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# Rich diversity of RNA viruses in the biological control agent, *Orius laevigatus*

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ABSTRACT

*Orius laevigatus* (Hemiptera, Anthocoridae) is a generalist predator extensively used for the biocontrol of diverse agricultural pests. Previous studies on *O. laevigatus* have focused on the improvement of insect genetic traits, but little is known about its association with microbes, especially viruses that may influence its production and efficacy. More than 280 RNA viruses have been described in other Hemiptera insects, in line with the continuous discovery of insect-specific viruses (ISVs) boosted by next-generation sequencing. In this study, we characterized the repertoire of RNA viruses associated with *O. laevigatus*. Its virome comprises 27 RNA viruses, classified within fourteen viral families, of which twenty-three viruses are specific to *O. laevigatus* and four are likely associated with fungal microbiota. The analysis of viral abundance in five *O. laevigatus* populations confirmed the presence of simultaneous viral infections and highlighted the ubiquitous presence and high abundance of one solinvivirus and three totiviruses. Moreover, we identified 24 non-retroviral endogenous viral elements (nrEVEs) in the genome of *O. laevigatus,* suggesting a long-term relationship between the host and its virome. Although no symptoms were described in the insect populations under study, the high diversity of viral species and the high abundance of certain RNA viruses identified indicate that RNA viruses may be significant for the applicability and efficacy of *O. laevigatus* in biocontrol programs.

## 1. Introduction

Viruses are obligate intracellular pathogens infecting a wide range of organisms, including insects. However, the study of the interactions between these two organisms has been mainly limited to arboviruses, which are transmitted by arthropod vectors and can cause human diseases (Young, 2018), viruses with clear symptomatology affecting insects with economic relevance as honeybees (Tantillo et al., 2015), and viruses with biocontrol application as baculoviruses (Lacey et al., 2015). Only recently, the use of high-throughput sequencing has expanded considerably the number of viruses that infect and replicate in insect cells, which are termed insect-specific viruses (ISVs) (Haoming et al., 2021; Käfer et al., 2019; Shi et al., 2016). Although some ISVs correlate with reduced insect fitness (Armién et al., 2023; Hernández-Pelegrín et al., 2023a; Yuan et al., 2020), most ISVs cause covert infections without obvious lethal symptoms for the host (Ryabov, 2017).

During the virus-host interaction, fragments of viral sequences can be integrated into the genome of the host forming non-retroviral endogenous viral elements (nrEVEs) (Bonning & Saleh, 2021). nrEVEs represent a record of past viral infections and reflect the evolutionary relationship between RNA viruses and their hosts (Harding et al., 2021). Moreover, few studies in *Aedes* mosquitoes have unravelled the antiviral role of certain nrEVEs, which is mediated by the Piwi-interacting RNA pathway (Blair, 2019; Suzuki et al., 2020; Tassetto et al., 2019). nrEVEs have been extensively studied in insects of health relevance as disease vector mosquitoes or insect pests (Crava et al., 2021; Hernández-Pelegrín et al., 2023b; Palatini et al., 2017; Pischedda et al., 2019; Russo et al., 2019; Whitfield et al., 2017). Instead, the presence and distribution of nrEVEs in the genomes of insect species reared for biocontrol purposes remains unexplored.

*Orius laevigatus* (Hemiptera; Anthocoridae), commonly known as the minute pirate bug, is a predatory insect of the order Hemiptera. It has acquired a high economic relevance as a biocontrol agent since it possesses a notably polyphagous diet, preying upon various insect taxa. In

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fact, *O. laevigatus* is commercialized for the biocontrol of agricultural pests, including thrips, aphids, mites, and small caterpillars. In this context, the polyphagous nature of *O. laevigatus* facilitates its exposure to RNA viruses, increasing the chances of host shift and the number of viruses associated with the species. To date, although RNA viruses have been previously described in other hemipteran species (Martinez-Mercado et al., 2022; Wang et al., 2016; Wu et al., 2018), the repertoire of RNA viruses associated with *O. laevigatus* remains unknown.

In this manuscript, we explored the current viral diversity found in *O. laevigatus* with different genetic backgrounds, and we identified traces of previous viral infections by studying the nrEVEs contained in the *O. laevigatus* genome. Overall, our results shed light on the past and present interactions between RNA viruses and *O. laevigatus* and discuss the potential of RNA viruses for the improvement of biocontrol strategies.

## 2. Materials and methods

### 2.1. Orius laevigatus populations

Five populations of *O. laevigatus* were employed for viral discovery: Agrobio (AB), Malaga (MA), Mix (MIX), Pamplona-Oviedo (PA-OV) and Polen 2 (*P*2). These laboratory populations originate from insects collected in diverse regions of Spain and were reared in the facilities of the Biocontrol Selection Lab of the Universidad Politécnica de Cartagena (Spain) under fixed conditions of temperature (26 °C), humidity (70 %) and light/dark cycles (16:8 L:D). Eggs of *Ephestia kuehniella* were provided as the diet for AB, MA, MIX and PA-OV while pollen was provided as diet for *P*2. The eggs of *E. kuehiniella* were provided by the biocontrol company Agrobio. All the *O. laevigatus* populations consumed the same mixture of eggs (obtained from the same *E. kuehniella* females). On the other hand, the pollen was commercial honeybee pollen (Hijas del Sol®). Agrobio strain (AB) is a standard commercial population of the company of the same name. Malaga strain (MA) derives from insects collected near to Malaga (southern Spain) in 2017. Mix strain (MIX) originates from the mixture of twenty-five wild populations collected around the Mediterranean area (see Balanza et al., 2019 for details) between 2012 and 2015. Pamplona-Oviedo strain (PA-OV) is a mixture of two wild populations collected in Pamplona and Oviedo (northern Spain) in 2021. Polen 2 (*P*2) is a strain artificially selected for many generations for a better fitness when feeding on a suboptimal food like pollen (Mendoza et al., 2022). All the strains were reared in the laboratory for several generations prior this study and no individuals from wild populations were re-introduced.

