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Crayfish pet trade as a pathway for the introduction of known and novel viruses

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ABSTRACT

Expanding international pet trade has emerged as one of the main introduction pathways of aquatic invasive species, with ornamental crayfish species commonly available on the EU and global markets. Besides most frequently studied crayfish pathogens, such as *Aphanomyces astaci* and white spot syndrome virus (WSSV), ornamental crayfish carry associated microbial communities, which may potentially lead to the emergence of known or even novel diseases following intentional or unintentional release of animals into the wild. This is especially problematic in the case of viruses, which represent an important, yet considerably understudied, group of crayfish pathogens. Here we analyzed viromes of hepatopancreas tissue of four crayfish species acquired in the international pet trade in Europe (*Procambarus clarkii, Procambarus alleni*, *Cherax holthuisi*, and *Cherax quadricarinatus*) using a high throughput sequencing based metagenomic approach. Seven different known viruses were identified, which were previously either directly associated with crayfish (WSSV, Cherax quadricarinatus reovirus, chequa iflavirus, athtab bunya-like virus) or with hosts from subphylum Crustacea or invertebrates associated with freshwater environment (Shahe ispoda virus 5, *Dicistroviridae* sp.). Additional sequences represented 8 potential novel and divergent RNA viruses, most similar to sequences belonging to members of *Picornavirales, Elliovirales, Reovirales, Hepelivirales, Tolivirales* and *Ghabrivirales* orders. We discuss our findings in relation to their phylogenetic relationships, geographical origins, and putative pathogenicity implications. The results highlight the need for further research into the risks related to disease emergence associated with the pet trade.

1. Introduction

The introduction and establishment of invasive species is one of the main components of global environmental change. They represent a growing threat to human well-being, the global economy and contribute to biodiversity loss, ecosystem degradation, and impairment of ecosystem services worldwide (Pyšek et al., 2020; Diagne et al., 2021; Cuthbert et al., 2024). These impacts are especially evident in freshwater ecosystems, which have often been extensively invaded (Keller et al., 2011). These ecosystems exhibit a high degree of endemism but also extinction rates (Dudgeon et al., 2006; Reid et al., 2019). Expanding international pet trade has emerged as one of the main introduction

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pathways of aquatic invasive species due to its long history and suboptimal efficiency of focused regulatory frameworks (Hulme et al., 2008; Patoka et al., 2018; Novák et al., 2020; Bláha et al., 2022). Recent studies have demonstrated that pet trade not only facilitates the potential for invasions (since pets may be either intentionally or unintentionally released into the wild), but also selectively favors invasive species (Chucholl et al., 2016; Gippet and Bertelsmeier, 2021). Ornamental species also carry associated microbial communities (Mrugała et al., 2015; Patoka et al., 2016; Ložek et al., 2021; Patoka and Patoková 2021) and can act as vectors for pathogens, which may be released with their hosts and cause the emergence of novel and known diseases in the wild (e.g., Kolby and Daszak,2016; Chinchio et al., 2020; Guilder et al., 2022; Prati et al. 2024). Therefore, pet trade is also an important pathway for the introduction of novel pathogens.

Freshwater crayfish (Decapoda: Astacidea) are an example of aquatic animals that are increasingly endangered (Bland 2017; Dewhurst-Richman et al., 2015). They are progressively declining in their native ranges due to the introduction and spread of invasive congeners, which originate partly from the pet trade (Yonvitner et al., 2020; Bláha et al., 2022). Crayfish from families Cambaridae (of North American origin) and Parastacidae (of Australian and New Guinean origin) are commonly available on the EU and global markets (Patoka et al., 2014; Yuliana et al., 2021; Lipták et al., 2023a; Olden and Carvalho 2024), with up to 60 crayfish species advertised as ornamentals and with formally undescribed taxa traded (Chucholl and Wendler 2017; Patoka et al., 2023; Patoka et al., 2025). Since its start in the mid-1990 s, ornamental crayfish trade and its popularity has been steadily increasing across continents (Chucholl, 2013; Faulkes, 2015; Bláha et al., 2022), leading to substantial rise of ornamental crayfish records in the wild (Panteleit et al., 2017; Weiperth et al., 2017; Weiperth et al., 2020; Patoka et al., 2025). A recent study by Bláha et al., (2022) recorded more than six different non-native crayfish species of ornamental origin established in a thermal spring in Hungary. Several recent studies have also clearly demonstrated that populations of such species are often carriers of *Aphanomyces astaci*, a pathogen causing crayfish plague responsible for the decimation of native crayfish populations across the Palaearctic (Mrugała et al., 2015; Mojžišová et al., 2024). An additional prominent crayfish pathogen, the white spot syndrome virus (WSSV) was also recorded in ornamental crayfish, but at a much lower incidence and in fewer species (Mrugała et al., 2015).

Besides known and most frequently studied crayfish pathogens, such as *A. astaci* and WSSV, ornamental crayfish may carry several novel pathogens, which may potentially lead to the emergence of novel diseases in both native and already established invasive crayfish (e.g., Wang et al., 2005; Edsman et al., 2015; Grandjean et al., 2019). This is especially problematic in the case of viruses, which represent an important group of crayfish microbial pathogens. Recent review of over 100 publications on potentially pathogenic viruses, bacteria, fungi and fungal-like microorganisms demonstrated that viruses are the least studied group of pathogens infecting crayfish, but also the group with the highest percentage of successfully determined pathogenicity status in aquaculture (Dragičević et al., 2021). Recent high throughput sequencing (HTS) based research on viromes of 13 economically important crustaceans revealed remarkable viral diversity, identifying 90 RNA viruses, 77 % of which were highly divergent from known viruses (Dong et al., 2024). Recent virome analysis of the red swamp crayfish (*Procambarus clarkii*), implementing the enrichment of viral particles before HTS, resulted in detection of 1729 viral operational taxonomic units, of which 92.71 % were reported for the first time (Guo et al., 2025). High virome diversity, comprising mostly novel viral sequences detected by HTS, was also observed in populations of freshwater crayfish in the wild, in both invasive signal crayfish (*Pacifastacus leniusculus*; Bačnik et al., 2021) and the native European noble crayfish (*Astacus astacus*; Zingre et al., 2023). For the vast majority of the detected viral sequences, the host range and impact on their hosts are unknown. Although viral infections do not necessarily cause mortality, they may exert damage to the host at some level of its biological organization (i.e., in an individual, organs, tissues, cells), which may induce adverse effects on host fitness (Casadevall and Pirofski, 2003). This means that ornamental crayfish that are released or escape into the wild can potentially spill-over known and unknown viruses and contribute to the emergence of novel diseases in wildlife (Prenter et al., 2004). To analyze the threat that ornamental crayfish may present in terms of introduction of known and unknown viruses, we analysed viromes of four ornamental crayfish species commonly available in the international pet trade using HTS-based metagenomic approach.

