Genomic characterization of viruses associated with the parasitoid Anagyrus vladimiri (Hymenoptera: Encyrtidae)

Yehuda Izraeli, David Lepetit, Shir Atias, Netta Mozes-Daube, Gal Wodowski, Oded Lachman, Neta Luria, Shimon Steinberg, Julien Varaldi, Einat Zchori-Fein, et al.

Cover page

Genomic characterization of viruses associated with the parasitoid *Anagyrus vladimiri* (Hymenoptera: Encyrtidae)

Yehuda Izraeli[1,3], David Lepetit[2], Shir Atias[3], Netta Mozes-Daube[3], Gal Wodowski[1,3], Oded Lachman[4], Neta Luria[4], Shimon Steinberg[5], Julien Varaldi[2], Einat Zchori-Fein[3] and Elad Chiel*[6]

[1]University of Haifa, Department of Evolutionary and Environmental Biology, Haifa, Israel,
[2]Laboratoire de Biométrie et Biologie Evolutive, Université Lyon 1, CNRS, Villeurbanne, France,
[3]Newe Ya'ar Research Center, Department of Entomology, ARO, Ramat Yishai, Israel,
[4]Volcani Research Center, Department of Plant Pathology and Weed Research, ARO, Rishon LeZion, Israel
[5]BioBee Sde Eliyahu Ltd, Emek Hamaayanot, Israel
[6]University of Haifa – Oranim, Department of Biology and Environment, Tivon, Israel

*** Correspondence:**
Elad Chiel
elad-c@sci.haifa.ac.il

Keywords: biocontrol agent, dicistrovirus, endosymbiont, iflavirus, mealybug, reovirus, virome

Running title: The Virome of a Biological Control Agent Parasitoid

Abstract

The knowledge on symbiotic microorganisms of insects has increased in recent years, yet relatively little data is available on non-pathogenic viruses. Here we studied the virome of the parasitoid wasp *Anagyrus vladimiri* (Hymenoptera: Encyrtidae), a biocontrol agent of mealybugs. By high-throughput sequencing of viral nucleic acids, we revealed three novel viruses, belonging to the families *Reoviridae* (provisionally termed AnvRV [Anagyrus vladimiri reovirus]), *Iflaviridae* (AnvIFV) and *Dicistroviridae* (AnvDV). Phylogenetic analysis further classified the AnvRV in the genus *Idnoreovirus*, and the AnvDV in the genus *Triatovirus*. The genome of AnvRV is comprised of 10 distinct genomic segments ranging in length from 1.5 to 4.2 Kbp, but only two out of the 10 open reading frames (ORFs) have a known function. AnvIFV and AnvDV each have one polypeptide ORF, which is typical to iflaviruses but very un-common among dicistroviruses. AnvRV was found to be fixed in a mass-reared population of *A. vladimiri*, whereas it's prevalence in field-collected wasps was ~15%. Similarly, the prevalence of AnvIFV and AnvDV were much higher in the mass rearing population than in the field population. Transmission electron micrographs of females' ovaries revealed clusters and viroplasms of *Reovirus*-like particles in follicle cells. AnvRV was not detected in the mealybugs, suggesting that this virus is truly associated with the wasps. The possible effects of these viruses on *A. vladimiri*'s biology, and on biocontrol agents in general are discussed. Our findings identify RNA viruses as potential players involved in the multitrophic system of mealybugs, their parasitoids and other members of the holobiont.

Importance

Different biological control approaches use industrially mass-reared natural enemy insects to reduce damage of arthropod pests. Such mass-reared cultures may be positively and/or negatively affected by various microorganisms, including viruses. Yet, current knowledge on virus diversity, especially in arthropods, is limited. Here, we provide the first virome characterization of a member of the wasps family Encyrtidae - the member being the parasitoid *Anagyrus vladimiri* - a commercially important natural enemy of mealybug pests. We describe the genome of three previously unknown RNA viruses, co-inhabiting this parasitoid, and elaborate on the prevalence of those viruses in individual wasps of both mass-reared and environmental origins. Microscopy images suggest that at least one of the viruses is transmitted maternally via the ovaries. We discuss

the genomic structure of the viruses and the possible relationship between those viruses and the *A. vladimiri* host, with implications on improvement of biocontrol of mealybug pests.

Introduction

Numerous arthropod species host symbiotic microorganisms that are integral to their life history, ecology and evolution (Zchori-Fein and Bourtzis, 2011). Such microorganisms engage in a wide range of symbiotic relationships, from parasitism to obligate mutualism, and may affect various biological features of their hosts (Zchori-Fein and Bourtzis, 2011; Drew et al., 2021). While the diverse interactions of bacteria, and to a lesser extent of fungi, with insects have been extensively studied, viruses associated with insects are much less explored. This is mainly due to paucity of knowledge of a great fraction of virus diversity as attested by recent large-scale or more focused surveys that unravelled numerous new virus lineages (Shi et al., 2016; Webster et al., 2016; Wu et al., 2020).

Among insects, it appears that endoparasitoids have special relationships with viruses. Those insects develop within an arthropod host, ultimately causing its death (Godfray, H. J., 1994). Most parasitoids belong to the order Hymenoptera. Apart from pathogenic viruses, they often harbour inherited viruses that may affect their phenotype. For instance, some viruses may affect their behaviour, as observed in the solitary parasitoid *Leptopilina boulardi*. In that case, the virus forces infected females to superparasitize (i.e., laying eggs in already parasitized hosts), thus favouring its horizontal transmission within the superparasitized host (Varaldi et al., 2003, 2005). The ssRNA iflavirus DcPV is also injected by the parasitoid *Dinocampus coccinellae* into its ladybeetle host during oviposition. The virus then replicates in the beetle's brain which may participate in turning the ladybeetle into a 'zombie' that guards the parasitoid cocoon from predation (Dheilly et al., 2015). Other true free-living viruses, such as the entomopoxvirus DlEPV found in some parasitoid wasps, may protect the wasp offspring from host immune reaction (Coffman et al., 2020). On the farther end of the pathogenic-mutualist axis, are some domesticated viruses that became integrated into the insect genome. The best-known example of this phenomenon is the case of polydnaviruses (PDVs) which are associated with wasps that parasitize caterpillars of butterflies and moths. The endogenized viral genes allow the production of viral-like particles which are injected together with the eggs inside the caterpillar host and suppress its immune system, hence allowing the successful development of the wasp larvae (Herniou et al., 2013). Evidently then, viruses have

diverse and important effects on the phenotype and ecology of endoparasitoids, and there are likely many more virus-insect associations that are yet to be unveiled. In the current study the viruses associated with *Anagyrus vladimiri* Triapitsyn (Hymenoptera: Encyrtidae), a solitary endoparasitoid wasp attacking mealybugs (Hemiptera: Pseudococcidae), were identified and characterized. *Anagyrus vladimiri* is mass-reared for commercial use in biological control programs to manage two major global pest species, which attack plants in dozens of families: the citrus mealybug, *Planococcus citri* (Risso) and the vine mealybug *P. ficus* (Signoret) (Bugila et al., 2015). The female parasitoid lays an egg into the body of the mealybug host, which continues to develop while the wasp larva feeds and develops inside it. After a few days the mealybug host dies, its cuticle hardens and turns into a 'mummy' and the parasitoid larva pupates inside. Eventually, the adult parasitoid chews a hole in the mummified host and emerges (Avidov et al., 1967).

Recently, we reported an analysis of the fungal and bacterial portion of microbiome inhabiting this population of *A. vladimiri*. We found that the symbiont *Wolbachia* is dominating the bacterial community and under some conditions it has minor negative effects on the fecundity of the parasitoid (Izraeli et al., 2020). The current research was launched to characterize the virome of *A. vladimiri*, annotate the viral genomes, assess their phylogenetic position, and test the prevalence of the various viruses in lab and field populations of the wasp.

Results

Electrophoresis characterization of Anagyrus vladimiri *virome*

Viral nucleic acids (VNAs) were purified from a pool of adults (from the mass-reared line, see Table 1 for details) and treated by RNaseA and DNaseI. At least nine bands ranging in size from ~1.5Kbp to ~4Kbp were clearly observed when VNAs were separated on non-denaturating gel by electrophoresis (Fig. 1, lane 3). VNAs were successfully digested by RNaseA (Fig. 1, lanes 8-11), but not by DNaseI (Fig. 1, lanes 13), suggesting that the virome of *A. vladimiri* is composed of RNA viruses only. For both our positive control (Phi6 dsRNA) and the VNAs from *A. vladimiri*, the RNase digestion was mitigated in high concentration of sodium citrate buffer (=high ionic strength) with low concentration of RNase, which is the expected outcome when the RNA is double-, and not single-stranded (Sorrentino et al., 1980; Khramtsov et al., 1997). These results suggest that the tested strain of *A. vladimiri* harbours at least one, dsRNA virus with nine segments (or more, since segments of similar size may co-migrate at the same position), or several non-segmented or segmented dsRNA viruses.

Sequencing the RNA virome of A. vladimiri

After reverse transcription, the VNAs of *A. vladimiri* were sequenced on an Illumina platform. We obtained 16 million of high quality paired-end reads.High sequence duplication levels were observed (>96%), suggesting that the genome sequence is complete (except for the extremities of untranslated regions), and as expected from the apparent low complexity of the sample (Fig. 1) which totalled approximately 20-30Kbp. De novo assembly of this dataset, resulted in only 30 contigs, 18 of which were shorter than 1,500bp.

Homology (by amino acid sequence identity) of 11 out of the 12 contigs longer than 1,500bp, as identified by BLASTx and PSI-BLASTp, were found to three RNA virus families; *Dicistroviridae*, *Iflaviridae* (both positive strand ssRNA, order: *Picornavirales*) and *Reoviridae* (segmented linear dsRNA, order: *Reovirales*) (Table 2, Fig. 2).

One contig was most similar to a *Dicistroviridae* member named Black queen cell virus infecting honeybees, with an average of 41% amino acid sequence identity. The contig is 8,726bp long, which is in the expected range for *Dicistriviridae* in general (8.5-10.2kb) and very close to the genome length of Black queen cell virus (8,550bp). We propose to name this newly discovered virus: Anagyrus vladimiri dicistrovirus (AnvDV).

One contig, 10,031bp long, had the highest similarity to different members of the family *Iflaviridae*, with 46% amino acid identity with the iflavirus member *Dinocampus coccinellae* paralysis virus used as a reference. The contig length is well within the expected range for *Iflaviridae* (8.8-9.7kb). We propose to name this newly discovered virus: Anagyrus vladimiri iflavirus (AnvIFV).