### 2.2. RNA sequencing and small RNA sequencing

Total RNA was extracted from pools of 20 adult individuals (10 males, 10 females) using TRIZOL reagent (TRItidy G, ITW Reagents), following manufacturer protocol. Library preparation was performed using the Truseq Total RNA library kit with Ribo-Zero for ribosomal RNA (rRNA) depletion (Illumina, San Diego, CA, USA). RNA sequencing was performed by NovaSeq 6000 platform rendering paired-end reads and a sequencing depth of 40 million reads per sample. All library construction and sequencing services were carried out by Macrogen (Seoul, Korea). Sequencing results are deposited in the Sequence Read Archive (SRA) of the National Centre of Biotechnology Information (NCBI) under the BioProject ID: PRJNA1116979 which contains the RNA data of the Agrobio (SAMN41553685), Pamplona-Oviedo (SAMN41553686), Málaga (SAMN41553687), Polen2 (SAMN41553688), and Mix (SAMN41553689) samples.

### 2.3. Viral discovery using de novo-assembled transcriptomic data

To describe the *O. laevigatus* RNA virome, we analysed the presence of RNA viruses in 5 *de novo* assembled transcriptomes individually obtained from AB, MA, MIX, PA-OV and *P*2 laboratory populations. Initially, the quality of the raw reads resulting from RNA sequencing was analysed using fastQC (https://www.bioinformatics.babra-ham.ac.uk/projects/fastqc/). The adaptors and low-quality sequences were then trimmed using Trimmomatic (Bolger et al., 2014), and the remaining reads were *de novo* assembled using Trinity v2.9.0 (Haas et al., 2013). Default parameters were used when no additional information is provided.

To detect the contigs of each *O. laevigatus* strain corresponding to RNA virus, a viral query was constructed including the protein sequences deposited in GenBank classified within the Riboviria domain (RNA viruses) and having invertebrate hosts. Then, the viral query was mapped against *O. laevigatus* contigs using tblastn (protein query against translated nucleotide database) with a threshold value of $1 \times 10^{-5}$. The putative viral contigs resulting from the analysis were manually filtered to remove (a) contigs smaller than 1000 bp in length, (b) contigs mapping to the reference genome of *O. laevigatus* after blastn with an e-value cut off of $1 \times 10^{-100}$, and (c) the contigs without open reading frames (ORFs) after evaluation with ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). The contigs that passed all these filters were considered as candidate RNA viruses associated with *O. laevigatus*.

### 2.4. Annotation and phylogenetic classification of the RNA viruses found in O. laevigatus

To study the relationships between newly discovered viruses and viruses present in public databases, we first performed blastx searches using the candidate *O. leavigatus* viruses as a query and the non-redundant protein sequences (nr) deposited on NCBI as database. The open reading frames contained in each virus were annotated with ORFfinder. The function of the proteins encoded by each open reading frame was characterized using a blastp search against the nr protein database on NCBI (Table S1).

Subsequently, to establish the phylogenetic relationships of each *O. leavigatus* virus, its RNA-dependent RNA polymerase (RdRp) protein sequence and those from its closest relatives were aligned using *T*-coffee mode "M−coffee" (Wallace et al., 2006) (Table S2). The alignment results were manually trimmed with BioEdit to eliminate the sequences that poorly aligned at the 5́ and 3́ ends. The curated alignments were loaded into IQtree2, which inferred the phylogenetic trees by applying the maximum-likelihood method, with default parameters, and 1000 ultrafast bootstrap (Minh et al., 2020). The results were visualized using iTOL (Letunic & Bork, 2021).

### 2.5. Characterization of nrEVEs repertoire

The reference genome of *O. laevigatus* was employed for nrEVEs characterization. The *O. laevigatus* reference genome was constructed using a pool of insects collected in the United Kingdom and is deposited in GenBank under the accession: GCA_018703685.1. nrEVEs were identified using a blastx search against the same protein database employed for viral discovery, with an e-value cut-off of $10^{-6}$. Then, putative nrEVEs were mapped to the NCBI non redundant (nr) protein database using blastx search with an e-value cut-off of $10^{-4}$ (Whitfield et al., 2017). nrEVEs sequences showing high similarity to eukaryotic proteins, retroviruses or transposable elements were discarded. For those nrEVEs that the second blast search confirmed its highest similarity to viruses, the top viral hit was considered for taxonomic classification. Finally, nucleotide similarity between nrEVEs and circulating viruses was determined by blastn comparisons with default parameters.