2. Materials and Methods

2.1. Animal collection and sampling

A total of 33 individuals of four commonly available ornamental crayfish species (Table 1) were obtained (Patoka et al., 2015; Yonvitner et al., 2020) from a Czech wholesaler importing aquatic organisms in October 2022 and in February 2023. The shipment with crayfish was obtained before entering the importer's culturing facility to prevent contamination with other locally present viruses. All purchased individuals were immediately transported on ice, in the original PS thermo-box to the laboratory of the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic. Crayfish were packed in individual plastic bags with some water and a piece of foam. As model species, we selected Everglades crayfish (*Procambarus alleni*; also called Florida crayfish), apricot crayfish (*Cherax holthuisi*), and redclaw crayfish (*Cherax quadricarinatus*).

In the lab, each crayfish individual was identified to the species level. In the collection of declared *P. alleni* individuals, seven individuals of red swamp crayfish (*Procambarus clarkii*) were also identified, which is an invasive species of EU concern according to the Invasive Alien Species Regulation (Regulation (EU), 2014, 2016) and therefore subject to restrictions in keeping, breeding and selling. Following species identification, we determined sex of each individual and performed dissection to obtain hepatopancreas tissue samples. Immediately before dissection, a rapid cut between the cephalothorax and abdomen was conducted, followed by swift cephalothorax shell removal. This resulted in rapid damage to the nerve cord. Albeit no institutional, national, or EU ethical guidelines exist for crayfish, the described procedure is in line with available guidelines for the humane killing of crayfish (Conte et al., 2021). Extracted hepatopancreas were isolated from each individual and washed with sterile distilled water. Then, left and right half-organs (Vogt 2019) were divided using sterile scalpel. Approximately 30 mg of the lowest part of the left half-organ was cut and dry-freezed for RNA extraction and sequencing (described below). All samples were stored at −80 °C until further analyses. All dissection equipment (scissors, tweezers, scalpels) and tables were sterilized between the dissection of each crayfish individual with 1 % sodium hypochlorite. Dissection equipment was additionally alcohol-flame sterilized.

2.2. RNA extraction and sequencing

Total RNA was extracted from the hepatopancreas of all collected crayfish using the NucleoSpin RNA kit (Macherey-Nagel, Germany), which includes DNase digestion. Before the extraction, approximately 30 mg of hepatopancreas was collected in a sterile 2 mL tube together with 2 mm solid glass beads (1040140500, Supelco, Merck), submerged

Table 1
Characteristics of purchased individuals used in the study. Information on species identity, number of individuals per sex, country of import, origin and population source (from wild populations or reared in aquaculture) is provided.

Species	No. of males/females	Country of import	Native Range	From: Wild/ Aquaculture
Cherax holthuisi	8/2	Indonesian New Guinea	Indonesian New Guinea – Aitinjo Lake	Wild
Cherax quadricarinatus	5/5	Indonesia (Java)	Australia/ New Guinea	Aquaculture
Procambarus alleni	4/2	Thailand	North America	Aquaculture
Procambarus clarkii[*]	5/2	Thailand	North America	Aquaculture

[*] imported as *P. alleni*.

in liquid nitrogen, and homogenized for 1 min at 30 Hz using a Tehtnica Millmix 20 (Domel, Slovenia). Following extraction, RNA concentration in each sample was measured using Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Then RNA samples were pooled by species, ensuring equal mass of RNA from each crayfish individual in the pooled sample. Isolation controls (30 μL nuclease-free water) were added to each of the performed extractions as one of the samples. Additionally, one pooled control sample was constructed by mixing equal amounts of isolation controls and spiked with synthetic luciferase RNA to ensure quality control, normalization, and standardization of sequencing data. Not to transport the animals or animal tissues, species identification, dissection and total RNA extraction were performed at the Faculty of Agriculture and Technology, University of South Bohemia in České Budějovice, Czech Republic. Four samples with approximately 1 μg of pooled total RNA and above described control were sent to Macrogen Europe for total RNA Sequencing; rRNA depletion was performed with Ribo-Zero (Human/Mouse/Rat) Gold Kit (Illumina, San Diego, CA, USA) followed by library preparation using TrueSeq Stranded Total RNA Sample Prep Kit (Gold) with pair-end (2 × 150 nts) sequencing on a NovaSeq6000 (DS-150) (Illumina).

2.3. Bioinformatic analysis

Sequencing datasets were examined, filtered, and analyzed according to procedures described previously (Bačnik et al., 2021). Briefly, sequencing datasets were first imported to CLC Genomics Workbench (version 23.0.3, Qiagen Bioinformatics, USA). Adapters of imported reads were trimmed and filtered using a quality filter (Limit = 0.05, no ambiguous nucleotides allowed) and size cutoff (reads shorter than 25 bp were discarded). Processed reads were exported from CLC Genomics Workbench and utilized for de novo assembly of longer contigs using SPAdes (version 3.15.5) with flag "–rnaviral" (Prjibelski et al., 2020). De novo assembled contigs were queried for similarity on protein level using Diamond BLASTx (Buchfink et al., 2015) with default setting parameters against NCBI nr database (2022). Taxonomic classification was performed using MEGAN6 (version 6.19.2) (Huson et al., 2016). To visualize the initial MEGAN classification of viral contigs across different taxa a chord diagram was constructed in R (version 4.4.0) (R Core Team, 2024) using circlize package (Gu et al., 2014).