Nine contigs were identified as homologous to nine different segments of a reovirus infecting the winter moth *Operophtera brumata* (ObIRV). Protein alignment to the known ObIRV segments as a reference by BLASTp, revealed that the coverage between the nine contigs to the nine segments ranges between 20-98%, while the amino acid sequence identity ranges between 19% and 37% in all segments (Table 2). The one remaining contig out of the 12 'long' ones, had no similarity according to BLASTx and Psi-BLASTp searches (contig k199_18). However, its length was similar to other contigs assigned to the reovirus, therefore it was added to the screening assay for all reovirus segments in individuals. Results of that assay (see below) strongly support the assumption that this contig is part of the reovirus genome, and together with the nine contigs identified by BLAST, this genome consists of 10 segments. We propose to name this newly discovered segmented virus: Anagyrus vladimiri reovirus (AnvRV).

Six out of the 18 contigs shorter than 1,500bp (contigs k119_1, k119_3, k119_5, k119_8, k119_9 and k119_13; Fig. 2), were also assigned to the *Iflaviridae* family by the BLASTx search (Table 2, Fig. 2). Aligning those to the long AnvIFV contig showed that the small contigs are actually parts of this long contig with >96% nucleotide identity. They were discarded from the analysis.

The nearly-complete gnome sequence of the three novel RNA viruses is available in GeneBank under BioProject PRJNA641546, with the following BioSample accession numbers __.

Annotation

Analysis of the recovered contigs predicted that all 12 'long' (>1,500bp) ones have a large open reading frame (ORF), covering >90% of their length, suggesting that they encode true functional genes. In contrast, most of the 18 'short' (<1,500bp) contigs had large portions of un-translated regions (UTRs), with the ORFs covering on average less than 60% (Table 2, Fig. 2).

Interestingly, the contig identified as AnvDV harbours only one ORF, unlike most known viruses of this family, which harbour two ORFs. Furthermore, no 'internal ribosome entry site' (IRES) - an RNA element typically found between the two ORFs of dicistroviruses (and enables the translation initiation of the second ORF) - was found along this contig. The genomes of the three

RNA viruses were further annotated by searching the predicted ORFs for conserved domains using the NCBI CDD tool. Both the AnvIFV and AnvDV were found to each harbour five domains encoding for: RNA-dependent RNA polymerase (RdRP) (accession: cd01699), RNA helicase (accession: pfam00910), capsid proteins (CRPV_capsid superfamily, found in many dicistroviruses) (accession: cl07393) and two 'rhv-like' domains (part of a capsid protein, named as 'drug-binding pocket' found in poliovirus, also a member of *Picornavirales* order) (accession: cd00205) (Fig. 3a).

No conserved domains were found for the 10 AnvRV segments. Nevertheless, two genes were inferred from the BLASTx searches by homology with the ObIRV genome: RdRP on segment no.1 and a polyhedrin protein on segment no.4 (Fig. 3b).

One conserved sequence motif (GAAGAKC) was found in the 3' end termini of the five out of 10 contigs assigned to AnvRV (supp. fig 2). No conserved sequences were found in the 5' end, suggesting that a few bases in the extremities of the contigs may be missing in our current assembly.

Phylogeny

AnvRV. Phylogenetic analysis of the RdRP encoding segment of the AnvRV revealed that its closest known relative is the 'Zoersel tick virus', identified from the tick *Ixodes ricinus.* This virus, together with three other close relatives, have no genus taxonomic classification. The closest classified virus is the ObIRV, which belongs to the genus *Idnoreovirus* (Fig. 4). Pairwise alignments of the AnvRV to those five viruses reveals a 34-43% RdRP amino acid sequence identity, while it's amino acid identity with all other viruses compared in the phylogenetic analysis was 27% and lower. As the common cut-off between ICTV verified genera is 30% amino acid identity (Matthijnssens et al., 2022), this suggests that the AnvRV, together with the four other unclassified relatives, belongs to the genus *Idnoreovirus* (Fig. 4).

AnvDV. The phylogenetic analysis for the AnvDV clearly show that this virus belongs to the genus *Triatovirus*, together with the Black queen cell virus (51% amino acid RdRP identity) and other relatives (Fig. 5).

AnvIFV. The phylogenetic analysis for the AnvIFV place this virus near the Nasonia vitripennis virus [accession ACN94442.1], Aedes vexans iflavirus [QGW51140.1](61-62% amino acid RdRP identity), and others. The unique Dinocampus coccinellae paralysis virus [YP_009111311.1], also clusters on the same branch with AnvIFV, and has 58% amino acid RdRP identity with it (Fig. 5).

Prevalence of viruses in lab and field samples

To assess the prevalence of the three RNA virus candidates in the different *A. vladimiri* populations, diagnostic PCRs with specific primers were used (Supp. table 1) on 20 individual females from each of the *Wolbachia*-carrying (W^+) and *Wolbachia*-free (W^-) lab-reared lines (Izraeli et al., 2020). In addition, 24 field-collected individuals were screened (see Table 1 for details).

All 40 lab-reared individuals harboured the AnvRV. The AnvIFV and the AnvDV were found in 33 and 25 specimens out of 40 individuals respectively (Fig. 6). All three viruses were also detected in field individuals, although the prevalence was lower, with three females carrying AnvRV (12.5%), 10 carrying AnvDV (42%), and two carrying AnvIFV (8%) out of 24 individuals (Fig. 6).

Interestingly, out of 10 field females carrying the AnvDV, only three lacked *Wolbachia*, and seven out of the eight females carrying *Wolbachia*, also carried the AnvDV. The co-occurrence of those two microbes was statistically significantly correlated (Fisher's exact test: p=0.002), suggesting a positive interaction between *Wolbachia* and AnvDV within *A. vladimiri* populations. However, AnvDV was present both in the *Wolbachia* positive and the *Wolbachia* negative strains in the lab.

Screening for the 10 Reovirus segments in individual A. vladimiri *wasps*

To test whether all the 10 Reovirus segments (nine identified by sequence homology, and one - contig K119_18 - suspected by its length and UTR/contig length ratio) belong to the same AnvRV genome, rather than to different RNA viruses, 24 individuals from two wasp lines were screened by a set of diagnostic PCRs. Those included 16 individuals from the W^+RV^+ line - a line harbouring the AnvRV as confirmed by PCR with primers targeting the RdRP segment, and 8 individuals from the 'Field-RV^-' line, as negative controls. Generally, the results were as expected (table 3); in 9 W^+RV^+ individuals all the segments were amplified, while in 6 'Field-RV^-' individuals, none were amplified, suggesting that all 10 segments belong to the same virus. However, in 7 individuals, one or two segments failed to amplify, and in two individuals from the negative population one single segment was detected (Table 3).

Presence of viruses in the mealybugs

To test whether any of the three RNA viruses originated from the mealybug hosts, four pools were tested for their presence: i) Mass-reared (MR) W^+ *A. vladimiri*, ii) Field-collected *A. vladimiri* line without the AnvRV (RV^-), iii) un-parasitized *P. citri*, and iv) *P. citri*, four days after parasitization

by MR *A. vladimiri*, (see Table 1 for details). AnvRV was not detected in the un-parasitized mealybugs, nor in the Field-RV$^-$ *A. vladimiri*, which were reared under the same conditions as the MR *A. vladimiri*, indicating that this virus was not acquired from the mealybugs, and is associated with *A. vladimiri*. In contrast, AnvIFV and AnvDV were detected in both *A. vladimiri* and un-parasitized mealybugs (albeit the bands for the mealybug samples were weak, they were confirmed by Sanger sequencing, with >99% identity), suggesting that those viruses may be acquired from the mealybugs or from the rearing environment, possibly the potato sprouts that the mealybugs feed on (Cheng et al., 2021), or even the various fungi that develop on this substrate (supplementary Fig. 1).

Virus particles in A. vladimiri ovaries

To validate the presence of viruses in the females' reproductive tissues, transmission electron microscopy (TEM) was carried on sections of *A. vladimir*i ovaries (from the W$^+$RV$^+$ line, see Table 1 for details). Images obtained from the three specimens tested showed clusters of round shaped viral particles, 60-65nm in diameter, organized either in viroplasm structures, or in dense clusters in the perinucleus area in the cytoplasm of follicle cells (Fig. 7). This pattern is typical to *Reoviridae* viruses (Shah et al., 2017).

Discussion

The viral community of the parasitoid *A. vladimiri*, an agriculturally important biological control agent of mealybugs, is dominated by two positive ssRNA viruses from the order *Picornavirales*, which have sequence similarity to viruses of economically important insects, and a dsRNA segmented genome, assigned to the *Idnoreovirus* genus of the *Reoviridae* family. The latter was localized in the ovaries of the *A. vladimiri*, and was not found in the mealybug hosts, suggesting it specifically infects the wasp.

As most insect viromes are yet to be explored, it was not surprizing to discover new viruses in this host (e.g. Shi et al. 2016). Nevertheless, to the best of our knowledge, other than this study there is only one available detailed report on viruses in wasps of the family Encyrtidae, which reported the genome sequence of one Picorna-like virus in the parasitoid *Diversinervus elegans* (Wu et al., 2021).

Most reoviruses reported to infect insects were found in Dipterans and in Lepidopterans (most of them in species that are important agricultural pests), and are assigned to the genera *Cypovirus* (16 accepted species, which include >75 isolates), and *Idnoreovirus* (7 accepted species, >10 isolates) (Matthijnssens et al., 2022). Some are reported to cause chronic diarrheal disease to larvae, with mild-severe symptoms, while many others have no apparent symptoms on their host (Matthijnssens et al., 2022).

Out of the few reports on reoviruses infecting parasitoids, even fewer have tested for phenotypic effects of the virus on its host. Renault et al., (2005) and Graham et al., (2008) both found no evidence for phenotypic effects of reoviruses inhabiting *Diadromus pulchelus* and *Phobocampe tempestiva* respectively. However, in some cases, reoviruses evidently cause significant effects on their parasitoid host. Renault et al., (2003) reported that during parasitization *Diadromus pulchellus* females inject a reovirus into the lepidopteran host together with the egg, where like the PDV effect, it inhibits the immune response and allows the full development of the parasitoid larva. In contrast, the cypovirus that infects both *Campoletis sonorensis* parasitoid and its lepidopteran host (in which it cannot replicate), seems to be beneficial to the lepidopteran as it reduces percent of mortality from parasitism (Deacutis, 2012).

Viruses of the families *Dicistroviridae* (15 accepted species, which include many more isolates) and *Iflaviridae* (15 accepted species, >80 isolates) are more commonly associated with insects, but similarly to reoviruses, in most cases their phenotype in the insect host is unknown (Valles et al., 2017a, 2017b). Reported effects of viruses from those families are mostly pathogenic (e.g., Acute bee paralysis virus, Drosophila C viruse, Cricket paralysis virus, for *Dicistroviridae*, and Varroa destructor virus1, Deformed wing virus for *Iflaviridae*). Conversely, at least one symbiotic relationship of those viruses with parasitoids was reported. The iflavirus DcPV aids the *Dinocampus coccinellae* to paralyze its ladybeetles host, as it replicates in the ladybeetle brain possibly leading to behaviour changes that protect the developing parasitoid (Dheilly et al., 2015). Another symbiotic relationship was found between a cripavirus (*Dicistroviridae*) and *Drosophila* flies, where the virus seems to increase the fecundity of individuals that carry it (Zhang et al., 2021).