### 2.6. Detection and quantification of RNA viruses and nrEVEs in different O. laevigatus populations

The relative abundance of the RNA viruses and the transcripts produced by the nrEVEs discovered in *O. laevigatus* was quantified using AB,

MA, MIX, PA-OV, and *P*2 RNA sequencing reads. These were mapped against the viral genomic sequences and the nrEVE sequences using Bowtie 2 v 2.3.5.1 (Langmead & Salzberg, 2012). Viral abundance and nrEVEs transcriptional levels were calculated using RSEM v 1.3.1 (Li & Dewey, 2011) with default parameters. The total number of reads mapping to each RNA virus or nrEVE transcript were normalized against the reads mapping to the cytochrome *c* oxidase subunit I (COX1) (accession number: OL753565) and reported in logarithmic scale. Results of viral abundance and nrEVE transcriptional levels in the different populations were visualized using the heatmap.2 function in the gplots v 3.1.1 package within the R software suite (Warnes et al., 2009). For RNA viruses, the fragments per kilobase of transcript per million fragments mapped (FPKM) were also calculated using RSEM v 1.3.1 (Li & Dewey, 2011).

## 3. Results

### 3.1. Twenty-seven putative RNA viruses are associated with Orius laevigatus

Sequences representing twenty-seven new RNA viruses classified within fourteen viral families were retrieved in the transcriptomes from the hemipteran *O. laevigatus* (Table 1). Positive single strand RNA viruses (+ssRNA) were the most abundant, with fourteen different viruses and nine viral families represented: *Nidoviridae* (n = 1), *Virgaviridae* (n = 2), *Picornaviridae* (n = 1), *Solinviviridae* (n = 1), Negevirus (n = 1), *Dicistroviridae* (n = 3), *Tombusviridae* (n = 1), *Narnaviridae* (n = 2) and *Ourmiaviridae* (n = 2). For negative single strand RNA viruses (−ssRNA), five viral genomes were discovered and classified within the *Rhabdoviridae* (n = 3) and *Bunyaviridae* (n = 2) families. Finally, eight viruses with double strand RNA genomes were classified within the *Totiviridae* (n = 4), *Reoviridae* (n = 3) and *Partitiviridae* (n = 1) families (Fig. 1, Table 1).

Eighteen of the viruses retrieved in *O. laevigatus* belonged to viral families containing monopartite genomes. These viruses presented complete open reading frames and displayed genome lengths similar to other viruses previously described for each viral family (Fig. 1, Table S1). Between the four viruses classified as *Totiviridae*, three presented the classic genomic organization with two overlapping ORFs (OleTV1, OlaeTV2, and OlaeTV3). Instead, the fourth virus presented a single ORF and was defined as toti-like virus (OlaeTLV1) (Table S1). The remaining nine viruses belonged to viral families containing multipartite genomes. Members of the *Bunyaviridae* family commonly have bi or tri-partite genomes consisting of RdRp (L), glycoprotein (M), and nucleocapsid (S). In *O. laevigatus,* one segment (L) was retrieved for OlaeBuV1 and two segments (L and M) for OlaeBuV2 (Fig. 1). Similarly, ourmiaviruses typically contain tri-partite genomes. In *O. laevigatus,* only the longest segment containing the RdRp function was retrieved for OlaeOuLV1 and OlaeOuLV2 (Fig. 1). Members of the *Partitiviridae* and *Tombusviridae* families contain bi-partite genomes encoding for RdRp and structural proteins, respectively. In *O. laevigatus,* both segments were identified for OlaeToLV1 while only the RdRp segment was

**Table 1**
List of the 27 RNA viruses described in *Orius laevigatus* clustered according to their genome and their viral classification.

| Type[1] | Virus classification[2] | Virus name & abbreviation[3] | | GenBank accession n° | Length (nt) |
|---|---|---|---|---|---|
| (−) ssRNA | *Rhabdoviridae* (F) | Orius laevigatus rhabdovirus 1 | OlaeRhV1 | PP908635 | 13,556 |
| | | Orius laevigatus rhabdovirus 2 | OlaeRhV2 | PP908636 | 10,996 |
| | | Orius laevigatus rhabdovirus 3 | OlaeRhV3 | PP908637 | 10,823 |
| | *Bunyaviridae* (F) | Orius laevigatus bunyavirus 1 | OlaeBuV1 | PP908621 | 8726 |
| | | Orius laevigatus bunyavirus 2 | OlaeBuV2 | PP908622 | 7203 |
| | | | | PP908623 | 3380 |
| (+) ssRNA | *Nidoviridae* (F) | Orius laevigatus *nido*-like virus 1 | OlaeNiLV1 | PP908630 | 16,758 |
| | *Virgaviridae* (F) | Orius laevigatus virga-like virus 1 | OlaeViLV1 | PP908656 | 11,505 |
| | | Orius laevigatus virga-like virus 2 | OlaeViLV2 | PP908657 | 11,314 |
| | *Picornaviridae* (F) | Orius laevigatus picorna-like virus 1 | OlaePiLV1 | PP908634 | 10,388 |
| | *Solinviviridae* (F) | Orius laevigatus solinvi-like virus 1 | OlaeSoLV1 | PP908649 | 10,006 |
| | Negevirus (T) | Orius laevigatus negev-like virus 1 | OlaeNeLV1 | PP908629 | 9763 |
| | *Dicistroviridae* (F) | Orius laevigatus dicistrovirus 1 | OlaeDiV1 | PP908624 | 9681 |
| | | Orius laevigatus dicistrovirus 2 | OlaeDiV2 | PP908625 | 8756 |
| | | Orius laevigatus dicistrovirus 3 | OlaeDiV3 | PP908626 | 7349 |
| | *Tombusviridae* (F) | Orius laevigatus tombus-like virus 1 | OlaeToLV1 | PP908651 | 2558 |
| | | | | PP908652 | 2095 |
| | *Narnaviridae* (F) | Orius laevigatus narnavirus 1 | OlaeNaV1 | PP908627 | 2798 |
| | | Orius laevigatus-associated narnavirus 2 | OlaeANaV2 | PP908628 | 2486 |
| | *Ourmiaviridae* (F) | Orius laevigatus-associated ourmiavirus 1 | OlaeAOuV1 | PP908631 | 2636 |
| | | Orius laevigatus-associated ourmiavirus 2 | OlaeAOuV2 | PP908632 | 2465 |
| dsRNA | *Totiviridae* (F) | Orius laevigatus totivirus 1 | OlaeTV1 | PP908653 | 7697 |
| | | Orius laevigatus totivirus 2 | OlaeTV2 | PP908654 | 6542 |
| | | Orius laevigatus totivirus 3 | OlaeTV3 | PP908655 | 5879 |
| | | Orius laevigatus toti-like virus 1 | OlaeTLV1 | PP908650 | 7617 |
| | *Reoviridae* (F) | Orius laevigatus reovirus 1 | OlaeRV1 | PP908638 | 4293 |
| | | | | PP908639 | 3893 |
| | | | | PP908640 | 3936 |
| | | | | PP908641 | 3632 |
| | | | | PP908642 | 2805 |
| | | | | PP908643 | 2452 |
| | | Orius laevigatus reovirus 2 | OlaeRV2 | PP908644 | 4083 |
| | | | | PP908645 | 4031 |
| | | | | PP908646 | 3485 |
| | | | | PP908647 | 2198 |
| | | Orius laevigatus reovirus 3 | OlaeRV3 | PP908648 | 2239 |
| | *Partitiviridae* (F) | Orius laevigatus-associated partitivirus 1 | OlaeAPaV1 | PP908633 | 2276 |