The contigs of all four samples that classified as viral in the Diamond-MEGAN analysis were imported into CLC Genomics Workbench for additional examination and assembly steps utilizing "de novo assembly" and verified using "map reads to reference" tools for extending shorter contigs and obtaining the average read coverage values. Assembly of contigs with BLASTx results is described in detail in Supplementary Information (Supplementary TAble S1). Virus-like contigs that were longer than 3500 nts were further subjected to phylogenetic analysis. In the case of virus-like contigs belonging to white spot syndrome virus, contigs longer than 1000 nts were subjected to further analysis. Potential open reading frames were predicted using SnapGene software (SnapGene Viewer 7.2.1) and manually selected based on genome organization of closely related viruses. Protein domain search was performed with InterproScan (Jones et al., 2014; accessed August 2024). To obtain pairwise similarities, virus-like sequences with the same best BLASTn match with high identity score (>90 %) were subjected to pairwise alignment using Needle (EMBL-EBI, accessed April 2024; Madeira et al., 2022). All sequencing datasets supporting the results of this study were deposited under PRJNA1142220 accession number in the NCBI BioProject database. The sequences of selected viral contigs identified in this study were deposited in the GenBank under the accession numbers listed in Tables 3 and 4.

2.4. Phylogenetic analyses

We extracted RNA dependent RNA Polymerase (RdRp) amino acid sequences from selected virus-like contigs and performed BLASTp search (NCBI-nr, February 2024). Most similar homologous sequences were retrieved and used for phylogenetic analyses. In addition, RdRp sequences of recognized viral species as well as such from previous studies (Chen et al., 2022; Shi et al., 2016) or from the RNA Viruses in Metatranscriptomes database (RVMT) (https://riboviria.org;accessed April 2024; Neri et al., 2022), belonging to taxa corresponding to discovered viruses (from *Orthornavirae* kingdom; *Elliovirales*, *Hepelivirales*, and *Ghabrivirales* orders; and *Spinareoviridae*, *Tombusviridae*, *Iflaviridae*, and *Dicistroviridae* families) were included in the respective phylogenetic analyses. Protein sequences were aligned using MAFFT software (v7.511) (Katoh & Standley 2013). Sequences included in the alignments are accessible in the Supplementary File Alignments SX1-SXn. Methods used for obtaining alignments of individual groups are described in detail in Supplementary Table S1. Where indicated (Supplementary TAble S1 phylogenetic_analysis), aligned sequences were trimmed manually using MEGA11 (11.0.13) (Tamura et al., 2021) and with TrimAI (v1.4.rev15) (Capella-Gutiérrez et al., 2009) using flag "-automated1". Obtained alignments were utilized for inferring Maximum likelihood trees using IQ-TREE (1.6.12) (Nguyen et al., 2015) employing the model selected by ModelFinder (Kalyaanamoorthy et al., 2017) and using ultrafast bootstrap method (Hoang et al., 2018) with 1000 bootstrap replicates to assess statistical support. Models used for inference of individual trees are described in detail in Supplementary TAble S1 phylogenetic_analysis.

In addition, as a preliminary analysis, virus-like contigs identified as picorna-like virus sequences (Virus-like contig 1, Virus-like contig 255, Virus-like contig 256 and Virus-like contig 257) were subjected to a preliminary phylogenetic analysis, where all GenBank sequences belonging to the order *Picornavirales* according to RVMT database (accessed April 2024) (Neri et al., 2022) were extracted and analyzed according to procedure described in the previous paragraph to obtain global positioning of virus-like sequences within *Picornavirales* order. In

the next step, only selected sequences belonging to the viral clusters in which our contigs were placed in the global phylogenetic tree were utilized to construct separate phylogenetic trees. All phylogenetic trees were visualized using iTOL (Letunic & Bork, 2021) and refined in Inkscape (Inkscape project 2023).

2.5. PCR detection of selected viruses in individual samples

Five known viruses detected in crayfish samples (white spot syndrome virus, Shahe isopoda virus 5, chequa iflavirus, athab bunya-like virus and Cherax quadricarinatus reovirus), with previously recorded associations with invertebrate hosts, were selected for RT-PCR confirmation in the individual crayfish samples. We constructed primer pairs that amplify the fragments of white spot syndrome virus and Shahe isopoda virus 5 (Supplementary Table S1pcr_primers) using NCBI Primer designing tool Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). For the detection of remaining viruses, primer pairs available from literature were utilized (Sakuna et al., 2017; Hayakijkosol et al., 2021; Nambiar et al., 2023; Supplementary TAble S1). cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosciences, Park Ave, NY, USA) following the manufacturer's recommendations and a prior denaturation of RNA for 5 min at 65 °C. Subsequently, 2 µl of cDNA were used as template for PCR with PPP Master Mix (Top-Bio, Czech Republic), with 1 µl of each 10 mM primer and PCR grade water up to a final volume of 25 µl. Cycling conditions were set according to the manufacturer's instructions and the corresponding annealing temperature for each primer pair (see Table S1). PCR products were stained with GelRed Nucleic Acid Gel Stain (Biotium, Inc.) and visualized with ultraviolet transillumination in 1–1.2 % agarose gels.

3. Results and discussion

3.1. The overview of known and novel viruses discovered in analyzed crayfish species

Sequencing of rRNA depleted total RNA yielded a high number of raw reads per sample, ranging from 3.64 to 3.68 × 10^8 (Table 2). Assembly of reads from pooled samples of *Procambarus clarkii*, *Procambarus alleni*, *Cherax holhuisi* and *Cherax quadricarinatus* resulted in 1,852,429 contigs in total. After diamond BLASTx search, a total of 325 contigs were classified as virus-like sequences. General sequencing and assembly information is provided in Table 2 and virus-like contigs are described in detail in Supplementary Table S1, together with results of BLASTx similarity searches.

Our analysis revealed a high diversity of known and novel viruses in crayfish species present in pet-trade, with most viral-like sequences classifying as sequences belonging to *Riboviria* realm, representing different RNA viruses. In the case of one species (*P. clarkii*), we also identified sequences of a dsDNA white spot syndrome virus (WSSV), one of the most studied crayfish pathogens, belonging to *Nimaviridae* family from *Varidnaviridae* realm. A few short viral-like contigs (<400 nts) similar to parvoviruses from *Monodnaviridae* realm were also detected but were not further analyzed. An overview of discovered viral-like sequences and their taxonomic classification is presented in Fig. 1.

For more detailed analysis we have chosen 27 long viral-like contigs for which association with crayfish was most likely, as described in the Materials and Methods section. A total of 18 viral-like contigs were highly similar (>88 % amino acid identities, obtained with BLASTx) to 7 different known viruses (Table 3), which were previously either directly associated with crayfish (WSSV, Cherax quadricarinatus reovirus, chequa iflavirus, athtab bunya-like virus; Sakuna et al., 2018; Liu et al., 2021; Edgerton et al., 2000) or with hosts from phylum Arthropoda (Shahe ispoda virus 5; Shi et al., 2016) or at least invertebrates associated with freshwater environment (*Dicistroviridae* sp.; Lu et al., 2022).