As mentioned above, in most cases the relationship of the virus with its insect host is unknown. As *A. vladimiri* wasps are reared in our lab for many generations, with no apparent disease symptoms, it can be assumed that under the tested rearing conditions (especially the AnvRV,

which is fixed in the mass-reared population), the virus is at least not pathogenic, or even may be beneficial to the wasp. Nonetheless, all scenarios are to be taken into consideration when speculating on the relationship of those three novel viruses with *A.vladimiri*, including the possibility that those viruses don't have any phenotypic effect, and are spread in the population through efficient vertical and or horizontal transmission (e.g. Lüthi et al., 2020; Martinez et al., 2016; Perreau & Moran, 2022) . Future study should address these possibilities.

Screening the wasp populations showed that while the two ssRNA viruses are not present in all individuals, the AnvRV is fixed in the lab-reared lines, suggesting either efficient transfer of this virus, whether vertical, horizontal or both (e.g. Varaldi et al., 2006). It may also suggest that the specific rearing conditions favour the carrying of the virus (e.g. Himler et al., 2011; Coffman et al., 2020). However, due to the low prevalence of the AnvRV in field populations of *A. vladimiri*, we conclude that the interaction between the two is not obligatory. Additionally, while the two ssRNA viruses were detected in virion extractions of unparasitized mealybugs, the AnvRV was not, meaning it is a wasp symbiont. On the other hand, the two ssRNA viruses may originate from the mealybug, or they can infect both the wasp and its host (e.g. Zhang et al., 2021)).

The TEM images of virus particles in follicle cells of *A. vladimiri*, resemble the typical pattern found in viruses from the family Reoviridae, which are known to be found in the cytoplasm, to be round shaped, 60-70nm long, and may be occluded in viroplasms (Shah et al., 2017). All species of this genus were detected only in insects (Matthijnssens et al., 2022) .Such a localization of a microbe in the ovary, strongly suggests vertical maternal transfer, which in turn may hint on long evolutionary relationships, and perhaps mutualism, between the two (Zchori-Fein and Bourtzis, 2011; Perlmutter and Bordenstein, 2020).

The evolutionary arms race between parasitoids and their hosts have led for several defence mechanisms that hosts use to escape parasitism. In response to invasion by an endo-parasitoid egg (or other foreign organisms), immune haemocytes in mealybugs may aggregate around the inserted egg, and form a melanized capsule, which eliminates its development (Blumberg, 1997). The rates of this encapsulation response to *A. vladimiri* parasitism is known to differ between *P. citri* and *P. ficus* hosts species (Blumberg et al., 1995; Suma et al., 2012). it can be speculated that AnvRV aids the wasp to overcome encapsulation, like DpRV2 does in the *Diadromus pulchellus* - *Acrolepiopsis assectella* system (Renault et al., 2003), but with different efficiency in different *Planococcus* hosts. If this speculation is indeed true then the different prevalence between lab and

311 field lines can be explained by the fact that the field population originated in vineyards where *P.*
312 *ficus* is the main host for *A. vladimiri*, whereas the lab population develops in *P. citri* hosts.
313 Overall results of the screening of all ten segments in *A. vladimiri* specimens, were conclusive and
314 indicate that all segments belong to the same virus. However, four segments failed to amplify in
315 between one-four out of 16 specimens tested. This might suggest that those four segments have
316 lower copy numbers than the other five. The detection of segment number four (k119_19) in two
317 specimens of the negative population can be explained by either technical error, or by
318 contamination.
319 From the pattern of *Wolbachia* and AnvDV prevalence in the 24 field collected females, where the
320 presence of those two microorganisms is somewhat correlated, it can be speculated that some
321 interaction takes place between those two. Reports on *Wolbachia* interaction with viruses focus on
322 associations with human viral pathogens vectored by insects and insect diseases caused by viruses.
323 For example, *Wolbachia* protects *Drosophila* flies from the Drosophila C virus (Family:
324 *Dicistroviridae*) (e.g. Hedges et al., 2008), and inhibits the Dengue Fever and Chikungunya
325 pathogens in humans (DENV and CHIKV, both also positive ssRNA viruses), (e.g. Hoffmann et
326 al., 2011; Walker et al., 2011). This effect is also detected in natural populations of *Drosophila*
327 and is however virus-specific (Cogni et al., 2021). Those inhibitions were generally correlated with
328 lower viral copy number of the viruses. In other cases, endosymbionts do correlate positively with
329 viral presence and transmission (e.g Gottlieb et al., 2010; Kliot et al., 2014), such as reported here
330 for *A. vladimiri*, where a significant positive correlation was found between the presence of the
331 bacterium and the AnvDV in the field population, and also a higher prevalence in the lab strain
332 infected by *Wolbachia*. This is intriguing since it could suggest a positive effect of *Wolbachia* on
333 virus transmission, may further indicate that this virus and *Wolbachia* interact and clearly needs to
334 be further investigated.
335 Interestingly, in oppose to most species of Dicistroviridae members, which have a 'Di-cistronic'
336 genome (the trait which is the source of the family's name), only one polypeptide ORF could be
337 identified in AnvDV. This is more similar to other families in the *Picornavirales* order, such as
338 *Iflaviridae* and *Picornaviridae*, however in those families the genes are in opposite order to the
339 order that was found in the AnvDV, where non-structural proteins are closer to the 3' end (Bonning
340 and Miller, 2010). As far as we know, such genomic arrangement was found only in a few viruses,
341 most of which are from aquatic invertebrates (Shi et al., 2016, see supplementary data #33; Cheng

et al., 2021; Wu et al., 2021). This rare finding suggests that this family has a broader diversity than previously known.

Conclusion

Most studies on viruses in insects focus on pathogenic interactions or transient in vectors to plants or animals. This is one of the first attempts to characterize the virome of a biocontrol agent, that overall seems to be healthy. The discovery of three new RNA viruses in a single population with no apparent diseases, highlights the richness of this hidden community of viruses. This community certainly deserves further investigation in order to clarify its transmission along generations and the phenotypic effects associated. If one (or more) of the three viruses influences the efficiency of *A. vladimiri* as a natural enemy, whether positively or negatively, this knowledge can be applied to improve the biological control of mealybug pests.

Methods

Insect rearing and field collections of parasitoids

Anagyrus vladimiri wasps were obtained from 'BioBee Sde Eliyahu Ltd' mass-rearing (MR) facility (Sde Eliyahu, Israel), and used to establish a *Wolbachia*-positive line (W^+), from which a *Wolbachia*-free line (W^-) was established by antibiotic treatments (Izraeli et al., 2020). The two lines were reared under controlled conditions of 26±1°C, 60±20% RH, and 16L:8D photoperiod regime on the citrus mealybugs *Planococcus citri*, feeding on sprouted potatoes *Solanum toberosum*.

Additional *A. vladimiri* individuals were collected in the field during the summer of 2020, using funnel insect traps placed in an unsprayed vineyard in northern Israel (32.7200N, 35.1903E). The traps (n=10), baited with mealybugs-infested potatoes, were retrieved from the field after one week and parasitized mealybugs were incubated in the lab. The *A. vladimiri* adults which emerged (emergence was achieved from three out of the 10 traps, the other seven were empty), were used either for screening viruses' prevalence (n=24, results in Fig. 3), and others were let to parasitize in the lab, establishing the 'Field' line (n=~20 female foundresses). One generation later, eight females of this Field line were individually placed in cups and allowed to oviposit in mealybugs. Mothers and three of their emerged offspring were then tested for the presence of the AnvRV and *Wolbachia*. All the cups in which both symbionts were not detected (n=7, since *Wolbachia* was detected in one mother) were pooled to establish an uninfected *A. vladimiri* line (termed hereafter 'Field-RV$^-$' line).

The origins of the five wasp lines, and the various experiments they were used for are summarized in Table 1.

Enzymatic characterization of A. vladimiris' *virome*

To characterize the viral community inhabiting *A. vladimiri*, viral nucleic acids (VNAs) were purified following (Martinez et al., 2016). Briefly, viral capsids were purified from 0.5 g of MR *A. vladimiri* wasps (pool of >1,500 individuals), by filtration, nuclease treatment (RNaseA and DNaseI) and centrifugation. To classify the genome type of potential viruses (RNA/DNA, double/single strand), VNAs were then extracted by SDS, subjected to different nuclease treatments, and then examined on an 0.8% agarose gel (Martinez et al., 2016). A non-treated VNA

sample, a dsRNA ladder (Phi6, Invitrogen, MA, USA), and a DNA ladder (1Kb+, Invitrogen, MA, USA) were used as controls.

Sequencing strategy

To further reveal all viral candidates inhabiting the wasp, VNAs were extracted from purified viral capsids using the All-In-One DNA/RNA Miniprep Kit (Biobasic, ON, Canada). Since the gel images produced in this process showed no indication of viral DNA molecules, only samples with RNA VNAs were further examined and sequenced. Libraries were prepared by the TruSeq RNA Ribo-Zero kit (Illumina, CA, USA) and sequenced by Illumina novaseq 6000 with 100 bp paired-end reads.

Since the sequence yield was very high (~16 million paired-end reads, where suspected viruses' genome size is up to 30 thousand bp), the data was randomly subsampled to three smaller datasets, including 10^5 paired reads, 10^6 paired reads and 2*10^6 paired reads. The subsampled datasets were *de-novo* assembled using 'Megahit' version 3.4 with default parameters. Comparing assembly results of the three subsampled datasets to the original full dataset, revealed that most of the contigs are identical, with a few additional small contigs and the 10^6 paired reads dataset was chosen as the most suitable for further analysis (average fold (sometimes termed 'depth') per bp = 4.2*10^4). The resulting contigs were searched for homologous similarity against both the nr and the swissprot databases using BLASTx, targeting viruses, bacteria and Hymenoptera (taxids 10239, 2, 7399 respectively). The following parameters were changed from default to increase sensitivity: matrix – BLOSUM45, Gap costs – 12;2. An additional PSI-Blastp analysis, using the protein sequences predicted by the ORFfinder online tool (RRID:SCR_016643), was used to identify similarity of contigs that Blastx failed to identify.

Annotation

All contigs were subjected to an open reading frame (ORF) search using ORFfinder online tool (RRID:SCR_016643), with start codon including alternative initiation codons. The length of the un-translated regions (UTR), included the 5' and 3'UTRs, was calculated as contig length minus the ORF length. As the ORFfinder tool may predict also false proteins, a calculation of UTR divided by the contig length was done to estimate the likeliness of the contig to harbour a 'true' protein, the higher the value the lower chance to have a 'true' protein.