[1] negative single strand RNA (−ssRNA), positive single strand RNA (+ssRNA) or double strand RNA (dsRNA).
[2] F=family, T=taxon.
[3] Viral names were defined as: name of the host, plus the root of the viral family, plus virus, plus an identification number (ordered from longest to shortest nucleotide sequence).

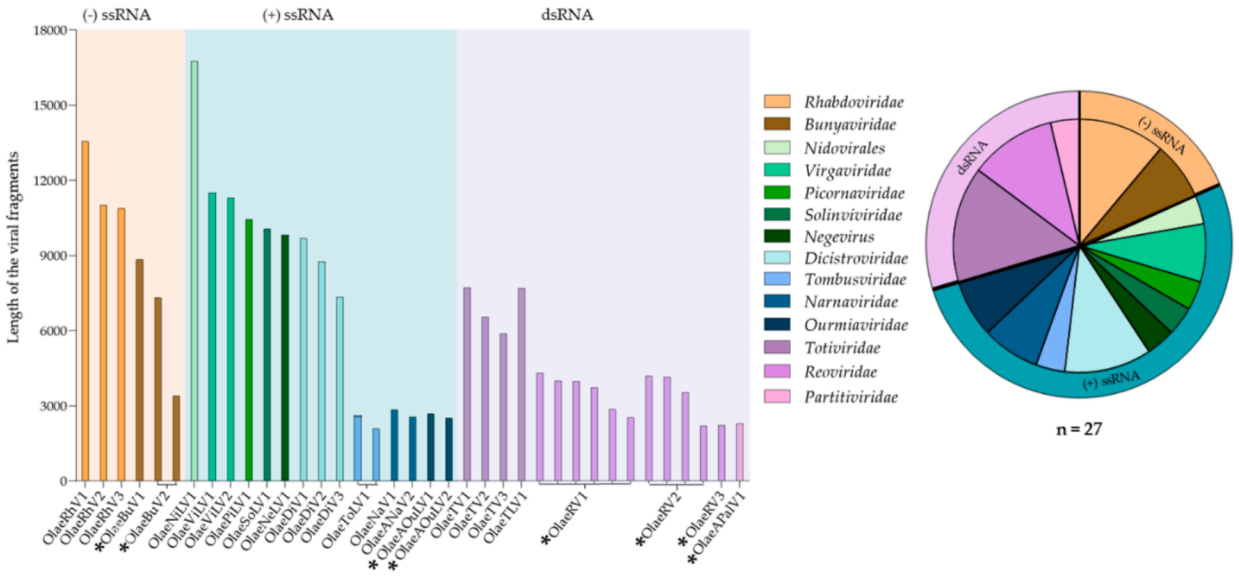


**Fig. 1.** Viral sequences retrieved in the transcriptomes of *O. laevigatus* and classified according to their length (left panel) and viral family (right panel). The symbol (*) on the viral names identifies those viruses which may be missing additional viral segments according to the genome size of closely related viral species.