Additional 9 virus-like contigs belonged to 8 potential novel viruses with BLASTx identity scores to the closest match ranging from 25 % to 62.56 % (Fig. 2, Table 4). These viral-like contigs, representing novel and divergent RNA viral sequences, were most similar to sequences belonging to members of *Picornavirales*, *Elliovirales*, *Reovirales*, *Hepelivirales*, *Tolivirales* and *Ghabrivirales* orders from *Orthornavirae* kingdom. Similarly as in the case of known viruses, the closest BLASTx matches for the analyzed novel virus-like sequences were associated with invertebrate hosts, with the majority associated with hosts from subphylum Crustacea. Most novel virus-like sequences had the highest identities to unclassified RNA viruses characterized in an extensive metagenomic study of invertebrate viromes (Shi et al., 2016). Detection of several novel RNA viral sequences in this study is in line with the previous studies focused on invertebrate and environmental viromes (Shi et al., 2016; Wolf et al., 2020; Chang et al.2021), including those analyzing crustacean and crayfish viromes (Bačnik et al., 2021; Zingre et al., 2023; Dong et al., 2024, Guo et al., 2025).

Multiple known viruses were detected in most of the analyzed species (*P. clarkii*, *P. alleni*, *C. quadricarinatus*), and only one known virus was detected in *C. holthuisi* (Table 3). This species is an endemic crayfish from the Aitinjo Lake in Southwest Papua Province, Indonesian New Guinea and was formally described relatively recently (Lukhaup and Pekny 2006). *C. holthuisi* had the most distinct virome composition with the highest count of previously unknown viruses (Table 4), possibly indicating its isolated distribution range. The vast majority (7) of novel viruses were recorded in *Cherax* species, while only one novel virus was recorded in *Procambarus* species (Table 3, Fig. 1).

3.2. Viruses detected in multiple crayfish species

Most of the reported viral sequences, including all novel virus sequences, were detected in only one of the four crayfish species (Fig. 1) and not in the other three. The exception to this were sequences belonging to two known viruses: Shahe isopoda virus 5 was detected in *P. alleni*, *P. clarkii* and *C. holthuisi*, and a virus from *Dicistroviridae* family in *P. alleni* and *P. clarkii*. Previous studies have shown that the two closely related species in our study, *P. clarkii* and *P. alleni*, overlap in native geographic range (Taylor et al., 2007) and are phylogenetically related (Johnson et al., 2011), which may shape virome composition

Table 2
General information about reads datasets obtained from pooled hepatopancreas samples of different crayfish species (Pc – *Procambarus clarkii*, Pa – *Procambarus alleni*, Ch – *Cherax holhuisi*, Cq – *Cherax quadricarinatus*; SRA – short reads archive).

Sample	Raw Reads	Reads after Trimming	Accession Number (SRA)	No. of Assembled Contigs	No. of Contigs Classified as Viral (Diamond)
Pc	368,882,472	368,413,742	SRR30063916	258,277	186
Pa	364,074,574	363,710,075	SRR30063915	212,065	56
Ch	366,535,356	365,996,342	SRR30063918	602,723	58
Cq	365,802,584	365,323,362	SRR30063917	779,364	25

