Byarlay[1,2 *]

[1]Agricultural Research Development Program, Central State University, Wilberforce, OH, USA

[2]Department of Agricultural and Life Sciences, Central State University, Wilberforce, OH, USA

[3]Department of Biology, York University, Toronto, ON, Canada

Author contact information

ZZ: zxzhao5@gmail.com

https://orcid.org/0000-0002-3627-5503

MCF: mfarrell@centralstate.edu

https://orcid.org/0000-0002-0081-3285

RP: rpudasaini@centralstate.edu

https://orcid.org/0000-0001-9957-1185

JM: JaylaMarvin832@gmail.com

SMR: sandra.rehan@gmail.com

https://orcid.org/0000-0002-6441-5155

HL-B: Hli-byarlay@centralstate.edu

https://orcid.org/0000-0001-6579-5172

***Corresponding Author**

Hli-byarlay@centralstate.edu

Abstract

Insect pollinators face growing challenges from viral pathogens that may contribute to population declines, yet comparative data on virome composition in managed versus wild bees remain limited. Using RNA-Seq-based metatranscriptomic analysis, we compared the virome of *Apis mellifera* L. and *Ceratina calcarata* Robertson collected from agricultural and non-agricultural landscapes. We identified multiple known bee pathogens including Black Queen Cell Virus and Deformed Wing Virus in *A. mellifera*, and discovered three novel insect viruses—two iflaviruses and one entomopoxvirus—associated with *C. calcarata*. Notably, one novel iflavirus was identified in *A. mellifera*. Our findings reveal species- and landscape-specific viral prevalence, highlight viral diversity beyond canonical bee pathogens, and provide genomic resources for future functional studies. These data offer new insights into virus-host dynamics and underscore the utility of virome profiling in understanding pollinator health at the molecular level.

Keywords: Honey bees, agricultural landscape, transcriptomics, bee virus, iflavirus, entomopoxvirus

Introduction

Animal pollinators have critical ecological functions in maintaining the diversity of global ecosystems [1, 2]. About 87.5% of flowering plants rely on animal pollinators for successful reproduction [3]. In agriculture, approximately 75% of crop species require pollinators, contributing about 30-35% of total crop production [4, 5]. Bees (Hymenoptera: Apidae) are considered the most abundant and important pollinators globally. Honey bee species (*Apis* spp.), with a global distribution due to domestication, contribute about 80% of total pollination and create about $700 million value annually in the US [6]. However, the ecological significance of non-*Apis* wild bee species shouldn't be neglected. Wild bees provide pollination services to many crops, even when managed honey bees are not available [7, 8]. Among the over 17,000 wild bee species worldwide, with a wide range of social structures from eusocial to solitary, eusocial species may be more susceptible to viral infections due to their high colony densities, frequent social interactions and genetic factors [19]. For last several decades, both wild and managed honey bees are suffering from colony declines [9-12], which not only causes economical losses to apiary industry, but more importantly, reduces pollination service and imposes potential risks for global food security.

The causes of bee populations decline are complex. Factors responsible for bee populations decline include habitat loss and land fragmentation, increased use of chemical pesticides and other agrochemicals, introduction of pests and pathogens, and climate change [11, 13]. Among these stressors, pathogenic viruses are considered the direct and one of the most significant hazards to both managed and wild bee populations (Ullah et al. 2020). Currently identified bee pathogenic viruses belong to positive-sense single-stranded RNA (+ss RNA) virus category. The majority of the pathogenic bee viruses are from two families under the order Picornavirales:

69 Iflaviridae and Dicistroviridae. Iflaviridae has only one genus: *Iflavirus*, which includes
70 Sacbrood virus (SBV), Deformed wing virus (DWV), Varroa destructor virus-1 (VDV-1),
71 and Slow bee paralysis virus (SBPV). While the family Dicistroviridae includes Black
72 queen cell virus (BQCV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV),
73 and Israeli acute paralysis virus (IAPV) [14]. Two additional pathogenic viruses of
74 bees are Chronic bee paralysis virus (CBPV), an unclassified virus with two genomic
75 RNA segments [15], and Lake Sinai viruses (LSVs), members of the order
76 Nodamuvirales [14], with eight identified lineages [16]. The symptoms caused by
77 pathogenic viruses vary, including body deformities, color changes, uncapped cells
78 with larvae or pupae, paralysis of adults, which eventually increase mortality and
79 lead to colony degradation. IAPV infections have been discovered association with
80 colony collapse disorder (CCD), which responsible for large-scale honey bee colony
81 loss in the US [17].
82 Despite managed honey bees, the pathogenic viruses can also infect wild bee
83 species [18-20]. For example, DWV has been reported to infect a wide range of
84 hymenopterans, including some social and solitary bees as well as several wasp
85 species [18, 20, 21]. Dolezal et al. [19] reported DWV and SBV were widely
86 detected in wild bees collected in Iowa, while IAPV, LSV and BQCV were also detected
87 in wild be species. Furthermore, DWV, BQCV and SBV can be deposited to pollen and
88 remain infective, which can contribute to inter-taxa transmission among bees, and
89 wasps [20]. These studies suggested viruses can be a potential factor responsible for
90 colony declines in wild bee species.
91 Although agriculture provides important foraging resources for bees, it is also
92 considered one of the major reasons for colony declines. It has converted natural
93 ecosystems to farmland primary for food and fiber productions. This process is
94 accompanied by loss of biodiversity and use of agrochemicals causing habitat loss,

95 fragmentation and degradation. Agriculture can also alter host-pathogen interactions
96 at multiple levels. A study demonstrated that the relevance of plant viruses is
97 negatively correlated with the biodiversity in agricultural landscape [22]. However,
98 whether agricultural activities affect viral prevalence in insects, especially in honey
99 bees and other bee species, is still unknown.