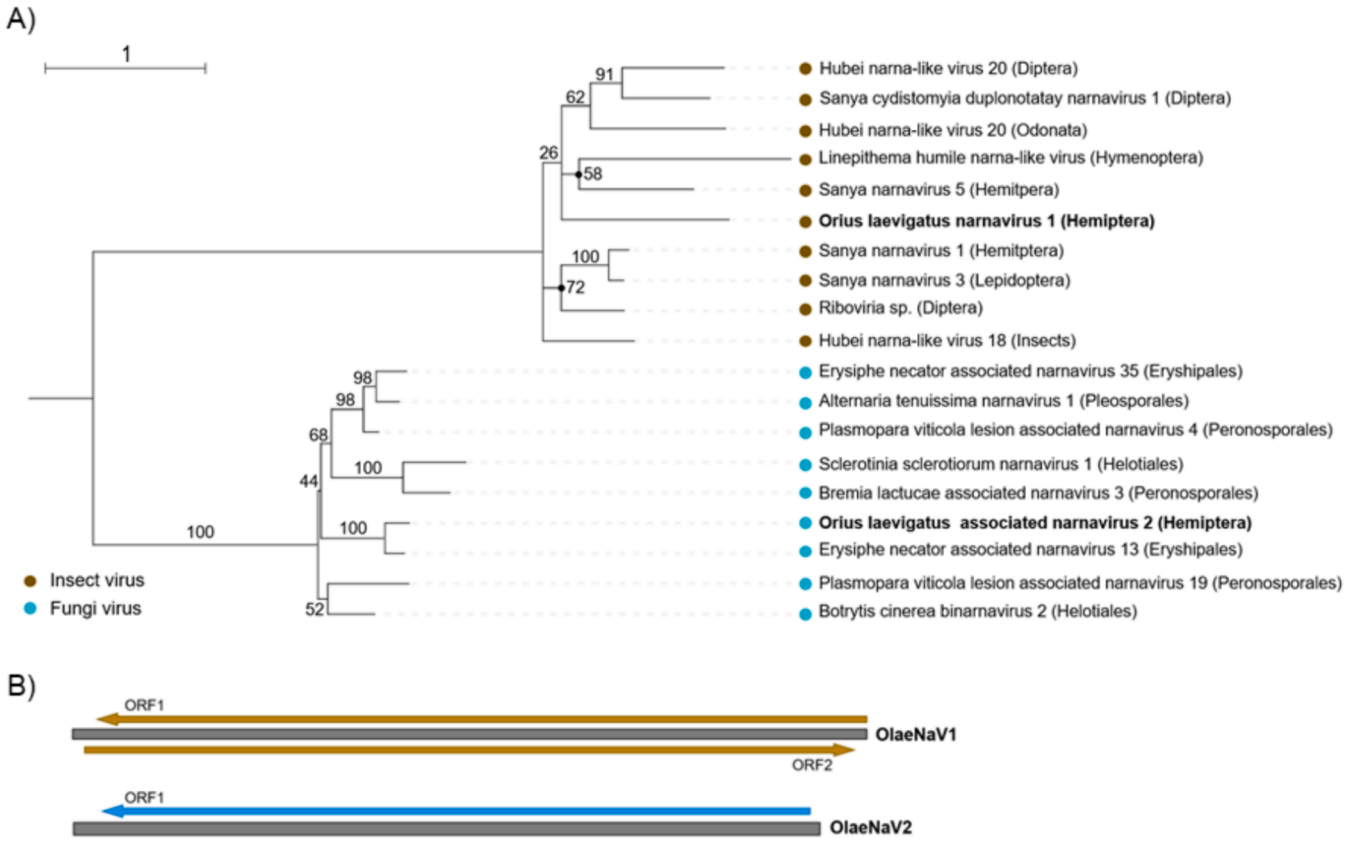


**Fig. 2.** Phylogenetic classification (A) and genome structure (B) of *Narnaviridae* members infecting *O. laevigatus*. Viral sequences were analysed at the protein level and the closest viral relatives were selected using Blastp (Table S2). Information about the main host of the viral sequences included in the analysis is shown in the coloured dots (see the legend). Results of the 1000 ultrafast bootstrap analysis performed using IQtree2 are included in the tree branches.

identified for OlaePaLV1 (Fig. 1). The *Reoviridae* family typically contains from 9 to 12 genome segments. In our analysis we retrieved eleven sequences classified as reovirus-like. They were finally grouped into three different reovirus genomes according to sequence similarity to other reoviruses (Table 1) and viral expression profiles (see below). Of these, six fragments were assigned to OlaeRV1, four to OlaeRV2 and only the RdRp segment to OlaeRV3 (Fig. 1).

### 3.2. Phylogenetic analysis revealed the primary hosts for the RNA viruses

To unravel whether *O. laevigatus* is the actual host of the newly described viruses, we performed a phylogenetic classification including