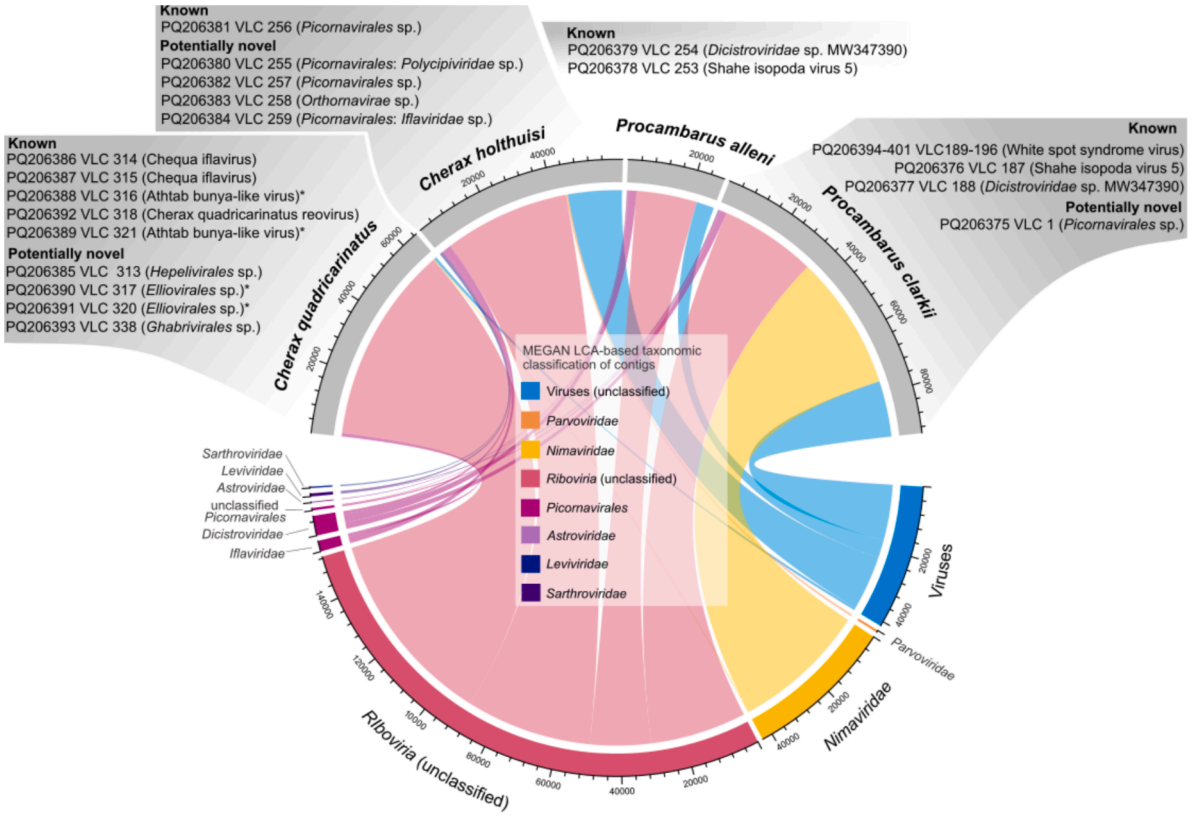

Fig. 1. Overview of viral sequences discovered in pooled hepatopancreas samples of analysed crayfish species. Chord diagram in the center represents a summary of results obtained with MEGAN-LCA based taxonomic classification based on diamond BLASTx similarity search for the de novo assembled viral-like contigs; ribbons connect virus-like contigs detected in specific crayfish species (top bands) with their initial taxonomic classification (bottom bands) and are color-coded according to the legend in the center. The length of the bands corresponds to cumulative contig lengths. For clearer representation, virus-like contigs classified below family level were grouped by their respective families or, when this was not possible, by higher taxonomic levels. Viral contigs classified as *Riboviria*, unclassified *Riboviria*, unclassified RNA viruses ShiM-2016, or those classified at the phylum level or higher are all grouped under *Riboviria (unclassified)*. Grey boxes connected to the crayfish species names at the top contain corresponding lists of virus-like contigs (VLC) that were selected for further analysis in this study and include their accession numbers, contig identificators and, in brackets, virus names or, where this was not possible, the lowest possible taxonomic classifications. Virus-like contigs marked with asterisk (*) likely represent separate genome segments of the same virus.

and virus transmission in animals (French et al., 2023; Jacquot et al., 2022). *P. clarkii* and *P. alleni*, which showed the highest overlap in detected known viruses in our study were also acquired from the same origin (Thailand aquaculture). Thus, both shared evolutionary traits and environmental factors probably contribute to the observed overlap.

3.2.1. *Known virus from Dicistroviridae family*

Two virus-like sequences (Virus-like contig 188, 4319 nts and Virus-like contig 254, 9053 nts) with high similarity to known arthropod infecting members of *Dicistroviridae* family were obtained from sequencing data with Virus-like contig 188 sequence extracted from *P. clarkii* samples and Virus-like contig 254 sequence extracted from *P. alleni* samples. BLASTn query returned *Dicistroviridae* sp. sequence (MW347390.1, 9343 nts) obtained from the water sample from river ports in China (Lu et al., 2022) as the closest match with 97.65 % and 97.34 % identity, respectively. Pairwise alignment of both sequences obtained in this study on the overlapping segment revealed 98.7 % identity between them. Phylogenetic tree illustrating relationship of these sequences with the most closely related known viral sequences (Supplementary Fig. S1) shows that most of the viral sequences placed in the same cluster in this analysis were associated with arthropod source materials Therefore, further research is needed to evaluate the association of this virus with crayfish as a host.

3.2.2. *Shahe isopoda virus 5*

Virus-like contigs with high similarity to Shahe isopoda virus 5 were recovered from *P. alleni* (Virus-like contig 253, 4360 nts) and *P. clarkii* (Virus-like contig 187, 4181 nts) hepatopancreas samples, sharing 98.07 % and 97.85 % identity on nucleic acid level (BLASTn) with Shahe isopoda virus 5 (KX883263, 4371 nts) isolated from channeled apple snail (*Pomacea canaliculata*) in China (Shi et al., 2016). Pairwise comparison of both sequences obtained in this study revealed 95.3 % identity on nucleic acid level between them. Phylogenetic analysis placed both contigs and Shahe isopoda virus 5 sequence in the cluster with tombus-like viruses associated with sponges and other organisms from the aquatic environments (Supplementary Fig. S2). A few short contigs (Virus-like contig 289 and 312) with high similarity to Shahe isopoda virus 5 were also detected in pooled hepatopancreas sample of *C. holthuisi*, however their length was shorter than 1000 nts and were therefore not included in more detailed analysis. RT-PCR analyses of individual samples confirmed detection of Shahe isopoda virus 5 in all six *P. alleni*, two out of seven *P. clarkii* and two out of ten *C. holthuisi* individuals (Supplementary Table S1). Since the same virus was previously associated with different source organisms; channeled apple snails (*Pomacea canaliculata*) and freshwater arthropods (Shi et al., 2016), and related viruses are associated with variety of different sources, such as sponge (*Halichondria panicea*; Waldron et al., 2020), the association of this virus with crayfish as a host is uncertain but cannot be ruled out. Assessing the host association of this virus is additionally challenging, because its sequences as well as sequences of related viruses were primarily identified through metagenomic studies. Shahe isopoda virus 5 is related to tombus-like viruses that are known to be extremely stable and

Table 3
Selected virus-like contigs of known viruses identified in this study with their respective lengths (nts), average read coverage and closest match according to BLASTx search (NCBI-nr) (Pc – *Procambarus clarkii*, Pa – *Procambarus alleni*, Ch – *Cherax holhuisi*, Cq – *Cherax quadricarinatus*).