100 The small carpenter bee, *Ceratina calcarata* Robertson (Hymenoptera: Apidae),
101 is a wild bee species native to the eastern part of North America. It is considered as
102 an indicator species of healthy ecosystems and important pollinators for several
103 native and agricultural plants [23]. Due to the feature of subsociality, *C. calcarata* is
104 used for studying pollinator ecology, behavior, evolution, genomics and nutrition
105 [24-27]. However, little is known about the virology of this species. The impact of
106 viruses on *C. calcarata* populations and whether the viral prevalence is related to
107 agricultural activities are not clear.

108 Here we presented our metagenomic studies on viruses associated with western
109 honey bee (*A. mellifera* L.) and *C. calcarata* in response to different agricultural
110 intensifications. Bees were collected from three landscape types representing levels
111 of agricultural intensification: conventional farming, organic farming and roadside
112 with no agricultural activities. The viral RNA was extracted from collected bee
113 samples and subjected to high-throughput sequencing. We analyzed known and
114 novel viruses associated with both bee species, which were highly specific to location
115 and bee species. Our study provides a new methodology and unique knowledge for
116 bee-virus interactions.

117 Results

118 *Reconstruct virome from viral RNA-Seq reads*

119 The RNA-Seq of extracted viral RNA generates approximately 17 to 36 million

120 raw read pairs for each *A. mellifera* and *C. calcarata* sample. After quality trimming
121 and partially removing reads from respective host genome (Amel HAv3.1
122 PRJNA471592 and Ccalc.v3 PRJNA791561), 5 to 25 million pairs were kept for each
123 sample (**Supplementary file S1**). Trinity *de nove* assembled 366,289 contigs for *A.*
124 *mellifera* and 203,317 contigs for *C. calcarata* virome, which consists of 188,433 and
125 91,314 non-redundant clusters. The assembled contigs were subject to BLASTN
126 searches against non-redundant databases (nr), which claimed 541 clusters and 1187
127 contigs from *A. mellifera* assembly potentially belong to viruses. In contrast, 62
128 clusters and 84 contigs from *C. calcarata* assembly were potentially from viruses. A
129 large portion of assembled contigs were from either host genes or the plants they
130 have visited.
131
132 *Viruses infecting bees and other insects detected in A. mellifera and C. calcarata*
133 *virome assemblies*
134 BLASTN search identified four known bee pathogenic viruses from *A. mellifera*
135 virome: BQCV, DWV, SBV, and LSV3 with fully assembled genome sequences. Two
136 other members of the LSV group: LSV1 and LSV2 were detected. But their sequences
137 were only partially assembled. Positive and negative strands were detected in all of
138 the above viruses except LSV2, from which only positive strand was detected. We
139 also detected *Apis mellifera* filamentous virus (AmFV) [28] and Hubei partiti-like virus
140 34 [29] in *A. mellifera*. The AmFV were partially assembled and the Hubei partiti-like
141 virus 34 demonstrated a fully assembled genome (**Table 1A**). In *C. calcarata*, BQCV
142 was the only detected bee pathogenic virus and appeared as partial-assembled as
143 negative-strand contig (**Table 1B**).
144
145 *Plant viruses discovered in A. mellifera and C. calcarata virome assemblies*

146 A range of plant viruses were detected in virome assemblies of both bee species.
147 In *A. mellifera,* white clover cryptic virus 1 (WCCV-1) and white clover cryptic virus 2
148 (WCCV-2) from genus *Alphapartitivirus* (family: Partitiviridae, +ssRNA), as well as
149 white clover mosaic virus (WCMV) from genus *Potexvirus* (family: Alphaflexiviridae,
150 +ssRNA) were fully assembled. Blackberry chlorotic ringspot virus (BCRV) from genus
151 *Ilarvirus* (family: Bromoviridae, +ssRNA) and *Raphanus sativus* cryptic virus 1 (RsPV1)
152 from Partitiviridae (+ssRNA) have one genomic segment fully assembled, while other
153 segments were either partially assembled or completely missing. Partially assembled
154 plant viruses include 7 +ssRNA viruses: peanut stunt virus (PSV) and tobacco ringspot
155 virus (TRSV) from Bromoviridae, clover yellow mosaic virus (ClYMV) from
156 Alphaflexiviridae, red clover nepovirus A (RCNVA) , tobacco streak virus (TSV), and
157 tomato ringspot virus (ToRSV) from genus *Nepovirus* (family: Secoviridae), red clover
158 vein mosaic virus (RCVMV) from Betaflexiviridae, and one -ssRNA virus: *Pinus flexilis*
159 virus 1 (PiFleV1) from Rhabdoviridae (**Table 1A**). However, the plant viruses in *C.*
160 *calcarata* virome were considerably less diverse compared to *A. mellifera*. WCCV-2
161 was the only fully assembled plant virus, while WCCV-1, BCRV, ToRSV, and WClMV
162 were partially assembled in *C. calcarata* (**Table 1B**).
163
164 *Novel viruses in A. mellifera and C. calcarata virome assemblies*
165 The genus *Iflavirus* (family: Iflaviridae) consists of +ssRNA viruses infecting
166 arthropods, including three bee pathogenic viruses SBV, DWV, and SBPV. Two novel
167 viruses under genus *Iflavirus*, referred to as unknown iflavirus 1 and unknown
168 iflavirus 2, were identified in *C. calcarata* virome assembly as positive strands. The
169 phylogenetic analysis using translated polyprotein amino acid sequence suggests
170 both viruses are in the same group as SBV. The unknown iflavirus-1 has the highest
171 identity with Darwin bee virus-2, an unclassified picornavirus infecting honey bee [30]