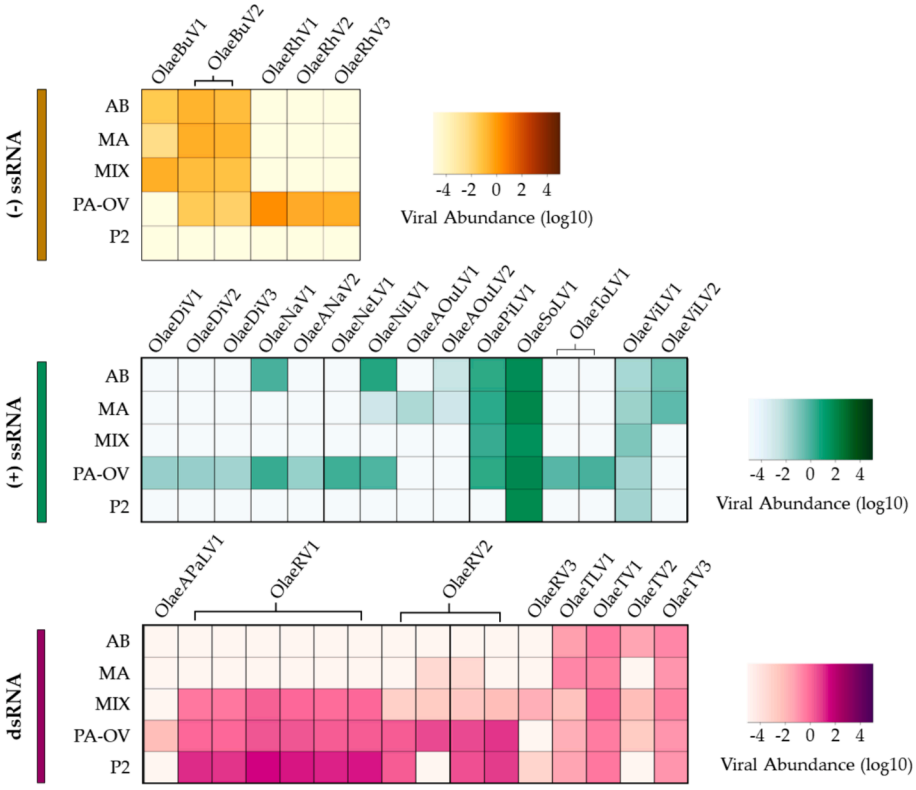


**Fig. 3.** Heatmaps showing the viral abundance of *O. laevigatus* RNA viruses in the 5 transcriptomic datasets. Viral relative abundance was calculated using the total counts of each virus and normalized to the COX-I mitochondrial gene counts.

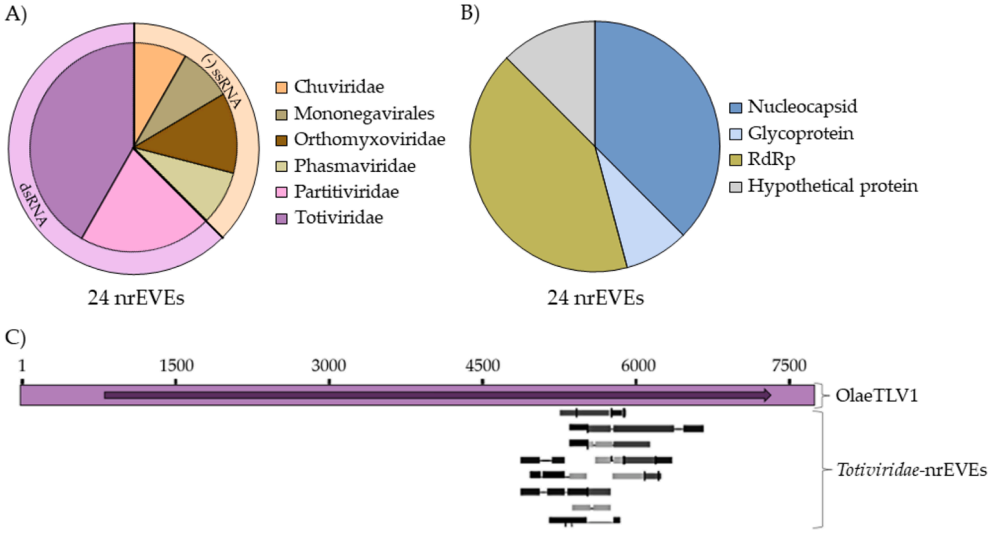


**Fig. 4.** Classification of nrEVEs according to their viral family (A) and viral function (B) and sequence similarity at protein level between *Totiviridae*-derived nrEVEs and OlaeTLV1 (C). Dark purple arrow represents the open reading frame of OlaeTLV1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*O. laevigatus* viruses and their closest viral relatives (Table S2). The phylogenetic analyses classified 23 out of the 27 identified RNA viruses together with previously described ISVs (Figure S1). The remaining four viruses were defined as simply 'associated' with *O. laevigatus*, since these viruses likely have a different host although they were identified in *O. laevigatus* transcriptomes. This is the case of Orius laevigatus-associated partiti-like virus 1 (OlaeAPaLV1), which phylogenetically classified with putative mycoviruses (Fig. S1E), despite the infection with partiti-like viruses has also been described in insects (Fig. S1E). Similarly, Orius laevigatus-associated ourmia-like virus 1 (OlaeAOuV1) and Orius laevigatus-associated ourmia-like virus 2 (OlaeAOuV2) clustered with putative mycoviruses (Fig. S1D), which aligns with previous reports describing members of the *Ourmiaviridae* family as infecting plant and fungal hosts. Regarding the *Narnaviridae* family, the phylogenetic classification revealed a close relation between Orius laevigatus narnavirus 1 (OlaeNaV1) and ISVs, and between Orius laevigatus-associated narnavirus 2 (OlaeANaV2) and mycoviruses (Fig. 2A). Interestingly, the analysis of their viral structure showed that insect-

specific narnaviruses including OlaeNaV1 contained an ambisense genome with two overlapping ORF, while putative mycovirus OlaeANaV2 contained one single ORF (Fig. 2B).

### 3.3. Viral abundance analysis evidenced the presence of simultaneous infections in O. laevigatus populations

The abundance of the 27 RNA viruses characterized in *O. laevigatus* was investigated in five *O. laevigatus* populations (Fig. 3). On average, 15 % (±5.9) of the total sequencing reads mapped to the 27 RNA viruses. Polen2 population presented the highest proportion of viral sequences (24 %) although it contained the lowest viral diversity with 8 different viral species identified (Fig. 3). Instead, the highest viral diversity was observed in the combination of Pamplona and Oviedo populations, which presented 21 different RNA viruses, including 8 RNA viruses exclusively identified in this dataset (Fig. 3).