412 The UTRs of the ten identified segments of AnvRV were searched for conserved sequence motifs
413 in the 5' and 3' end termini. This was done by subjecting those UTR sequences to a BLAST search
414 against themselves, with the automatic adjusted parameters for short sequences of NCBI BLASTn
415 suite.

416 Further annotation analysis of the viral genomes was done by subjecting all the ORFs to the NCBI
417 batch-CDD tool (Marchler-Bauer et al., 2015) , against the CDD v.3.19 database to search for
418 conserved domains. For relevant viruses a search for internal ribosome entry sites (IRES) was done
419 by the IRESite online tool (IRESite: The database of experimentally verified IRES structures,
420 2022) using the NUC4.4 matrix.

421 *Phylogeny*

422 To construct phylogenetic trees of the three novel viruses, the RdRP amino acid sequence of each
423 virus was aligned together with ~20 sequences of other viruses from the corresponding family.
424 Representative sequences were chosen from a BLASTp search and from the supplementary
425 material of Shi et al. 2016, to represent the whole family (or subfamily for the AnvRV). As the
426 AnvDV and AnvIFV belong to the same *Picornavirales* order, one tree was constructed for both
427 of them together. Multiple sequence alignment was conducted by Clustal Omega and a maximum
428 likelihood tree was constructed with the LG substitution model using PhyML V.3 (Sievers et al.,
429 2011) . Branch support was measured by approximate likelihood ratio tests (SH-aLRT; Guindon
430 et al., 2010). The outgroups chosen for the AnvRV tree were two mammal infecting viruses from
431 the subfamily *Sedoreovirinae*, and a poliovirus sequence was used for the *Picornavirales* tree.

432 *Virion purification from* Anagyrus vladimiri *and* Planococcus citri

433 To test whether any of the three RNA viruses originate from the mealybug hosts, virions were
434 purified from pools of: i) Mass-reared (MR) W^+ *A. vladimiri* (~0.7 g), ii) Field-collected *A.*
435 *vladimiri* line without the AnvRV (RV^-) (~0.2 g), iii) un-parasitiezed *P. citri*,(~0.7 g) and iv) *P.*
436 *citri*, four days after parasitization by MR *A. vladimiri*, (~0.3 g) (see Table 1 for details).
437 Purification was done according to Luria et al., (2020), with minor modifications. Briefly, the
438 insects were homogenized in a TBE buffer, supernatant was filtered through a 0.45 µm membrane,
439 placed on a 30% sucrose cushion, and subjected to ultracentrifugation at 242,922 g for 2 h. Then,
440 RNA was extracted from the pellet using the Viral RNA extraction kit (Bioneer, Daejeon, South

Korea). Detection of the three viruses (AnvRV, AnvIFV and AnvDV) in those samples was carried by cDNA synthesis and PCRs as described below.

PCR detections of the three viruses

Detection of RNA viruses in *A. vladimiri* individuals was obtained by RNA extraction (RNeazy plus kit, Qiagen, Düsseldorf, Germany) according to manufacturer protocol, with addition of Dithiothreitol to homogenizing buffer, to inhibit degradation by RNase. RNA samples were reverse-transcribed to cDNA using the RT-PCRbio kit (PCR Biosystems, London, UK), with no-template and no-enzyme controls. cDNA was used as templates for diagnostic PCR to identify presence/absence of the three viruses. Specific primers were designed for each virus and each viral genomic segment according to the contig sequences identified from the RNA-seq data (Supp. table 1).

Transmission electron microscope (TEM)

Anagyrus vladimiri females from the W^+RV^+ line (see Table 1 for details) were used to test for the presence of viruses in the reproductive tissues of the parasitoids. Dissected *A. vladimri* ovaries were fixed overnight with 1% Paraformaldehyde, 2.5% Glutaraldehyde in 0.1 M Cacodylate buffer pH 7.4 containing 5 mM CaCl2, washed in 0.1 M cacodylate, post fixed for 1 hour in with 1% OsO4 and 5 mM CaCl2 in Cacodylate buffer. Then the samples went through dehydration in ethanol and embedded in epon 812 (EMS). The blocks were cut with a diamond knife using a UC7 ultramicrotome (Leica,Wetzlar, Germany). Sections of ~75nm were collected on grids, contrast stained for 10 min with 2% uranyl acetate and visualized in a TEM (L120C, Talos, Thermo Fisher Scientific, MA, USA).

References

Avidov, Z., Rosseler, Y., and Rosen, D. (1967). Studies on an Israel strain of *Anagyrus pseudococci* (Girault) [Hym., Encyrtidae]. II. Some biological aspects. *Entomophaga* 12, 111–118. doi: 10.1007/BF02370607.

Blumberg, D. (1997). Parasitoid encapsulation as a defense mechanism in the Coccoidea (Homoptera) and its importance in biological control. *Biological Control* 8, 225–236. doi:10.1006/BCON.1997.0502.

Blumberg, D., Klein, M., and Mendel, Z. (1995). Response by encapsulation of four mealybug species (Homoptera: Pseudococcidae) to parasitization by *Anagyrus pseudococci*. *Phytoparasitica* 23, 157–163. doi:10.1007/BF02980975.

Bonning, B. C., and Miller, W. A. (2010). Dicistroviruses. *Annual Review of Entomology* 55, 129–150. doi:10.1146/ANNUREV-ENTO-112408-085457.

Bugila, A. A. A., Franco, J. C., da Silva, E. B., and Branco, M. (2015). Suitability of five mealybug species (Hemiptera, Pseudococcidae) as hosts for the solitary parasitoid *Anagyrus sp. nr. pseudococci* (Girault) (Hymenoptera: Encyrtidae). *Biocontrol Science and Technology* 25, 108–120. doi:10.1080/09583157.2014.952711.

Wu, C. Y., Zhao, Y. J., and Zhu, J. Y. (2021). Genome sequence of a novel member of the order *Picornavirales* from the endoparasitoid wasp *Diversinervus elegans*. *Archives of Virology* 166, 295–297. doi: 10.1007/s00705-020-04824-y.

Cheng, R. L., Li, X. F., and Zhang, C. X. (2021). Novel Dicistroviruses in an unexpected wide range of invertebrates. *Food and Environmental Virology* 13, 423–431. doi:10.1007/s12560-021-09472-2.

Coffman, K. A., Harrell, T. C., and Burke, G. R. (2020). A mutualistic Poxvirus exhibits convergent evolution with other heritable viruses in parasitoid wasps. *Journal of Virology* 94, e02059-19. doi:10.1128/JVI.02059-19.

Cogni, R., Ding, S. D., Pimentel, A. C., Day, J. P., and Jiggins, F. M. (2021). *Wolbachia* reduces virus infection in a natural population of *Drosophila*. *Communications Biology* 4, 1327. doi:10.1038/s42003-021-02838-z.

Deacutis, J. (2012). The characterization and biological effects of a novel cypovirus on the *Heliothis virescens* and *Campoletis sonorensis* host-parasitoid system. University of Kentucky.

Dheilly, N. M., Maure, F., Ravallec, M., Galinier, R., Doyon, J., Duval, D., et al. (2015). Who is the puppet master? Replication of a parasitic wasp-associated virus correlates with host behaviour manipulation. *Proceedings of the Royal Society B: Biological Sciences* 282, 20142773. doi:10.1098/rspb.2014.2773.

Drew, G. C., Stevens, E. J., and King, K. C. (2021). Microbial evolution and transitions along the parasite–mutualist continuum. *Nature Reviews Microbiology* 19, 623–638. doi:10.1038/s41579-021-00550-7.

Godfray, H. J. C. (1994). *Parasitoids : Behavioral and Evolutionary Ecology.* Princeton University Press.

Gottlieb, Y., Zchori-Fein, E., Mozes-Daube, N., Kontsedalov, S., Skaljac, M., Brumin, M., et al. (2010). The transmission efficiency of tomato yellow leaf curl virus by the whitefly *Bemisia tabaci* is correlated with the presence of a specific symbiotic bacterium species . *Journal of Virology* 84, 9310–9317. doi: 10.1128/jvi.00423-10.

Graham, R. I., Rao, S., Sait, S. M., Attoui, H., Mertens, P. P. C., Hails, R. S., et al. (2008). Sequence analysis of a reovirus isolated from the winter moth Operophtera brumata (Lepidoptera: Geometridae) and its parasitoid wasp *Phobocampe tempestiva* (Hymenoptera: Ichneumonidae). *Virus Research* 135, 42–47. doi:10.1016/j.virusres.2008.02.005.

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology* 59, 307–321. doi:10.1093/SYSBIO/SYQ010.

Hedges, L. M., Brownlie, J. C., O'Neill, S. L., and Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science* 322, 702–702. doi:10.1126/science.1162418.

Herniou, E. A., Huguet, E., Thézé, J., Bézier, A., Periquet, G., and Drezen, J.-M. (2013). When parasitic wasps hijacked viruses: genomic and functional evolution of polydnaviruses. *Philosophical Transactions of the Royal Society B: Biological Sciences* 368, 20130051. doi:10.1098/RSTB.2013.0051.

Himler, A. G., Adachi-Hagimori, T., Bergen, J. E., Kozuch, A., Kelly, S. E., Tabashnik, B. E., et al. (2011). Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* 332, 254–256. doi:10.1126/science.1199410.

Hoffmann, A. A., Montgomery, B. L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P. H., Muzzi, F., et al. (2011). Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 476, 454–457. doi:10.1038/nature10356.

IRESite: The database of experimentally verified IRES structures (2022). http://www.iresite.org/IRESite_web.php?page=blastsearch&search_type=blast_iress [Accessed January 30, 2022].

Izraeli, Y., Lalzar, M., Mozes-Daube, N., Steinberg, S., Chiel, E., and Zchori-Fein, E. (2020). *Wolbachia* influence on the fitness of *Anagyrus vladimiri* (Hymenoptera: Encyrtidae), a bio-control agent of mealybugs. *Pest Management Science* 77, 1023–1034. doi:10.1002/ps.6117.

Khramtsov, N. v., Woods, K. M., Nesterenko, M. v., Dykstra, C. C., and Upton, S. J. (1997). Virus-like, double-stranded RNAs in the parasitic protozoan *Cryptosporidium parvum*. *Molecular Microbiology* 26, 289–300. doi:10.1046/j.1365-2958.1997.5721933.x.

Kliot, A., Cilia, M., Czosnek, H., and Ghanim, M. (2014). Implication of the bacterial endosymbiont *Rickettsia* spp. in interactions of the whitefly *Bemisia tabaci* with tomato yellow leaf curl virus. *Journal of Virology* 88, 5652–5660. doi: 10.1128/jvi.00071-14.

Luria, N., Smith, E., Lachman, O., Laskar, O., Sela, N., and Dombrovsky, A. (2020). Isolation and characterization of a novel cripavirus, the first Dicistroviridae family member infecting the cotton mealybug *Phenacoccus solenopsis*. *Archives of Virology* 165, 1987–1994. doi:10.1007/s00705-020-04702-7.