Known viruses										
Source (Sample)	Contig Name	Lowest determined taxonomic rank	Name	Isolate	GenBank Accession No.	Contig Length	Average coverage	BLASTx Results		
								Closest Protein Hit Name and Corresponding Viral Name	e-Value	Identities*
PC	Virus-like contig 189	*Whispovirus*	White spot syndrome virus	Proclav4	PQ206394	5215	2326.44	wsv277 [Shrimp white spot syndrome virus]	0	791/791 (100 %)
PC	Virus-like contig 190	*Whispovirus*	White spot syndrome virus	Proclav4	PQ206395	1808	94.78	ORF167 [White spot syndrome virus]	0	600/602 (99 %)
PC	Virus-like contig 191	*Whispovirus*	White spot syndrome virus	Proclav4	PQ206396	1795	81.38	ORF398 [White spot syndrome virus]	0	572 / 572 (100 %)
PC	Virus-like contig 192	*Whispovirus*	White spot syndrome virus	Proclav4	PQ206397	1593	119.15	ORF398 [White spot syndrome virus]	0	530 / 530 (100 %)
PC	Virus-like contig 193	*Whispovirus*	White spot syndrome virus	Proclav4	PQ206398	1209	112.38	wsv360 [Shrimp white spot syndrome virus]	0	401 / 402 (99 %)
PC	Virus-like contig 194	*Whispovirus*	White spot syndrome virus	Proclav4	PQ206399	1150	89.48	wsv465 [Shrimp white spot syndrome virus]	0	278 / 278 (100 %)
PC	Virus-like contig 195	*Whispovirus*	White spot syndrome virus	Proclav4	PQ206400	1097	83.39	DNA polymerase [White spot syndrome virus]	0	364 / 364 (100 %)
PC	Virus-like contig 196	*Whispovirus*	White spot syndrome virus	Proclav4	PQ206391	1051	69.85	ORF398 [White spot syndrome virus]	0	349 / 349 (100 %)
PC	Virus-like contig 187	*Tombusviridae*	Shahe isopoda virus 5	Proclav2	PQ206376	4181	310.26	hypothetical protein 2 [Shahe isopoda virus 5]	0	877/937 (93.6 %)
PC	Virus-like contig 188	*Dicistroviridae*	Dicistroviridae sp.	Proclav3	PQ206377	4319	106.96	polymerase polyprotein [Dicistroviridae sp.]	0	1191 / 1229 (96.91 %)
PA	Virus-like contig 254	*Dicistroviridae*	Dicistroviridae sp.	Proallv2	PQ206379	9053	338.60	polymerase polyprotein [Dicistroviridae sp.]	0	1702 / 1747 (97.42 %)
PA	Virus-like contig 253	*Tombusviridae*	Shahe isopoda virus 5	Proallv1	PQ206378	4360	1962.97	hypothetical protein 2 [Shahe isopoda virus 5]	0	876/937 (93.49 %)
CH	Virus-like contig 256	*Picornavirales*	Picornavirales sp.	Chehouv2	PQ206381	6700	183.68	hypothetical protein [Hubei tetragnatha maxillosa virus 1]	0	1955/2221 (88 %)
CQ	Virus-like contig 315	*Iflaviridae*	Chequa iflavirus	Chequav3	PQ206387	9129	5469.24	polyprotein [Chequa iflavirus]	0	2953/2981 (99.06 %)
CQ	Virus-like contig 314	*Iflaviridae*	Chequa iflavirus	Chequav2	PQ206386	9324	1419.67	polyprotein [Chequa iflavirus]	0	2755/2985 (92.29 %)
CQ	Virus-like contig 316	*Elliovirales*	Athtab bunya-like virus	Chequav4	PQ206388	6809	704.13	RNA-dependent RNA polymerase [Athtab bunya-like virus]	0	2210/2218 (99.64 %)
CQ	Virus-like contig 321	*Elliovirales*	Athtab bunya-like virus	Chequav4	PQ206389	3582	1321.81	glycoprotein N [Athtab bunya-like virus]	0	792/802 (98.75 %)
CQ	Virus-like contig 318	*Spinareoviridae*	Cherax quadricarinatus reovirus	Chequav6	PQ206392	4174	1843.02	RNA-dependent RNA polymerase [Cherax quadricarinatus reovirus]	0	483/484 (99.79 %)

* number of matching amino acids / number of total amino acids of the closest protein hit (percentage of amino acid identity).

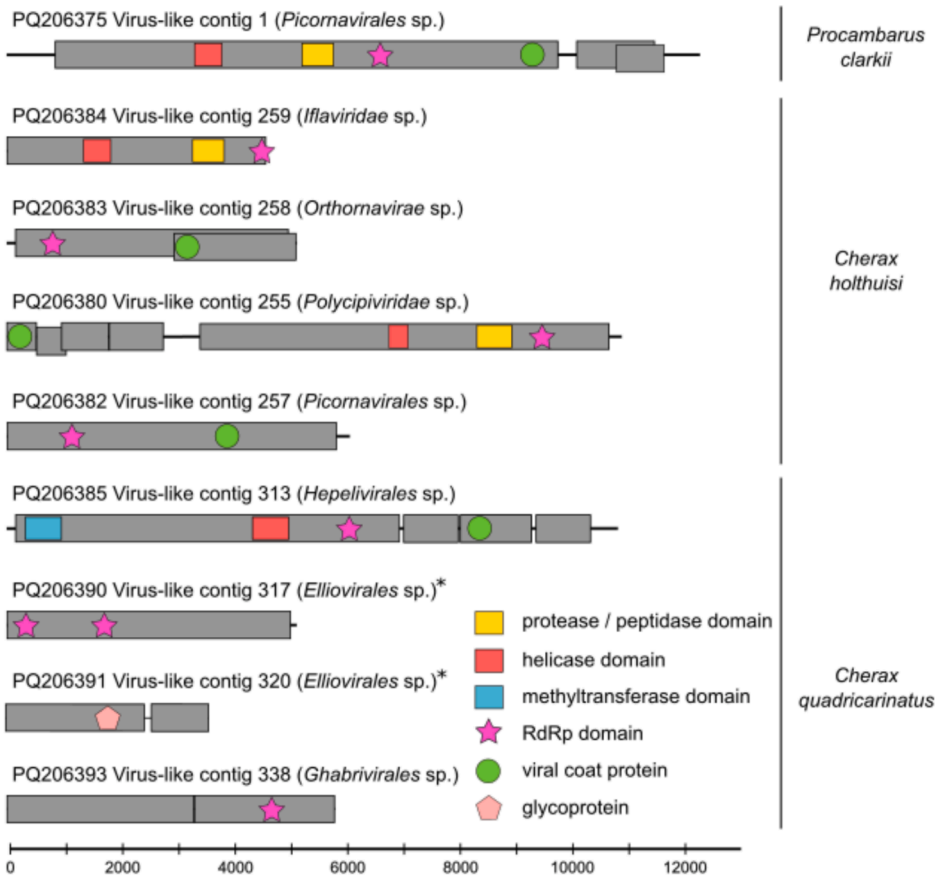

Fig. 2. Genome organization of potential novel virus-like sequences recovered in this study. Grey bars represent putative coding sequences, whereas symbols represent domains associated with viral replication and capsid structure. Virus-like contigs marked with asterisk (*) likely represent separate genome segments of the same virus.

thus ubiquitous in environmental samples, such as water (Koch et al., 2020). Its detection in crayfish and/or in other species could thus be a consequence of their release from original host and long-term persistence in water.