172 (**Figure 1A**). This virus was also detected in *A. mellifera* virome assembly as
173 a negative strand, suggesting that it can infect and replicate in honey bees.
174 In *C. calcarata*, we detected a novel poxvirus (a double-strand DNA virus). Its
175 DNA-dependent RNA polymerase RPO132 is partially assembled. Phylogenetic
176 analysis showed this virus is closely related to the Yalta virus, an uncategorized
177 poxvirus from *Drosophila melanogaster* and *Diachasmimorpha* entomopoxvirus
178 (DIEPV), from parasitoid wasp *Diachasmimorpha longicaudata*. The three viruses
179 form a sister cluster to other entomopoxviruses (EPVs) (genus: *Alphaentomopoxvirus*,
180 *Betaentomopoxvirus*, and *Deltaentomopoxvirus*), while all the vertebrate poxviruses
181 (genus: *Avipoxvirus*, *Capripoxvirus*, and *Parapoxvirus*) are clustered into a separate
182 group. The result suggests this novel virus belongs to the subfamily
183 Entomopoxvirinae (EPV) that primarily infect insects (**Figure 1E**).
184 Three additional novel plant viruses were detected in the *A. mellifera* virome,
185 including one from the genus *Nepovirus* (unknown nepovirus 1, family: Secoviridae),
186 one from family Partitiviridae (unknown parititivirus 1, family: Partitiviridae), and one
187 of genus *Amalgavirus* (unknown Amalgavirus 1, family: Amalgaviridae). Nepovirus
188 has two positive-strand genomic RNAs [31]. For unknown nepovirus 1, the open
189 reading frame (ORF) of polyprotein RNA2 was fully assembled. However, the
190 correspondent RNA1 encoding viral coat protein was not detected. The phylogeny of
191 the polyprotein sequence shows that it is most closely related to Blueberry leaf
192 mottle virus and Grapevine Bulgarian latent virus, forming a separate cluster (**Figure**
193 **1B**). Similarly, only RNA1 encoding RNA-dependent RNA polymerase (RdRp) was fully
194 assembled for another potential plant virus: unknown parititivirus 1. The
195 phylogenetic analysis of RdRp showed that it is clustered with several uncategorized
196 partitivirus viruses associated with plant: *Polygonatum kingianum* cryptic virus 1,
197 Panax cryptic virus 3, and *Dichroa partitivirus* 1. This cluster is a sister group to

198 plant-infecting genus *Alphapartitivirus* (**Figure 1D**). The genome of unknown
199 amalgavirus 1 was fully assembled with two complete ORF: the ORF1+2 fusion
200 protein responsible for genome replication, and a coat protein. The phylogeny of
201 fusion proteins showed that it is the most closed to Vicia cryptic virus M (VCV-M) and
202 Medicago sativa amalgavirus 1 (MsAV1) and have a clear separation with
203 *Zygosaccharomyces bailii* virus Z (ZbV-Z), a yeast-infecting virus belongs to the genus
204 *Zybavirus* (**Figure 1C**).
205 In addition, other viruses, including phages and a human virus: Merkel cell
206 polyomavirus (Family: Polyomaviridae) were identified in both virome assemblies.
207 The viral genome consists of circular double-stranded RNA (dsRNA), which resulted in
208 the assembly of two identical full-length viral sequences into one contig in *C.*
209 *calcarata* virome. In *A. mellifera*, only partial sequence was assembled. How the
210 Merkel cell polyomavirus was introduced into virome and its effects in both species,
211 are still unknown.
212
213 *Abundance of viruses in response to different agricultural landscapes*
214 The distributions of the above detected viruses were highly site-specific. In *A.*
215 *mellifera*, among all identified insect viruses, five known bee viruses: BQCV, DWV,
216 LSVs, SBV, and AmFV were detected at all of the experimental sites regardless
217 of agricultural practices, with AmFV only marginally detected in conventional farm C1.
218 In contrast, the novel unknown ilfavirus 1 was only detected in samples from organic
219 farm O2 (**Figure 2A**). For five known bee viruses, the edgeR did not show any
220 significant differences at virus level. However, a separate 2834-bp LSV3 segment,
221 containing ORFs of two viral proteins, was significantly increased in bees collected
222 from organic sites compared to bees collected from roadside site (FDR: 0.04). The
223 Hubei partiti-like virus was also identified at all sites except conventional farm C1