Lüthi, M. N., Vorburger, C., and Dennis, A. B. (2020). A Novel RNA Virus in the parasitoid wasp *Lysiphlebus fabarum*: Genomic structure, prevalence, and transmission. *Viruses* 12, 59. doi: 10.3390/V12010059.

Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., et al. (2015). CDD: NCBI's conserved domain database. *Nucleic Acids Research* 43, D222–D226. doi:10.1093/NAR/GKU1221.

Martinez, J., Lepetit, D., Ravallec, M., Fleury, F., and Varaldi, J. (2016). Additional heritable virus in the parasitic wasp *Leptopilina boulardi*: Prevalence, transmission and phenotypic effects. *Journal of General Virology* 97, 523–535. doi:10.1099/jgv.0.000360.

Matthijnssens, J., Attoui, H., Bányai, K., Brussaard, C. P. D., Danthi, P., Vas, M., et al. (2022). ICTV Virus Taxonomy Profile: Reoviridae. *Journal of General Virology*.

Perlmutter, J. I., and Bordenstein, S. R. (2020). Microorganisms in the reproductive tissues of arthropods. *Nature Reviews Microbiology* 18, 97–111. doi:10.1038/s41579-019-0309-z.

548 Perreau, J., and Moran, N. A. (2022). Genetic innovations in animal–microbe symbioses. *Nature Reviews*
549 *Genetics* 23, 23–39. doi: 10.1038/s41576-021-00395-z.

550 Renault, S., Bigot, S., Lemesle, M., Sizaret, P.-Y. Y., and Bigot, Y. (2003). The cypovirus Diadromus
551 pulchellus RV-2 is sporadically associated with the endoparasitoid wasp *D. pulchellus* and modulates
552 the defence mechanisms of pupae of the parasitized leek-month, *Acrolepiopsis assectella*. *Journal of*
553 *General Virology* 84, 1799–1807. doi:10.1099/vir.0.19038-0.

554 Renault, S., Stasiak, K., Federici, B., and Bigot, Y. (2005). Commensal and mutualistic relationships of
555 reoviruses with their parasitoid wasp hosts. *Journal of Insect Physiology* 51, 137–148.
556 doi:10.1016/j.jinsphys.2004.08.002.

557 Shah, P. N. M., Stanifer, M. L., Höhn, K., Engel, U., Haselmann, U., Bartenschlager, R., et al. (2017).
558 Genome packaging of reovirus is mediated by the scaffolding property of the microtubule network.
559 *Cellular Microbiology* 19, e12765. doi:10.1111/cmi.12765.

560 Shi, M., Lin, X. D., Tian, J. H., Chen, L. J., Chen, X., Li, C. X., et al. (2016). Redefining the invertebrate
561 RNA virosphere. *Nature* 540, 539–543. doi:10.1038/nature20167.

562 Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation
563 of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems*
564 *Biology* 7, 539. doi:10.1038/MSB.2011.75.

565 Sorrentino, S., Carsana, A., Furia, A., Doskocil, J., and Libonati, M. (1980). Ionic control of enzymic
566 degradation of double-stranded RNA. *Biochimica et Biophysica Acta* 609, 40–52. doi:10.1016/0005-
567 2787(80)90199-9.

568 Suma, P., Mansour, R., Russo, A., La Torre, I., Bugila, A. A. A., and Franco, J. C. (2012). Encapsulation
569 rates of the parasitoid *Anagyrus* sp. nr. *pseudococci*, by three mealybug species (Hemiptera:
570 Pseudococcidae). *Phytoparasitica* 40, 11–16. doi:10.1007/s12600-011-0199-8.

571 Valles, S. M., Chen, Y., Firth, A. E., Guérin, D. M. A., Hashimoto, Y., Herrero, S., et al. (2017a). ICTV
572 virus taxonomy profile: Dicistroviridae. *Journal of General Virology* 98, 355–356.
573 doi:10.1099/JGV.0.000756.

574 Valles, S. M., Chen, Y., Firth, A. E., Guérin, D. M. A., Hashimoto, Y., Herrero, S., et al. (2017b). ICTV
575 virus taxonomy profile: Iflaviridae. *Journal of General Virology* 98, 527–528.
576 doi:10.1099/JGV.0.000757/CITE/REFWORKS.

577 Varaldi, J., Boulétreau, M., and Fleury, F. (2005). Cost induced by viral particles manipulating
578 superparasitism behaviour in the parasitoid *Leptopilina boulardi*. *Parasitology* 131, 161–168.
579 doi:10.1017/S0031182005007602.

580 Varaldi, J., Fouillet, P., Ravallec, M., López-Ferber, M., Boulétreau, M., and Fleury, F. (2003). Infectious
581 behavior in a parasitoid. *Science* 302, 1930. doi:10.1126/science.1088798.

582 Varaldi, J., Gandon, S., Rivero, A., Patot, S., Fleury, F., Gandon, S., et al. (2006). "A newly discovered
583 virus manipulates superparasitism behavior in a parasitoid wasp," in Insect Symbiosis, ed. Bourtzis,
584 K., and Miller, T. A. Taylor and Francis, (Boca Raton, FL: CRC Press), 141–162.
585 doi:10.1201/9781420005936-11.

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., et al. (2011). The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* 476, 450–453. doi:10.1038/NATURE10355.

Webster, C. L., Longdon, B., Lewis, S. H., and Obbard, D. J. (2016). Twenty-Five new viruses associated with the Drosophilidae (Diptera). *Evolutionary Bioinformatics* 12, 13–25. doi:10.4137/EBO.S39454.

Wu, H., Pang, R., Cheng, T., Xue, L., Zeng, H., Lei, T., et al. (2020). Abundant and diverse RNA viruses in insects revealed by RNA-Seq analysis: Ecological and evolutionary implications. *mSystems* 5, 1–14. doi:10.1128/msystems.00039-20.

Zchori-Fein, E., and Bourtzis, K. (2011). *Manipulative Tenants; Bacteria Associated with Arthropods*. Boca Raton: CRC Press. doi:10.1201/b11008.

Zhang, J., Wang, F., Yuan, B., Yang, L., Yang, Y., Fang, Q., et al. (2021). A novel cripavirus of an ectoparasitoid wasp increases pupal duration and fecundity of the wasp's *Drosophila melanogaster* host. *The ISME Journal* 15, 3239-3257. doi:10.1038/s41396-021-01005-w.

Tables

Table 1. Summary of *Anagyrus vladimri* lines used in this study.		
line name	*source*	*experiments used for*
MR	'Biobee' mass-rearing facility	Virome analysis
W^+RV^+	'Biobee' mass-rearing facility, and further reared in our lab. Wasps carry *Wolbachia* and AnvRV.	Virus prevalence screening, Reovirus segment screening, Transmission electron microscopy
W^-RV^+	MR wasps that fed on antibiotics (Izraeli *et al.* 2020) Wasps carry AnvRV but not *Wolbachia*.	Virus prevalence screening
Field	Field collections, north Israel	Virus prevalence screening, Reovirus segment screening (negative controls)
Field-RV^-	Iso-female lines of the field collected wasps	Presence of viruses in mealybugs

600 **Table 2.** Virome analysis of *A. vladimiri.* Details for all 30 contigs resulted from RNAseq.

contig name	contig length	ORF length	UTRs length	UTR/contig length	BLASTx hit	taxonomic group	amino acid identity (%)
k119_28	10031	9173	858	0.085535	YP_009111311.1	Iflaviridae	47
k119_29	8726	7829	897	0.102796	NP_620564.1	Dicistroviridae	38
k119_27	4250	4148	102	0.024	YP_392501.1	Reoviridae	33
k119_21	3936	3788	148	0.037602	QBA09478.1	Reoviridae	29
k119_25	3620	3521	99	0.027348	APG79179.1	Reoviridae	25
k119_19	3388	3257	131	0.038666	YP_392504.1	Reoviridae	45
k119_14	2163	1985	178	0.082293	YP_392505.1	Reoviridae	22
k119_18	1963	1388	575	0.292919		Reoviridae*	
k119_22	1880	1814	66	0.035106	QYV43128.1	Reoviridae	
k119_20	1865	1739	126	0.06756	AWA82241.1	Reoviridae	24
k119_12	1620	1526	94	0.058025	YP_392507**	Reoviridae	
k119_17	1535	1418	117	0.076221	QBA09478.1	Reoviridae	34
k119_24	1067	107	960	0.899719	XP_017796731.1	Hymenoptera	
k119_30	887	223	664	0.748591		NA	
k119_9	809	743	66	0.081582	YP_009342053.1	Iflaviridae	39
k119_10	782	311	471	0.602302		NA	
k119_16	711	0	711	1		NA	
k119_8	701	611	90	0.128388	YP_009342053.1	Iflaviridae	40
k119_15	571	437	134	0.234676	CAB0037418.1	Hymenoptera	
k119_2	564	389	175	0.310284	XP_015596585.1	NA	
k119_4	560	230	330	0.589286	EZA52908.1	Hymenoptera	
k119_7	451	320	131	0.290466	CAA84692.1	Phage	
k119_13	432	203	229	0.530093	YP_009342053.1	Iflaviridae	
k119_6	412	83	329	0.798544		NA	
k119_5	394	356	38	0.096447	QFP98431.1	Iflaviridae	36
k119_3	375	371	4	0.010667	YP_009342053.1	Iflaviridae	
k119_0	358	143	215	0.600559	ABD33943.1	Picornaviriales	
k119_23	351	86	265	0.754986	AGF91671.1	Phage	
k119_26	306	296	10	0.03268	QGJ03578.1	Phage	
k119_1	218	164	54	0.247706	YP_009342053.1	Iflaviridae	

601 *concluded as part of the genome of AnvRV by the segment screening assay (see table 3)

602 **this hit was found only by using the PSI-BLASTp algorithm

603 **Table 3.** Screening of ten AnvRV segments in 24 individuals from two lines of *A. vladimiri;* 16
604 females from the MR population, and eight from Field population. '0' denotes absence, '+' denotes
605 presence of the viral genomic segment by diagnostic PCR. Empty boxes denote untested samples.