3.3. *Viruses detected in Procambarus clarkii*

Samples of *P. clarkii* were imported from aquaculture in Thailand and sold under different name (*P. alleni*). According to EU regulation on invasive alien species (Regulation (EU) No 2014; 2016), *P. clarkii* is on the list of species of Union concern and its keeping, trade and import is prohibited. It was recently reported that despite this ban, the species remains present in hobby aquaria (Patoka et al., 2018; Bláha et al., 2022) and its new populations continuously appear in the wild (Lipták et al., 2024; Karaouzas et al., 2024). Recent global study of crayfish online trade also identified that *P. clarkii* continues to be routinely present in the European marketplaces, and the reliance on self-reported taxonomy by marketplaces is a key challenge that obscures species identification and hinders regulation enforcement for prohibited species (Olden and Carvalho 2024). Mislabeling of taxa and especially of *P. clarkii* in pet trade in EU was also highlighted in a recent study by Oficialdegui et al., (2025), which calls out for expert involvement and application of molecular identification in the pet trade at border controls and post-border controls. Here we also observed an example of this taxonomical misidentification in pet-trade, additionally emphasizing the need to strengthen education, stakeholder capacity in species identification, and regulatory efforts in this sector (Lipták et al., 2023b) as well as involvement of research institutions (Oficialdegui et al., 2025). The virome of *P. clarkii* was recently characterized and a rich array of new viruses were identified (Guo et al., 2025). In *P. clarkii* we detected several virus-like contigs and further analyzed those corresponding to three known viruses and one putative novel virus belonging to the order *Picornavirales*. Two of the known viruses (*Dicistroviridae* sp. and Shahe isopoda virus 5) were detected also in other species, as described above, while WSVV was detected only in *P. clarkii*.

3.3.1. *White spot syndrome virus*

Several contigs (108) recovered from pooled samples of *P. clarkii* hepatopancreas corresponded to WSSV, a dsDNA virus from *Nimaviridae* family. Additional mapping of reads to chimeric WSSV reference genome (MG264599.1; Dantas et al., 2018) showed that 125,518 reads mapped to the reference genome, covering around 36 % of the genome sequence, while the longest contig assembled (Virus-like contig 189) was 5214 nts long. Analyzed contigs shared high similarity with different isolates of WSSV according to BLASTn analysis. Recovering a fragmented assembly of a viral genome, covering only part of the genomic sequence, is likely a consequence of recovering only expressed parts of this DNA virus by RNA sequencing. Phylogenetic analyses including either only the longest contig or a concatenated sequence of longer contigs did not bring informative results, i.e., it resulted in poorly supported clades in the tree (data not shown). This is likely a consequence of a weak phylogenetic signal in the recovered parts of the viral genome.

WSSV is one of the major crustacean pathogens, adversely affecting crustacean populations in the wild and limiting aquaculture productivity (Bateman and Stentiford 2017; Liu et al., 2021; Xue et al., 2022). WSSV has a broad host range that includes many crustacean species both in marine and freshwater environment (Escobedo-Bonilla et al., 2008;

Table 4
Selected virus contigs of potential novel crayfish-associated viruses identified in this study with their respective lengths (nts) and closest match according to BLASTx search (NCBI-nr) (Pc – *Procambarus clarkii*, Pa – *Procambarus alleni*, Ch – *Cherax holhuisi*, Cq – *Cherax quadricarinatus*). * number of matching amino acids / number of total amino acids of the closest protein hit (percentage of amino acid identity).

Novel virus-like sequences									
Source (Sample)	Contig Name	Lowest determined taxonomic rank	Proposed name	Isolate	GenBank Accession No.	Contig Length	Average coverage	BLASTx Results	
								Closest Protein Hit Name and Species	e-Value	Identities*
PC	Virus-like contig 1	*Picornavirales*	Picornavirales sp.	Proclav1	PQ206375	12,324	952.77	nonstructural polyprotein [Robinvale bee virus 6]	5×10^{-24}	112/421 (26.6 %)
CH	Virus-like contig 259	*Iflaviridae*	Iflaviridae sp.	Chehouv5	PQ206384	4611	183.68	polyprotein [Chequa iflavirus]	0	969/1549 (62.56 %)
CH	Virus-like contig 258	*Orthomavirae*	Orthomavirae sp.	Chehouv4	PQ206383	5161	6152.04	RdRp [Beihai permutotetra-like virus 1]	1×10^{-117}	252/711 (35.44 %)
CH	Virus-like contig 255	*Polycipiviridae*	Polycipiviridae sp.	Chehouv1	PQ206380	10,934	192.53	RNA-dependent RNA polymerase [Riboviria sp.]	0	730/2015 (36 %)
CH	Virus-like contig 257	*Picornavirales*	Picornavirales sp.	Chehouv3	PQ206382	6100	1875.16	polyprotein [Macrobrachium rosenbergii virus 1]	1×10^{-22}	154/614 (25 %)
CQ	Virus-like contig 313	*Hepelivirales*	Hepelivirales sp.	Chequav1	PQ206385	10,866	32359.68	RdRp [Wenling hepe-like virus 4]	6×10^{-130}	312/906 (34.44 %)
CQ	Virus-like contig 317	*Elliovirales*	Elliovirales sp.	Chequav5	PQ206390	5157	313.03	RNA-dependent RNA polymerase [Athtab bunya-like virus]	0	752/1620 (46.42 %)
CQ	Virus-like contig 320	*Elliovirales*	Elliovirales sp.	Chequav5	PQ206391	3621	719.31	glycoprotein N [Athtab bunya-like virus]	0	378/751 (50.33 %)
CQ	Virus-like contig 338	*Ghabrivirales*	Ghabrivirales sp.	Chequav7	PQ206393	5844	215.29	hypothetical protein 3 [Beihai sea slater virus 3]	2×10^{-100}	261/845 (31 %)

Stentiford et al., 2009; Bateman and Stentiford 2017), including crayfish (Bateman et al., 2012). It is characterized by high mortality and fast spread, especially in aquaculture environments, where it leads to devastating economic losses (Liu et al., 2021). Previous studies have shown that recorded occurrences of WSVV in the wild crayfish populations are scarce (Baumgartner et al., 2009; Sasson et al., 2024). The virus was detected previously in pet-traded crayfish; in imported *C. quadricarinatus* specimens from Australia (Mrugała et al., 2015) and in *P. clarkii* individuals from Singapore (Longshaw et al., 2012). In the recent study by Sasson et al., (2024), 50 % of store-bought crayfish (*Procambarus troglodytes* and *P. clarkii*) were infected with WSSV. In our study, WSSV was detected in two out of seven *P. clarkii* individuals by RT-PCR (Supplementary TAble S1). This result could be affected by the mentioned limited ability of detection due to isolation procedures (RNA isolation). The record of WSSV in analyzed samples of *P. clarkii* is concerning. This is not solely because WSSV is classified as a reportable disease by the World Organization for Animal Health (WOAH, 2023), necessitating immediate notification of outbreaks to curb further spread, but also due to the significant impact WSSV can have on crayfish health, potentially leading to substantial losses in crayfish production and wild populations' declines.