224 (**Figure 2A**). Its full-length sequence was significantly higher in bee samples collected
225 from organic farms compared to bee samples collected from both conventional and
226 roadside sites (FDR: 0.006 to conventional, 0.003 to roadside).
227 The presence and levels of plant viruses were highly dependent on the sampling
228 locations. For instance, TSV and PiFleV1 were detected only at one separate roadside
229 site each, while RCNVA and TRSV were found only at one conventional and one
230 organic site, respectively. RCVMV was identified across three conventional sites, but
231 at relatively low levels. WCIMV, WCCV-2, and ToRSV were expressed at all
232 experimental sites (**Figure 2B**). WCCV-1 was the only significant plant virus detected
233 by edgeR. The levels of both genome segments decreased significantly in organic
234 sites, compared to both conventional (FDR, RNA1: 0.011, RNA2: 0.006) and roadside
235 sites (FDR, RNA1: 0.006, RNA2: 0.003). The viral levels of BRCV at roadside sites were
236 either very low or undetected, but the overall level is too low to perform statistical
237 analysis in edgeR.
238 *C. calcarata* hosted relatively fewer viruses compared to *A. mellifera*. The
239 presence of insect viruses was also highly site-specific. BQCV and unknown
240 entomopoxvirus 1 were only found at one conventional site, whereas two novel
241 ilfavirus were detected in roadside sites and conventional farm C1. None of the
242 viruses were detected from bee samples collected from organic sites, conventional
243 farm C3, and roadside R2 (**Figure 2C**). Regarding the plant viruses, WCCV-1 and
244 WCCV-2 had a similar pattern, being detected only at conventional farms, while
245 WCIMV and ToRSV were detected in few bee samples collected from roadside sites as
246 well (**Figure 2D**). However, their levels were not statistically significant. Similarly,
247 none of the plant viruses were detected in bee samples collected from organic sites.
248

Discussion

The metagenomic approach based on viral RNA-Seq was adopted for identifying and quantifying known viruses, as well as discovering novel viruses. The present study surveyed the abundance of viruses associated with two bee species. Findings show that the virome compositions were site-specific. However, the *A. mellifera* carried a wider range of both insect and plant viruses than *C. calcarata*. The results also indicate that the *A. mellifera* may transmit plant diseases more effectively than *C. calcarata*.

The Honey bee viruses in A. mellifera and spillover *to C. calcarata*

The levels of BQCV, DWV and SBV were in high levels in *A. mellifera* virome at all experimental sites, suggesting the high prevalence of these viruses in *A. mellifera*. In contrast, none of the acute bee paralysis virus complex (ABPV, KBV and IAPV) were detected. This result is consistent with other studies in the Northeast of the US [20, 32]. LSV is a highly variable, multi-strain bee virus that does not have any significant symptoms but is likely to weaken colonies. The LSV contigs assembled in this study are most closely related to LSV1, LSV2 and LSV3. The LSV3 was detected from all experimental sites and the only LSV with full-length genome sequence assembled. The LSV3 was originally identified in Slovenia [33] and has been detected in the Western US [34]. The study confirmed LSV3 has been spread to the New England area, in the US.

Two known viruses associated with *A. mellifera* were also detected: AmFV and Hubei partiti-like virus 34. The AmFV is a weak pathogen in *A. mellifera* and rarely causes significant symptoms. Its infection has been reported related to BQCV and DWV [28, 35]. We detected AmFV from all sites, while its viral loads were from extremely low at conventional farm C1 to medium-high at conventional farm C3. The

275 Hubei partiti-like virus 34 was first discovered as an arthropod RNA virus [36] and
276 was reported associated with hymenopteran species, including *A. mellifera* and Asian
277 hornet (*Vespa velutina*) in East Asia and South Europe [29, 37]. The current study
278 reported the low to moderate infection of Hubei partiti-like virus 34 from all sites
279 except conventional farm C3 site. The potential impact of this virus on honey bee
280 health is still unknown.

281 The spillover of managed honey bee pathogen to wild bees has been considered
282 as a threat to native bee populations [38, 39]. In genus *Ceratina*, DWV was found
283 spillover to two species *C. dupla* [20] and *C. smaragdula* [40]. The current study
284 showed that, given the high prevalence of both BQCV and DWV in *A. mellifera*, BQCV
285 may have spilled over to *C. calcarata*, while no evidence of DWV spillover was
286 observed. BQCV was only detected from conventional farm C2 in relatively low
287 prevalence and viral load, indicating that this spillover to *C. calcarata* is not a
288 common scenario.

289

290 *Potential viruses infecting A. mellifera and C. calcarata*

291 The primary hosts for iflaviruses are arthropods. We discovered two closely
292 related novel ifla-like viruses. Unknown iflavirus 1 was detected in both *A. mellifera*
293 and *C. calcarata*, while the unknown iflavirus 2 was detected only in *C. calcarata*. The
294 prevalence of both viruses was low, however, the viral load of unknown ilfavirus 2
295 was slightly higher in *C. calcarata* samples collected from conventional farm C1 and
296 roadside site 1, compared to the unknown ilfavirus 1 in the same samples.