Contig	**Homologous ObIRV segment**	**Mass-reared population**																**Field population**							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	1	2	3	4	5	6	7	8
k119_27	segment1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
k119_21	segment2	+	+	+	0	+	+	+	+	+	+	0	+	+	+	0	0	0	0	0	0	0	0	0	0
k119_25	segment3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
k119_19	segment4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+
k119_14	segment5	+	+	+	0	+	+	+	0	+	+	+	+	0	+	+	+	0	0	0	0	0	0	0	0
k119_20	segment6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
k119_12	segment7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
k119_17	segment8	+	+	+	+	+	0	+	+	+	+	+	+	0	+	+	+	0	0	0	0	0	0	0	0
k119_22	segment10	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
k119_18	Unknown	+	+	+	+	+	+	+	+	+				0	+	+	+	0	0	0	0	0	0	0	0

606

Figure legends

Fig 1. Electrophoresis pattern (using a 0.8% agarose gel) of VNAs extracted from *A. vladimiri*, which were treated by RNase and DNase separately. 'AV' denotes VNAs of *A. vladimiri*. 'SSC' denotes sodium citrate buffer, which was used in 0.01x and 2x ionic strengths. '10'/'0.1' denotes high/low concentration (µg/ml) of RNase respectively. Details by lane numbers: 1-3; no digestion treatment. 1; 1Kb ladder (1KB+, Invitrogen, MA, USA), 2; Phi6 – a ladder made of viral dsRNA (Invitrogen, MA, USA), 3; *A. vladimiri* VNA. 4-11; Digestion by RNase A in four buffer/enzyme concentrations. 4-7; Phi6 ladder as a control for the enzymatic activity of RNase. 8-11; AV digestion. 12-13; Digestion by DNase I, 12; 1Kb – a DNA ladder as a control for the enzymatic activity of DNase. 13; AV digestion.

Fig 2. Virome analysis of *A. vladimiri*. The assembled 30 contigs were assigned to seven taxonomic groups according to the best BLASTx hit. **A)** Average depth per taxonomic group, calculated as: ((sum number of reads*100bp)/sum length of contigs). **B)** Columns represent the size of each of the 30 contigs (bp length). Purple crosses represent the proportion of both 5' and 3' untranslated regions (UTRs) to the contigs' full length, as predicted by the ORF finder. High proportion suggests insufficient protein products, while low proportion suggests a true protein is translated from this contig. See table 2 for more details about each contig.

Fig 3. Genome annotation of the three novel RNA viruses. **A)** AnvDV and AnvIFV. White box indicates location of ORF, colored boxes indicate identified protein domains. Numbers on scale are in bp according to the full contig. RdRP, RNA-dependent RNA polymerase. **B)** Annotation of the segmented AnvRV. The ten segments are drawn to scale, with boxes indicating the ORF location on the full segment, colors represent the proteins encoded from those segments as assigned from homology to known sequences of the ObIRV genome.

Fig 4. Phylogenetic analysis of AnvRV (colored), based on amino acid sequence similarities of the RdRP genome segment (~1,200aa long). Representative viruses of all nine genera in the subfamily Spinareovirinae are shown. The icons of organisms on the tips represent the taxonomic group of the host in which the virus was found. The ● symbol indicate viruses that have not been classified to the genus level. The human Rotavirus B and the bovine Bluetongue virus (Family: *Reoviridae*, subfamily: *Sedoreovirinae*) were used as an outgroup. The tree was inferred by maximum-likelihood using the LG substitution model, the numbers on branches indicate the results of approximate likelihood ratio tests for branch support (aLRT-SH test; Guindon et al. 2010). Scale bar: 0.7 substitutions per site.

Fig 5. Phylogenetic analysis of A. vladimiri iflavirus (AnvIFV) and A. vladimiri dicistrovirus (AnvDV) (both colored), based on amino acid sequence similarities of the RdRP gene (~300aa long). The human poliovirus (order: *Picornavirales*, family: *Picornaviridae*) was used as an out-group. The icons of organisms on the tips represent the taxonomic group of the host in which the virus was found (however, in some cases the viruses can be found in hosts from additional arthro-pod orders, such as Cricket paralysis virus). The 'star of David' symbol signs the virus that is reported to have a mutualistic relationship with its insect host. The phenotype of all other viruses is either unknown or pathogenic. The tree was inferred by maximum-likelihood using the LG

substitution model, the numbers on branches indicate the results of approximate likelihood ratio tests for branch support (aLRT-SH test; Guindon et al. 2010). Scale bar: 0.5 substitutions per site.

Fig 6. The multi-infection status of three lines of *A. vladimiri* individuals by the three RNA viruses and *Wolbachia*. Boxes on the same horizontal axis denote the infection status of the four microbes in the same individual; blue fill=detected, no fill=undetected. Detection of the AnvRV, which has a segmented genome, was done with the primers targeted to the largest segment, encoding for the RdRp gene (see supp. table 1 for all primer pairs used). W+: wasps from the *Wolbachia*-infected line, W-: wasps from *Wolbachia*-uninfected line.

Fig 7. Transmission electron microscope images of AnvRV particles in *A. vladimiri* follicle cells. **A)** a cluster of virus particles (black arrow), **B)** a viroplasm (Vp), **C)** high magnification of a small cluster of virions. N, nucleus; M, mitochondria. Scale bars; A) 500 nm, B) 500nm, C) 100nm.

Data availability

Accession numbers to be received.

Conflict of interest declaration

Author SS was employed by BioBee Sde Eliyahu Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

The authors would like to thank Prof. A. Dombrovsky for useful advice, E. Erel for insect rearing consultancy and Dr. L. Shaulov for preparation and visualization of ovary slides in TEM.

Funding

This research was supported by the ISRAEL SCIENCE FOUNDATION (grant No. 397/21) to E. Z-F and E.C.

Figures

Fig 1

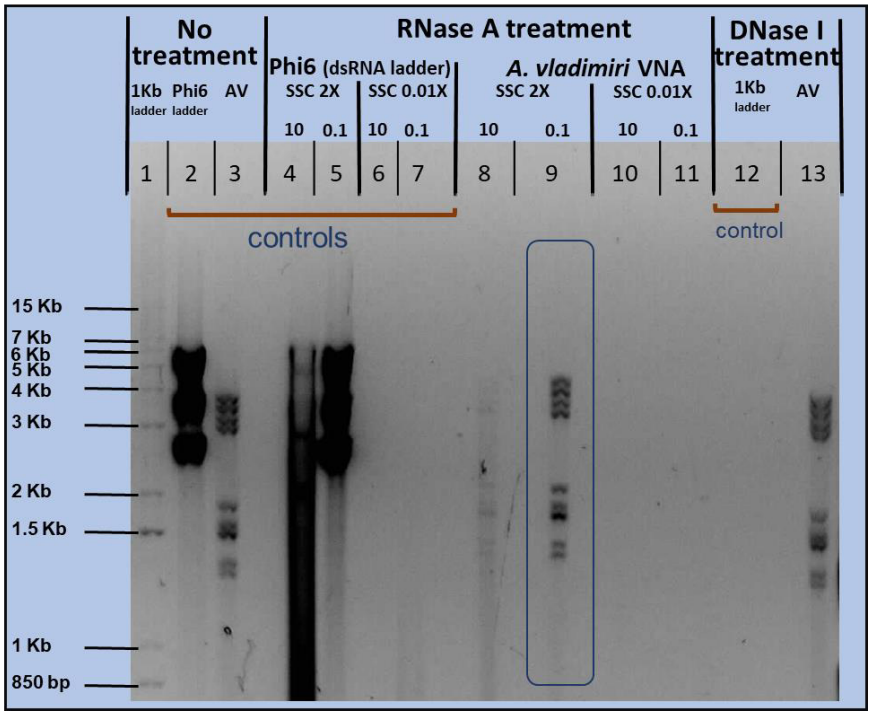

Fig 1. Electrophoresis pattern (using a 0.8% agarose gel) of VNAs extracted from *A. vladimiri*, which were treated by RNase and DNase separately. 'AV' denotes VNAs of *A. vladimiri*. 'SSC' denotes sodium citrate buffer, which was used in 0.01x and 2x ionic strengths. '10'/'0.1' denotes high/low concentration (µg/ml) of RNase respectively. Details by lane numbers: 1-3; no digestion treatment. 1; 1Kb ladder (1KB+, Invitrogen, MA, USA), 2; Phi6 – a ladder made of viral dsRNA (Invitrogen, MA, USA), 3; *A. vladimiri* VNA. 4-11; Digestion by RNase A in four buffer/enzyme concentrations. 4-7; Phi6 ladder as a control for the enzymatic activity of RNase. 8-11; AV digestion. 12-13; Digestion by DNase I, 12; 1Kb – a DNA ladder as a control for the enzymatic activity of DNase. 13; AV digestion.

Fig 2

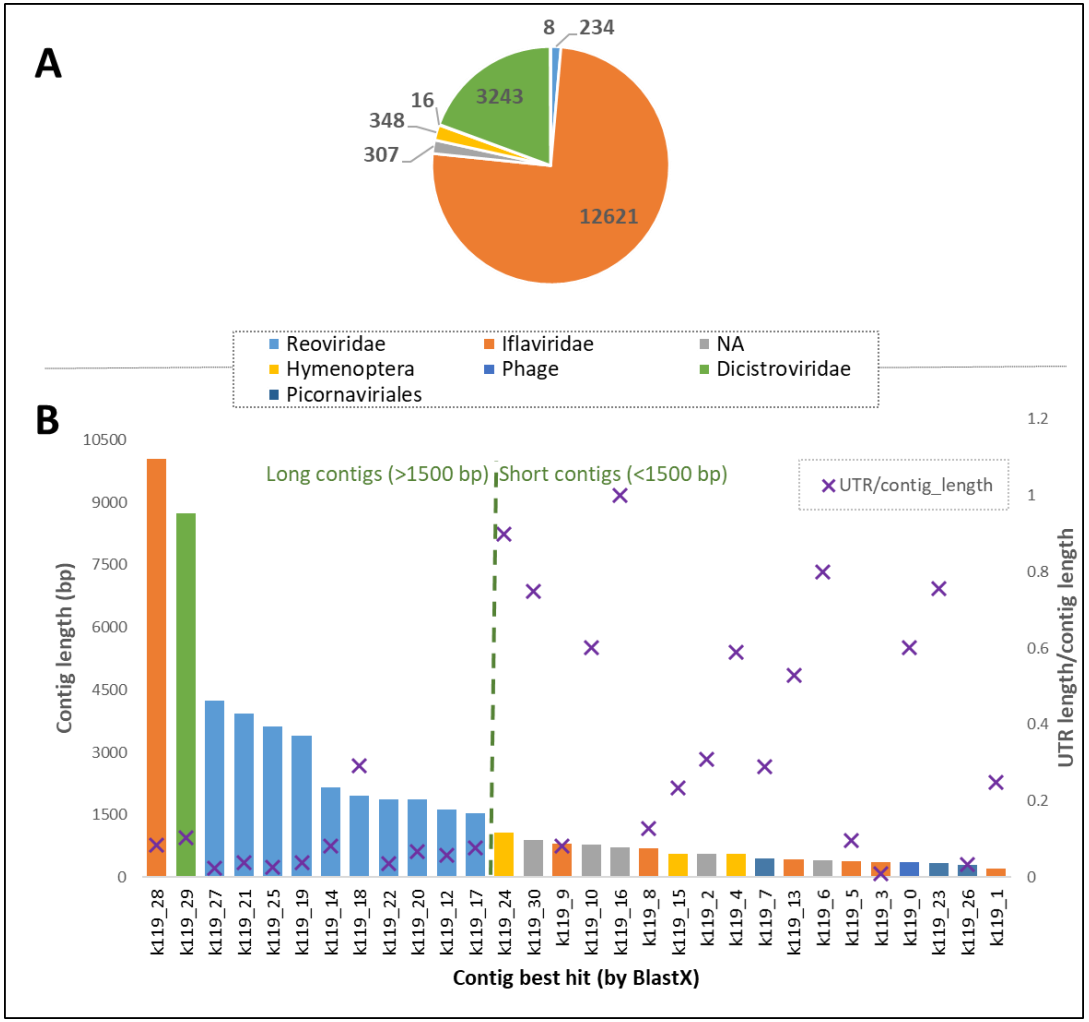

Fig 2. Virome analysis of *A. vladimiri*. The assembled 30 contigs were assigned to seven taxonomic groups according to the best BLASTx hit. **A)** Average depth per taxonomic group, calculated as: ((sum number of reads*100bp)/sum length of contigs). **B)** Columns represent the size of each of the 30 contigs (bp length). Purple crosses represent the proportion of both 5' and 3' untranslated regions (UTRs) to the contigs' full length, as predicted by the ORF finder. High proportion suggests insufficient protein products, while low proportion suggests a true protein is translated from this contig. See table 2 for more details about each contig.