OlaeSoV1, OlaeTLV1, OlaeTV1, and OlaeTV3 were retrieved in all the analysed datasets, suggesting that these viruses may constitute the core RNA virome of *O. laevigatus* (Fig. 3). Between them, OlaeSoV1 presented the highest viral abundance, representing on average a 10 % of the total RNA sequencing reads. Instead, the low prevalence and abundance assessed for OlaANaV2, OlaeAOuV1, OlaeAOuV2 and OlaeAPaLV1, which were phylogenetically related to mycoviruses (Fig. 2, Figure S1), reinforces the idea that these viruses do not infect *O. laevigatus* as their main host (Fig. 3). In this context, the identification of these viruses may be a consequence of the presence of fungi, which range between 0,02 % and 1,7% of the total reads in the *O. laevigatus* samples. In line with this hypothesis, the percentage of reads mapping those hypothetical fungal viruses is lower than 0.005 %.

Regarding multipartite viruses, the viral RNA profiles observed in the different *O. laevigatus* populations confirmed the proper characterization of multipartite viruses. For instance, the two segments for the OlaeBuV1 and OlaeToLV1 and the six segments for OlaeRV1 were always detected together in *O. laevigatus* samples (Fig. 3). The four viral segments described for OlaeRV2 were detected in the MIX and PA-OV populations while only three and two OlaeRV2 segments were detected in *P*2 and MA populations, respectively (Fig. 3).

### 3.4. The presence of nrEVEs reflects the evolutionary relationship between RNA viruses and O. laevigatus

A total of 24 nrEVEs were annotated in the reference genome of *O. laevigatus* (Fig. 4, Table S3). The majority of nrEVEs derived from the *Totiviridae* family (n = 10) in correlation with the high number of totiviruses identified in *O. laevigatus* populations. These nrEVEs displayed sequence similarity to the viral RNA-dependent RNA polymerase (RdRp), and presented incomplete ORFs, or ORFs with smaller length than the original RdRp gene. nrEVEs deriving from the *Paritiviridae* (n = 5) family of dsRNA viruses were also identified. Differently from *Totiviridae*-derived nrEVEs, *Partitiviridae* nrEVEs mainly derived from viral nucleocapsid or hypothetical proteins. In addition, among negative ssRNA viruses, nine nrEVEs were classified within the Mononegavirales order (n = 2), and the *Chuviridae* (n = 2), the *Orthomyxoviridae* (n = 3), and the *Phasmaviridae* (n = 2) families (Fig. 4A). These nrEVEs originated from structural viral proteins as the nucleocapsid or the glycoprotein (Fig. 4B, Table S3).

To explore the possible antiviral role of nrEVEs, we investigated the sequence similarity between the nrEVEs and the circulating viruses described in *O. laevigatus*. At the protein level, nine nrEVEs showed more than 45 % similarity with OlaeTLV1 (Fig. 4C). Among them, nrEVE_*Toti_10* displayed the highest similarity with OlaeTLV1, with 88 % aminoacidic identities and 72 % nucleotide identities along a 616 bp region of the 676 bp nrEVE. Regarding nrEVEs transcriptional activity, no transcripts derived from *Totiviridae*-related nrEVEs were identified. Overall, less than 40 % of the nrEVEs were transcriptionally active, and only 21 % were consistently transcribed in the 5 populations under analysis (Figure S2).

## 4. Discussion

The number of covert RNA viruses identified in insects is continuously increasing (Edgar et al., 2022; Shi et al., 2016). In this study, we expanded the repertoire of RNA viruses associated with hemipteran insects by describing 27 new RNA viruses in adults of the biocontrol agent *Orius laevigatus* (Hemiptera). The association between hemipteran insects and RNA viruses has been previously documented in different species as the whitefly *Bemisa tabaci* (21 RNA viruses), the brown planthopper *Nilaparvata lugens* (17 RNA viruses), the flower bug O*rius insidious* (13 RNA viruses), or the tomato bug *Nesidiocoris tenuis* (2 RNA viruses) (Dong et al., 2018; Haoming et al., 2021; Qi et al., 2023; Xu et al., 2017). The RNA viruses described in *O. laevigatus* grouped within fourteen viral families of positive single-strand, negative single-strand, and double-strand RNA viruses. Other viruses belonging to these viral families had been previously identified in insects. This is the case of picornaviruses, which are the most abundant viral species in insects. Similarly, mononegavirus, totivirus and partitivirus showed high prevalence in other hemipteran insects (Haoming et al., 2021).

The large set of viruses identified in *O. laevigatus* compared to other hemipteran insects (Haoming et al., 2021; Qi et al., 2023) may be a consequence of its feeding habits. *O. laevigatus* is a generalist predator feeding on thrips, whiteflies, aphids, or spider mites; and can also feed on pollen under laboratory conditions (Mendoza et al., 2022). In this context, it is essential to determine which viruses are specific from *O. laevigatus*. The phylogenetic classification showed that 23 new RNA viruses branched together with previously described ISVs. These may include viruses infecting *O. laevigatus,* but also viruses infecting the eggs of the Mediterranean flour moth *E. kuehniella,* which are provided as a diet for AB, MA, MIX, and PA-OV laboratory populations. Between the 23 ISVs, thirteen were detected in *P*2 and two other *O. laevigatus* populations feeding on pollen (Fig. 3, and data not shown), indicating that *E. kuehniella* is not the host of these viruses. The remaining ten ISVs were exclusively detected in *O. laevigatus* populations feeding on *E. kuehniella* eggs, questioning which is the primary host of these viruses. Based on the common origin of the *E. kuehniella* eggs employed in the rearing of the different populations, ISVs infecting the eggs would be detected in the four populations feeding on *E. kuehniella* diet. However, this condition is not met for any of the ISVs, suggesting that *O. laevigatus* is the primary host of these ISVs. In addition to ISVs, we identified four viruses branched with previously described mycoviruses: two ourmiaviruses (OlaeAOuV1, OlaeAOuV2), one partiti-like virus (OlaeAPaLV1), and one narnavirus (OlaeANaV2) (Esteban & Fujimura, 2008; Ghabrial et al., 2008; Turina et al., 2017). Based on this result, these four viruses were defined as *O. laevigatus*-associated viruses.