297 The EPVs are double-stranded DNA (dsDNA) viruses that infect insects and
298 predominantly insect pathogens. The unknown entomopoxivirus 1 detected in *C.*
299 *calcarata* are more closely related to Yalta viruses and DIEPV than other
300 entomopathogenic viruses. Although the role of Yalta virus is still unknown, the

301 DIEPV has been found replicated in both parasitoid wasp *D. longicaudata* and its host
302 fruit fly (*Anastrepha suspensa)*. The replication of virus is highly virulent to the host
303 fruit fly and no negative impact to wasp host, suggesting a mutualistic symbiotic
304 relationship of DIEPV to parasitoid wasp [41]. Although both *C. calcarata* and *D.*
305 *longicaudata* are from order Hymenoptera, no such parasitoid relationship was
306 observed in *C. calcarata.* Whether this novel entomopoxivirus primary infects *C.*
307 *calcarata* or just an overspill event to *C. calcarata* yet to be studied.
308 Although our findings reveal the presence of viral sequences in bee samples, it does
309 not confirm active infection or pathogenicity in these bees. Some of the viral
310 sequences may originate from environmental exposure, gut contents, or external
311 body surfaces, rather than systemic infection. Therefore, the present study does not
312 infer a direct relationship between viral presence and bee health or stress levels
313 (Olgun et al., 2020).
314
315 *Bees carrying plant viruses*
316 Many plant viruses can travel within or attach to pollen grains [42, 43], including
317 BRCV, TSV, PSV, WCCV-1, WCCV-2, TRSV, ToRSV, and WClMV as observed in the
318 present study However, only TSV, TRSV, and ToRSV have been proven vertically and
319 horizontally transmitted by pollen. The rest were only detected by RNA-Seq [43].
320 These findings show that pollinators such as *A. mellifera* posing potential risks due to
321 these viruses. However, none of the detected viruses were on the list of known
322 honey bee-transmitted viruses, according to Fetters et al [43]. TRSV was reported
323 infect honey bee [44], however whether cross-kingdom infection assist transmitting
324 diseases is unknown. On the other hand, bees are a powerful tool for monitoring
325 plant diseases [45]. The plant viruses detected in *A. mellifera* resembles to the results
326 of other metagenomic studies of bees and pollen at family and order levels [45, 46],

while the species composition is determined by plant and prevalence of plant viruses at the location.

Compared to *A. mellifera*, plant viruses in *C. calcarata* are much less diverse. Previous studies have shown that pollen from clover and rose is a major food resource for bees [47], and white clover pollen significantly increases the fitness of *C. calcarata* [23]. Consistent with that, among four plant viruses detected in *C. calcarata* virome, WCCV-1, WCCV-2, WClMV primarily infect white clover (*Trifolium repens*), and the host range of ToRSV include rose and red clover (*T. pratense*). None of the detected viruses originated beyond *C. calcarata's* foraging range, suggesting that *C. calcarata* may be an ideal bee species for monitoring white clover-associated pathogens within localized areas.

Material and method

Sample collection

Both honey bee (*A. mellifera*) and small carpenter bee (*C. calcarata*) samples were collected from sites representing three different types of land use: conventional farm, organic farm and roadside. Honey bees and the small carpenter bees were collected by sweep nets from May to July 2018 in Strafford County, New Hampshire. We collected both species within three conventional farms and three organic farms. The roadside samples were collected in the urbanized area within the University of New Hampshire campus. The detailed about locations and GPS coordinates are described in previous publications [48, 49]. At least ???? bees were collected at each sampling site, and to minimize age-related variation, all bee samples were collected from forager bees. Bee samples were flash-frozen in liquid nitrogen and temporarily stored at -80 °C.

352 *RNA extraction, sequencing and data cleaning*

353 The viral RNA was extracted from *A. mellifera* and *C. calcarata* individually by
354 Quick-RNA viral kit (ZYMO RESEARCH, Irvine, CA). DNase I (ZYMO RESEARCH)
355 in-column digestions were applied to remove genomic and environmental DNA. For
356 mRNA library generation, 200ng of total RNA was treated with NEBNext Poly mRNA
357 Magnetic Isolation Module E7490L (New England Biolabs, USA) following the
358 manufacturer protocol. Subsequently, the isolated mRNA was fragmented for 10
359 minutes. cDNA was synthesized and amplified for 12 PCR cycles using NEBNext Ultra
360 II Directional (stranded) RNA Library Prep Kit E7760L for Illumina (New England
361 Biolabs, USA) with NEBNext Multilex oligos indexes kit E6440S following the
362 manufacturer directions. Distributions of the template length and adapter-dimer
363 contamination were assessed using an Agilent 2100 Bioanalyzer and High Sensitivity
364 DNA kit (Agilent Technologies, Inc). The concentration of cDNA libraries was
365 determined using Invitrogen Qubit dsDNA HS reagents and read on a Qubit
366 Fluorometer (Thermo Fisher), and the cDNA libraries were paired-end 150bp format
367 sequenced on a Novaseq SP system (Illumina, San Diego, CA). In total, 17 honey bee
368 samples and 16 *C. calcarata* samples were sequenced (**Supplementary File S1**). Raw
369 reads were subject to adapter and quality trimming by Trimmomatic [50]. Sample
370 metadata from collection was used to assign each sample to either *A. mellifera* or *C.*
371 *calcarata*. To remove endogenous transcripts from bee hosts, the reads were
372 respectively aligned to honey bee and small carpenter bee [25] genomes (including
373 mitochondrial sequences) using Bowtie2 [51]. Read pairs that were concordantly
374 aligned to bee genomes were considered as endogenous transcripts and removed
375 from further analysis.