Fig 3

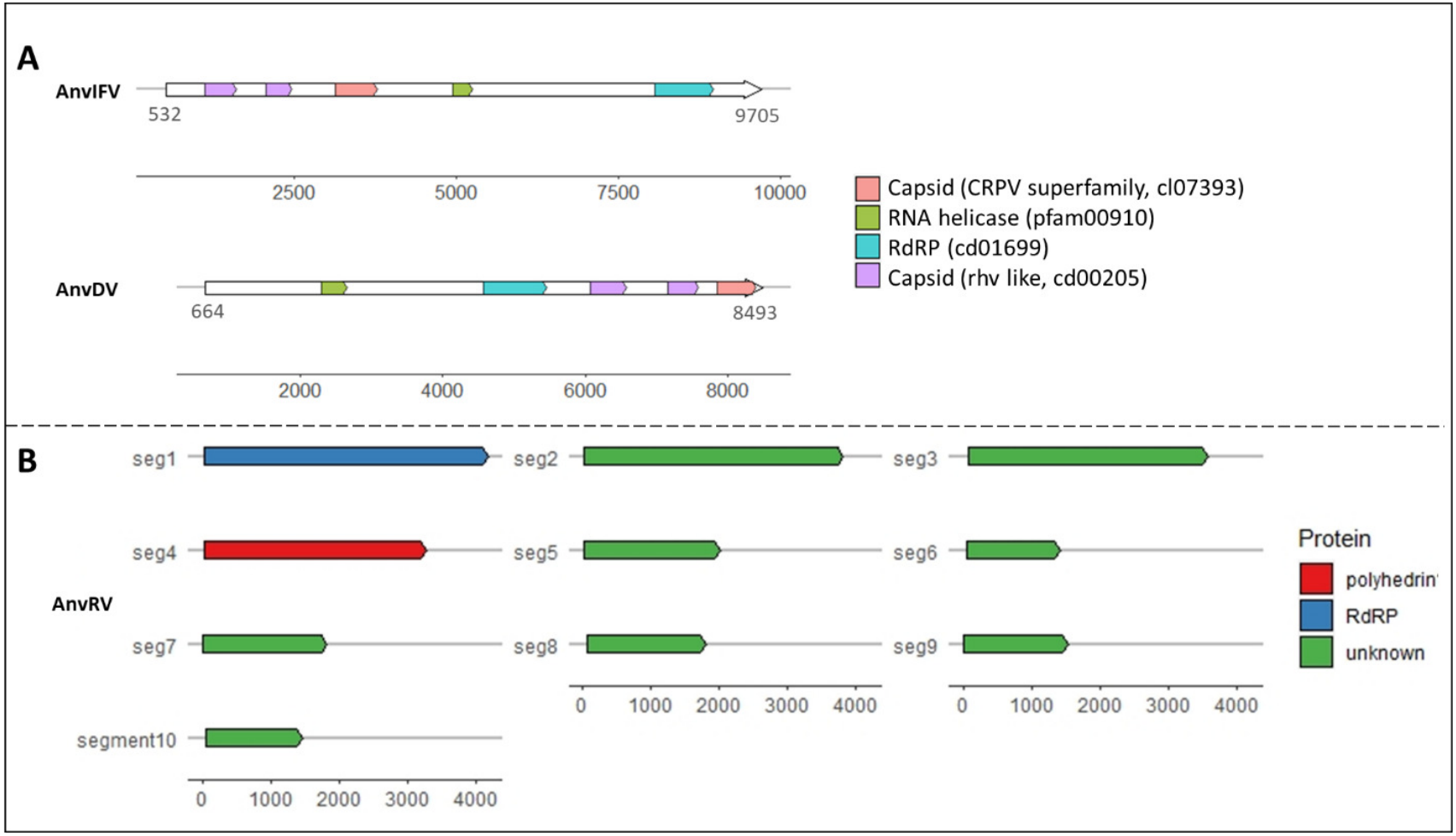

Fig 3. Genome annotation of the three novel RNA viruses. **A)** AnvDV and AnvIFV. White box indicates location of ORF, colored boxes indicate identified protein domains. Numbers on scale are in bp according to the full contig. RdRP, RNA-dependent RNA polymerase. **B)** Annotation of the segmented AnvRV. The ten segments are drawn to scale, with boxes indicating the ORF location on the full segment, colors represent the proteins encoded from those segments as assigned from homology to known sequences of the ObIRV genome.

Fig 4

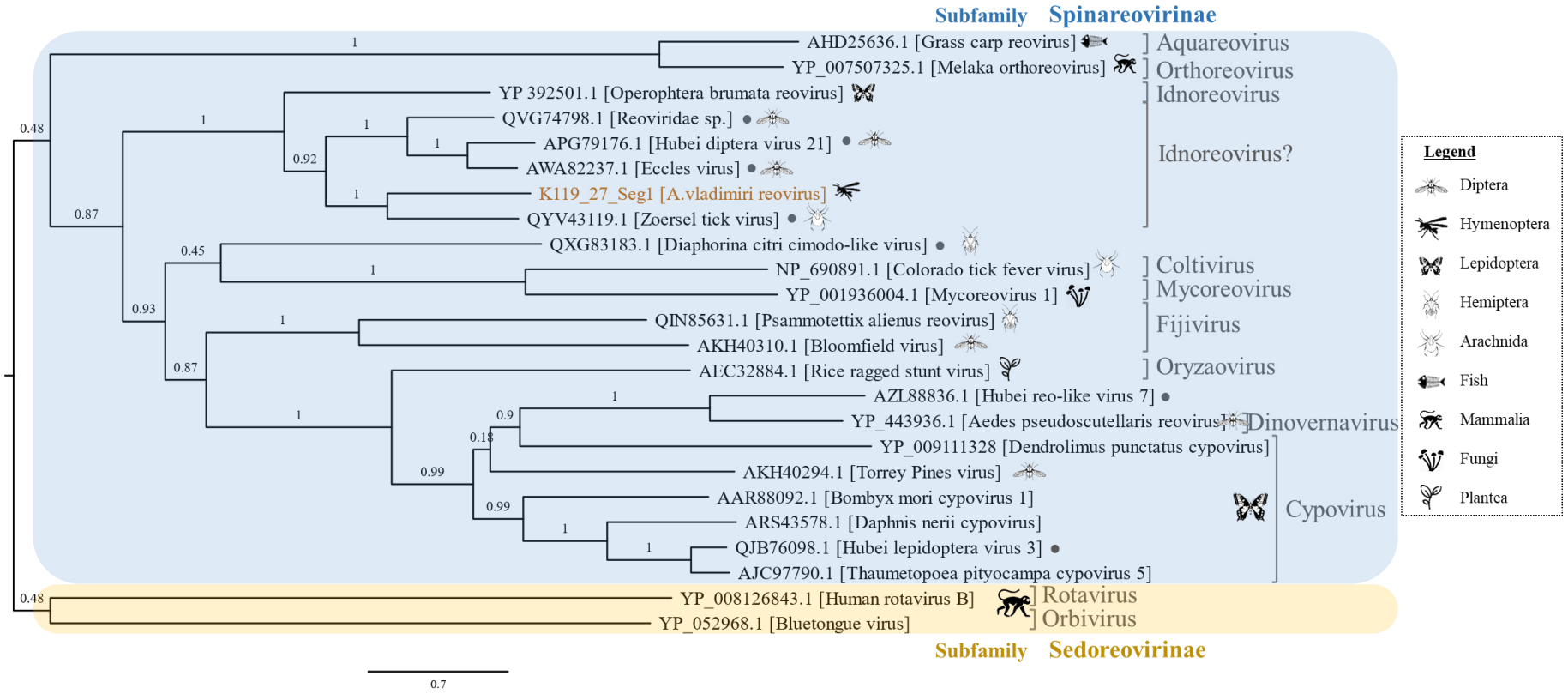

Fig 4. Phylogenetic analysis of AnvRV (colored), based on amino acid sequence similarities of the RdRP genome segment (~1,200aa long). Representative viruses of all nine genera in the subfamily Spinareovirinae are shown. The icons of organisms on the tips represent the taxonomic group of the host in which the virus was found. The ● symbol indicate viruses that have not been classified to the genus level. The human Rotavirus B and the bovine Bluetongue virus (Family: *Reoviridae*, subfamily: *Sedoreovirinae*) were used as an outgroup. The tree was inferred by maximum-likelihood using the LG substitution model, the numbers on branches indicate the results of approximate likelihood ratio tests for branch support (aLRT-SH test; Guindon et al. 2010). Scale bar: 0.7 substitutions per site.