To gain additional detail on the distribution of RNA viruses we investigated the viral abundance in five laboratory populations. Sixteen RNA viruses were detected in more than one laboratory population under analysis and four of them (OlaeTV1, OlaeTV2, OlaeTLV1, and OlaeSoV1) were widespread in *O. laevigatus* laboratory populations. On the other side, eleven RNA viruses were exclusively detected in one laboratory population or sample. Specifically, ten of them were only detected in the PA-OV sample, which presented the highest number of 22 different viral species. Regarding viral abundance, 20 out of the 23 hypothetical ISVs were defined as abundant, since they presented normalized read counts (FPKM) higher than 50 in at least one of the populations (Viljakainen et al., 2023). Between them, OlaeSoV1 showed the highest viral abundance in all populations representing between the 6 % and the 15 % of the total reads. Instead, the group of non-abundant viruses included three dicistroviruses only detected in PA-OV populations (OlaeDiV1, OlaeDiV2, and OlaeDiV3), and three out of the four viruses phylogenetically grouped with fungi-related viruses (OlaeAOuLV1, OlaeAOuLV2, and OlaeAPaLV1).

While circulating viruses represent a record of current infections, the

endogenous viral elements (EVEs) represent an archive of the history of viral-host interactions (Crava et al., 2021; Horie et al., 2010; Palatini et al., 2017; Russo et al., 2019; Whitfield et al., 2017). Twenty-four EVEs derived from non-retroviral RNA viruses (nrEVEs) were identified in the reference genome of *O. laevigatus*. The presence of additional nrEVEs specific from any of the populations under study cannot be discarded and would entail that new nrEVEs have been introduced after the separation of the populations. Regarding nrEVEs characterization, nrEVEs are classified within the same viral families as circulating viruses, with the *Totiviridae* family having the highest number of RNA viruses (n = 4) and nrEVEs (n = 10). Previous studies in *Aedes* mosquitoes have suggested an antiviral role of the nrEVEs through the Piwi-interacting RNA pathway. This antiviral role depends on the sequence similarity between the EVEs and the genomes of circulating viruses infecting the host (Miesen et al., 2016). In *O. laevigatus,* nrEVEs and RNA viruses shared low nucleotide identities. In fact, nrEVE_*Toti_10* and OlaeTLV1 displayed the highest similarity (72 % nucleotide identities), suggesting that nrEVEs do not exert an antiviral function through RNA interference.

The broad and historical interaction observed between RNA viruses and *O. laevigatus* opens the question about the function of these viruses. The RNA viruses detected caused covert infections since no phenotypical effects were observed in the insects during the analysis and those colonies are continuously reared in the laboratory without obvious symptoms of disease. The most abundant virus retrieved in *O. laevigatus* was the OlaeSoV1, which was detected in all the populations under analysis and represented 10 % of the total RNA reads on average. Although the effect of solinviviruses (Picornavirales) on insect health remains unexplored, previous studies have shown detrimental effects associated with the presence of other picornaviruses in mass-reared insects (Fujiyuki et al., 2005; Hernández-Pelegrín et al., 2023a; Yuan et al., 2020). Alternatively, the high prevalence and abundance of OlaeSoV1 and the *Totiviridae* species may have a positive effect on the host or be required for proper insect development (Coffman et al., 2020). In both scenarios (positive or negative effect), the presence of RNA viruses may influence the efficiency of *O. laevegatus* as a biological control agent. First, RNA viruses are considered a risk for insect mass-rearing in which the specific rearing conditions can lead to disease outbreaks that compromise the success of the industry (Eilenberg et al., 2015; Maciel-Vergara & Ros, 2017; Slowik et al., 2023). Second, RNA viruses could influence the effectiveness of biocontrol by altering the predatory behaviour of *O. laevigatus*. In this line, infection with certain ISVs enhances the parasitism performance of parasitic wasps (Coffman et al., 2020; Hernández-Pelegrín et al., 2023b; Roossinck & Bazán, 2017). Third, field conditions may enhance the effect of RNA viruses on the host, in comparison to the controlled conditions encountered in the laboratory. In this scenario, the obtention of virus-free colonies will play a key role in deciphering the function of RNA viruses (Morrow et al., 2023).

Overall, our study provides valuable insights into the viral landscape of a relevant and commercialized biocontrol agent. We described 27 RNA viruses in transcriptomes of *O. laevigatus,* from which 23 were defined as ISVs. Moreover, the presence of 24 nrEVEs in the *O. laevigatus* genome confirmed the long-term relation between *O. laevigatus* and RNA viruses. Despite the absence of observable symptoms, the high proportion of reads classified as viral reads highlights the potential role of RNA viruses on the applicability and efficacy of *O. laevigatus* in insect biocontrol.

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## CRediT authorship contribution statement

**Luis Hernández-Pelegrín:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Amador Rodríguez-Gómez:** Methodology, Investigation. **Ana Belén Abelaira:** Methodology, Investigation. **Ma. Carmen Reche:** Methodology, Investigation. **Cristina Crava:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Fang Shiang Lim:** Writing – review & editing, Methodology, Investigation. **Pablo Bielza:** Supervision, Resources, Funding acquisition. **Salvador Herrero:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2024.108175.

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