376

377 *Virome assembly and viral identification*

378 To reconstruct the virome of *A. mellifera* or *C. calcarata,* we first merged all the
379 pre-cleaned reads from the same organism to increase the sequence coverage. Then
380 two *de novo* assembler: Trinity [52] and metaSPAdes [53] were used with default
381 setting. The results were comparable; however, the total running time for Trinity was
382 lower. Therefore, we chose Trinity for the subsequent analyses.
383 Despite the pre-cleaning process, the assembled virome still contains host
384 sequences. To identify virus sequences, we used BLASTN to search assembled contigs
385 to default nr/nt database with E-value cutoff of 1e-7. Contigs with first three top hits
386 to viral sequence from database was retained as viral contigs. For further virus
387 identification, we examined the blast results of contigs >500 nt. A contig was
388 assigned as a known virus if it aligned stringently (E-value = 0) to existing viruses. If
389 the alignment is less stringent (0<E-value<1e-7), it was assigned as a novel virus. The
390 "full-length" or "partial" labels were based on the completeness and intactness of
391 viral protein of each viral contig. For potential novel viral contigs, ORF finder and
392 BLASTP were used to translate and predict viral protein. MEGA 11 [54] was used to
393 construct Maximum-likelihood phylogenetic tree based on the sequence of
394 homologous proteins of known and novel viruses.
395
396 *Abundance of viruses and differential analysis between landscapes*
397 To quantify the abundance of each contigs, the clean reads were mapped to
398 virome assembly of respective species using Bowtie 2 [51]. Multiple alignment was
399 allowed to obtain maximum sensitivity. The read counts were obtained by
400 FeatureCounts [55] and were normalized and converted to count per million reads
401 (CPM) by R edgeR package [56]. We also used edgeR to conduct differential analysis
402 between different landscape types. Prior to that, each contig, including endogenous
403 and viral, was filtered based on its expression level [57]. Only contigs with sufficiently

high expression levels were retained for statistical tests. The contigs with FDR less than 0.05 were considered significantly difference between landscapes. The analysis then focused on viral contigs as defined by the viral identification step.

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Author contributions:

HL-B and SMR designed the experiment. SMR conducted the field studies and provided the samples. MCF and JM prepared samples for RNA-seq. ZZ conducted data analysis and wrote the first draft of the manuscript. All authors reviewed the final manuscript.

Figures and Tables:

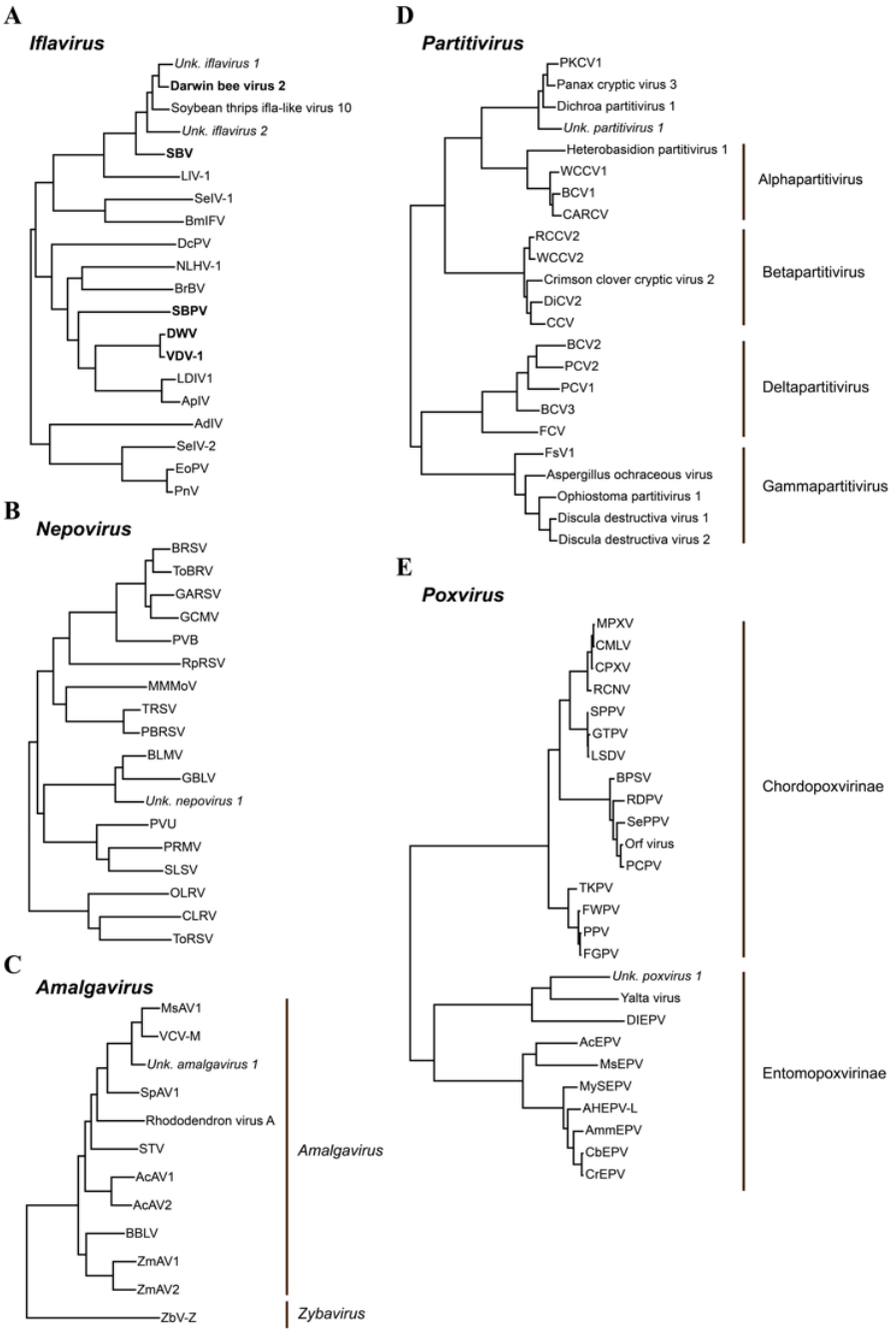

Figure 1. Phylogenetic trees of novel viruses discovered from *A. mellifera* and *C. calcarata* virome assemblies by Maximum-likelihood analysis of sequences of viral proteins: **(A)** polyprotein (iflavirus), **(B)** polyprotein from RNA2 (neprovirus), **(C)** RNA-dependent RNA polymerase (amalgavirus), **(D)** RNA-dependent RNA polymerase from RNA1 (partitivirus), and **(E)** RNA-dependent RNA polymerase submit RPO132 (poxvirus). The names in bold correspond to honeybee-associated viruses.

432 Abbreviations and protein accessions of known viruses are included in **Supplementary File S2**.

433

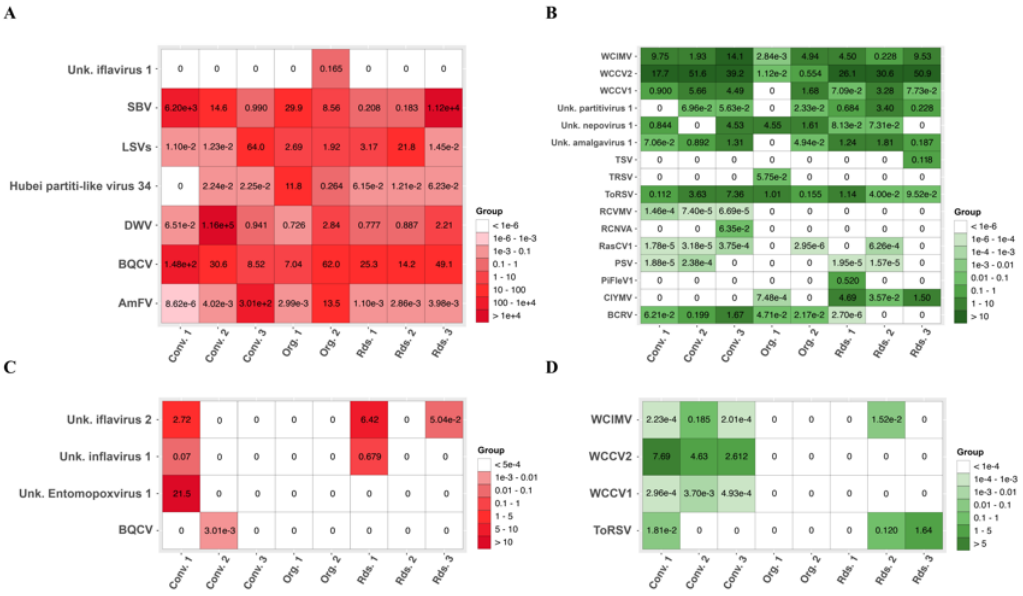

Figure 2. Heatmaps demonstrating the relative abundance of **(A)** insect viruses in *A. mellifera*, **(B)** plant viruses in *A. mellifera*, **(C)** insect viruses in C. *calcarata*, and **(D)** plant viruses in *C. calcarata* at each sampling site. Average normalized count-per-million reads (cpm) from RNA-Seq was calculated for each virus of each site and used in the heatmap. White color indicates the virus is undetected on that site. Potential insect and plant viruses were included.