Fig 5

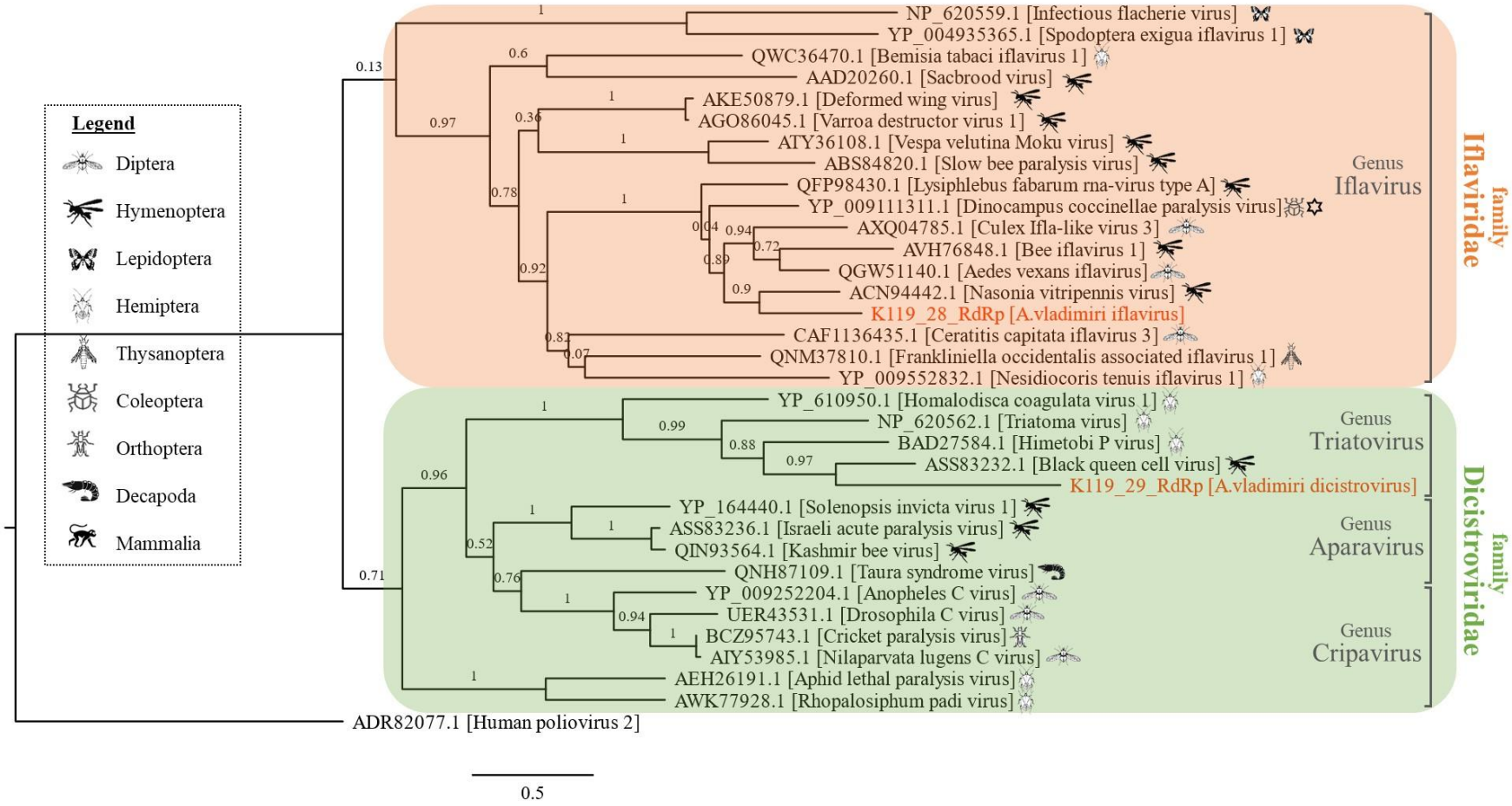

Fig 5. Phylogenetic analysis of A. vladimiri iflavirus (AnvIFV) and A. vladimiri dicistrovirus (AnvDV) (both colored), based on amino acid sequence similarities of the RdRP gene (~300aa long). The human poliovirus (order: *Picornavirales*, family: *Picornaviridae*) was used as an outgroup. The icons of organisms on the tips represent the taxonomic group of the host in which the virus was found (however, in some cases the viruses can be found in hosts from additional arthropod orders, such as Cricket paralysis virus). The 'star of David' symbol signs the virus that is reported to have a mutualistic relationship with its insect host. The phenotype of all other viruses is either unknown or pathogenic. The tree was inferred by maximum-likelihood using the LG substitution model, the numbers on branches indicate the results of approximate likelihood ratio tests for branch support (aLRT-SH test; Guindon et al. 2010). Scale bar: 0.5 substitutions per site.

Fig 6

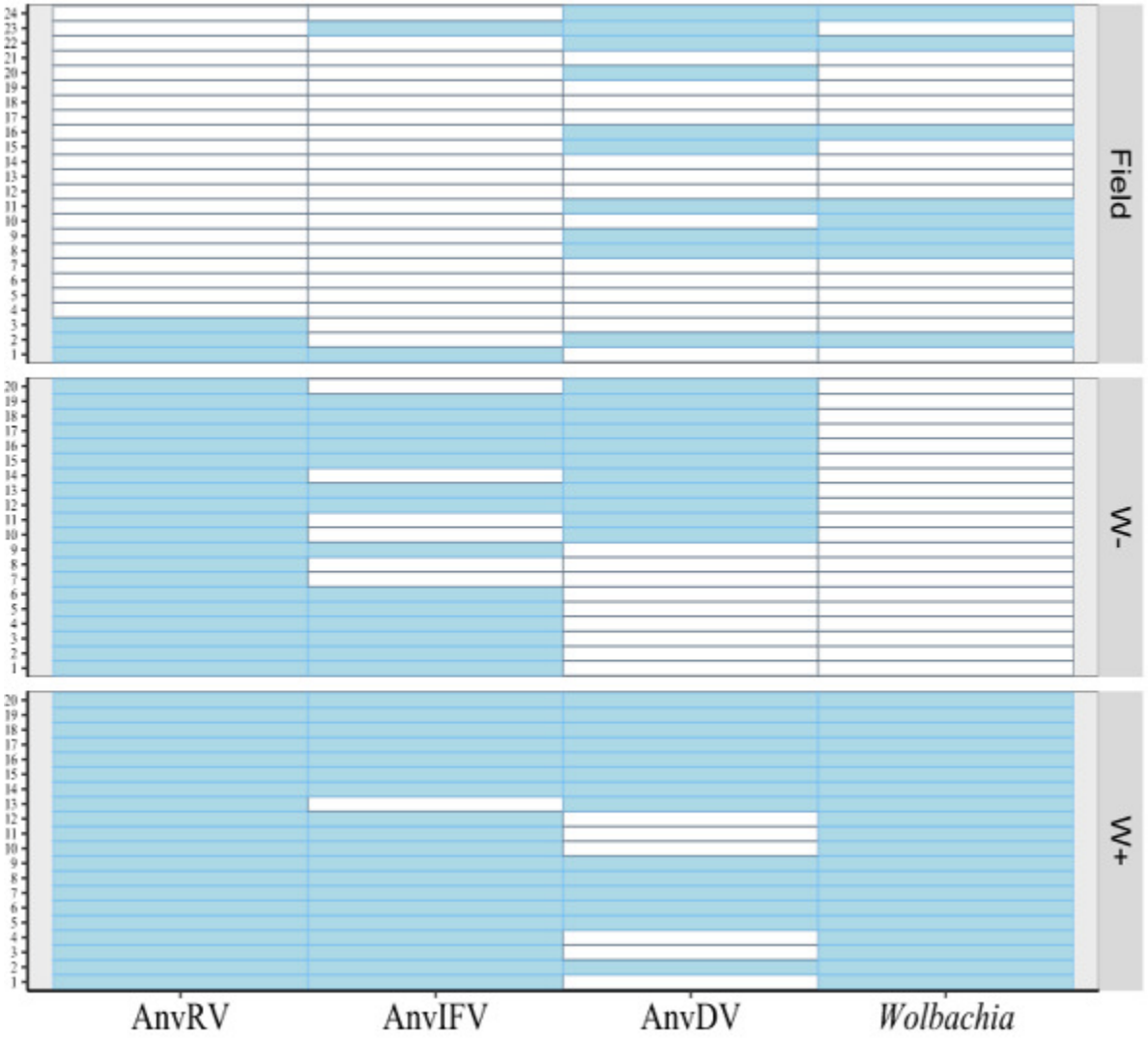

Fig 6. The multi-infection status of three lines of *A. vladimiri* individuals by the three RNA viruses and *Wolbachia*. Boxes on the same horizontal axis denote the infection status of the four microbes in the same individual; blue fill=detected, no fill=undetected. Detection of the AnvRV, which has a segmented genome, was done with the primers targeted to the largest segment, encoding for the RdRp gene (see supp. table 1 for all primer pairs used). W+: wasps from the *Wolbachia*-infected line, W-: wasps from *Wolbachia*-uninfected line.

Fig 7

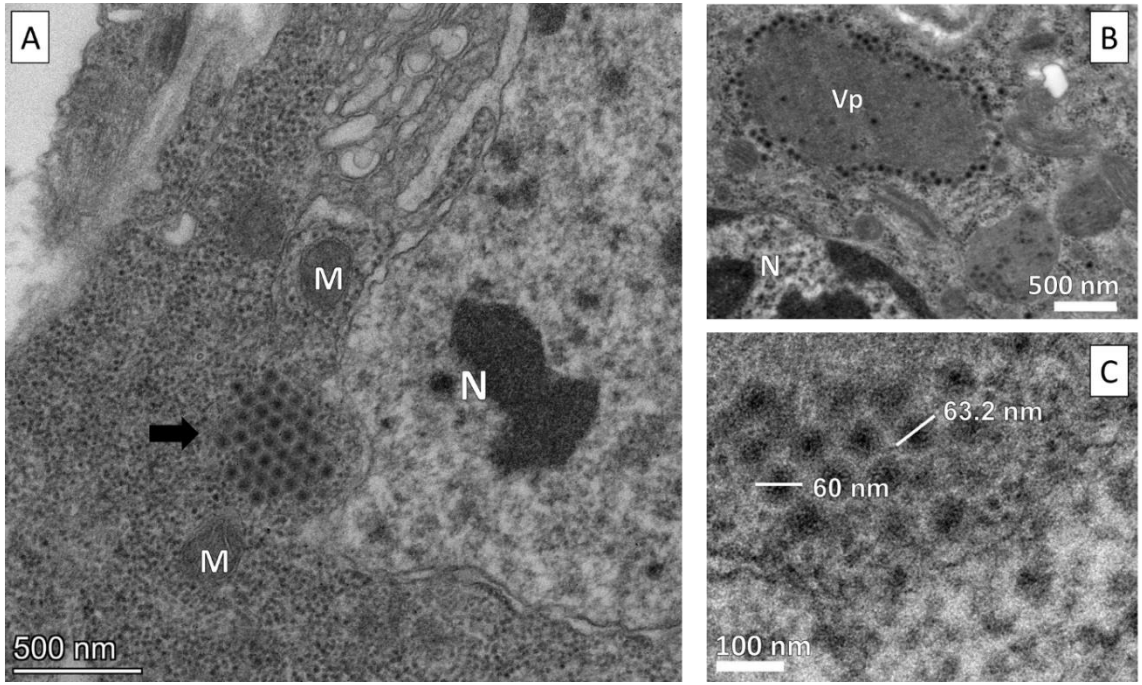

Fig 7. Transmission electron microscope images of AnvRV particles in *A. vladimiri* follicle cells. **A)** a cluster of virus particles (black arrow), **B)** a viroplasm (Vp), **C)** high magnification of a small cluster of virions. N, nucleus; M, mitochondria. Scale bars; A) 500 nm, B) 500nm, C) 100nm.