441 **Table 1**. List of identified insect and plant viruses in **(A)** *A. mellifera* and **(B)** *C. calcarata* virome

442 assemblies, assembled from RNA-Seq data

443 **A**

Virus name	Abbr.	Type	Family	Genus	Host	Assemble status	Strand
Black queen cell virus	BQCV	(+)ssRNA	Dicistroviridae	*Triatovirus*	Bees	Full	+/-
Deformed wing virus	DWV	(+)ssRNA	Iflaviridae	*Iflavirus*	Bees	Full	+/-
Sacbrood virus	SBV	(+)ssRNA	Iflaviridae	*Iflavirus*	Bees	Full	+/-
Lake Sinai virus 3	LSV3	(+)ssRNA	Sinhaliviridae	*Sinaivirus*	Bees	Full	+/-
Lake Sinai virus 1	LSV1	(+)ssRNA	Sinhaliviridae	*Sinaivirus*	Bees	Partial	+/-
Lake Sinai virus 2	LSV2	(+)ssRNA	Sinhaliviridae	*Sinaivirus*	Bees	Partial	+
Apis mellifera filamentous virus	AmFV	dsDNA	*unclassified*	*unclassified*	Bees	Partial	+/-
Hubei partiti-like virus 34	-	RNA	*unclassified*	*unclassified*	Bees	Full	+
Blackberry chlorotic ringspot virus	BCRV	(+)ssRNA	Bromoviridae	*Ilarvirus*	Plants	RNA1: Full RNA2: Partial RNA3: Partial	+ - +
Tobacco streak virus	TSV	(+)ssRNA	Bromoviridae	*Ilarvirus*	Plants	RNA1: Partial RNA2: Partial RNA3: Partial	+/- +/- +
Peanut stunt virus	PSV	(+)ssRNA	Bromoviridae	*Cucumovirus*	Plants	RNA1: Partial RNA2: Partial RNA3: Partial	+ + -
Raphanus sativus cryptic virus 1	RasCV1	dsRNA	Partitiviridae	*unclassified*	Plants	RNA1: Full *RNA2: Missing*	-
White clover cryptic virus 1	WCCV-1	dsRNA	Partitiviridae	*Alphapartitivirus*	Plants	RNA1: Full RNA2: Full	- -

White clover cryptic virus 2	WCCV-2	dsRNA	Partitiviridae	*Alphapartitivirus*	Plants	RNA1: Full RNA2: Full	- -
Red clover nepovirus A	RCNVA	(+)ssRNA	Secoviridae	*Nepovirus*	Plants	RNA1: Partial RNA2: Partial	+ +/-
Tobacco ringspot virus	TRSV	(+)ssRNA	Secoviridae	*Nepovirus*	Plants	RNA1: Partial RNA2: Partial	+/- -
Tomato ringspot virus	ToRSV	(+)ssRNA	Secoviridae	*Nepovirus*	Plants	RNA1: Partial RNA2: Partial	+/- -
White clover mosaic virus	WClMV	(+)ssRNA	Alphaflexiviridae	*Potexvirus*	Plants	Full	+/-
Clover yellow mosaic virus	ClYMV	(+)ssRNA	Alphaflexiviridae	*Potexvirus*	Plants	Partial	+/-
Red clover vein mosaic virus	RCVMV	(+)ssRNA	Betaflexiviridae	*Carlavirus*	Plants	Partial	+/-
Pinus flexilis virus 1	PiFleV1	(-)ssRNA	Rhabdoviridae	*unclassified*	Plants	Partial	+/-

B

Virus name	Abbr.	Type	Family	Genus	Host	Assembly status	Strand
Black queen cell virus	BQCV	(+)ssRNA	Dicistroviridae	*Triatovirus*	Bees	Partial	-
Tomato ringspot virus	ToRSV	(+)ssRNA	Secoviridae	*Nepovirus*	Plants	RNA1: Partial RNA2: Partial	+/- +/-
White clover cryptic virus 1	WCCV-1	dsRNA	Partitiviridae	*Alphapartitivirus*	Plants	RNA1: Full *RNA2: Missing*	-
White clover cryptic virus 2	WCCV-2	dsRNA	Partitiviridae	*Alphapartitivirus*	Plants	RNA1: Full RNA2: Full	+ -
White clover mosaic virus	WClMV	(+)ssRNA	Alphaflexiviridae	*Potexvirus*	Plants	Partial	+/-

448 **Table 2.** List of novel viruses detected in *A. mellifera* and *C. calcarata* virome assemblies assembled

449 from RNA-Seq data

450

Virus	Potential host	Length (bp)	Viral protein ORF status	Detected in
Merkel cell polyomavirus	Vertebrate	3330	Partial	*A. mellifera*
		10809	Full	*C. calcarata*
Unknown iflavirus 1	Arthropods	1574	Partial	*A. mellifera*
		616	Partial	*C. calcarata*
Unknown iflavirus 2	Arthropods	7343	Full	*C. calcarata*
Unknown nepovirus 1	Plants	6501	Full	*A. mellifera*
Unknow partitivirus 1	Plants	1895	Full	*A. mellifera*
Unknown amalgavirus 1	Plants	3425	Full	*A. mellifera*
Unknown entomopoxvirus 1	Arthropods	2543	Partial	*C. calcarata*

451

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