3.3.2. Novel picorna-like virus

Virus-like contig 1 (12324 nts), likely representing a near complete sequence of a novel picorna-like virus, was detected in pooled sample of *P. clarkii*. BLASTx query returned Robinvale bee virus 6 (AWK77870.1) obtained from a honeybee *(Apis mellifera*) in Australia (Roberts et al., 2018) as the closest match with 26.6 % sequence identity. Phylogenetic analysis clustered virus-like contig 1 with unclassified members of *Picornavirales* order within family-level cluster f.0012 according to the RVMT global RNA virus phylogenetic analysis (Neri et al., 2022; Fig. 3). This cluster does not contain any viruses that would currently be classified by International Committee on Taxonomy of Viruses (ICTV) to lower taxonomic levels. The viruses that clustered together with virus-like contig 1 in our analysis were previously associated with diverse source materials, predominately arthropods, including crustaceans and crayfish (Shi et al., 2016).

3.4. Viruses detected in Procambarus alleni

Viral-like contigs recovered from *P. alleni* dataset were mostly short (Supplementary TAble S1) and only two were selected for further analysis. Those belonged to two known viruses with unclear association with crayfish as a host, Shahe isopoda virus 5 and a virus from the family *Dicistroviridae*. Both were also found in other species and are discussed above in section "Viruses detected in multiple crayfish species".

The lower viral diversity found in *P. alleni* compared to other crayfish species analyzed in this study (Fig. 1) could be due to a combination of factors. First, it could be a consequence of shorter length of assembled contigs that were not further analyzed here. If this is not a case or additionally, *P. alleni* may have lost its native viral community during translocation (from its native range in North America to Thailand and to aquaculture) due to bottlenecks and/or the inability of native viruses to adapt to the new ecological conditions (Colautti et al., 2004). These processes could have been combined with changes in susceptibility to the local virus community (Kelly et al., 2009). In addition, this species was sampled from aquaculture, where practices such as selective breeding may have contributed to reducing the viral load and diversity in this species (Nguyen 2024). Finally, we analyzed the lowest number of individuals of this species (six *P. alleni* individuals versus seven to ten individuals of other species), which could lead to an underrepresentation of viral diversity.

3.5. Viruses detected in Cherax quadricarinatus

In pooled sample of *C. quadricarinatus* individuals obtained from

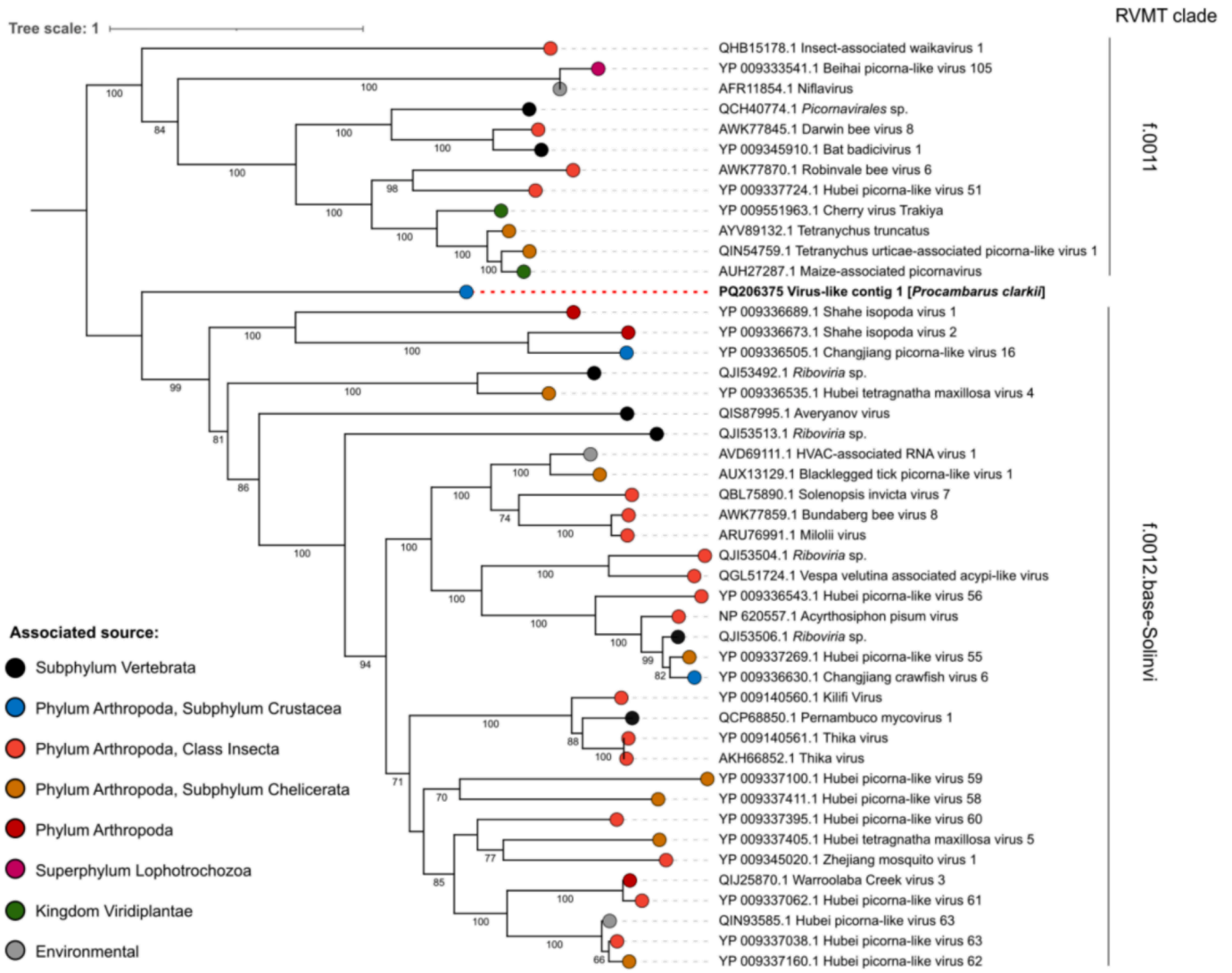

Fig. 3. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences of Virus-like contig 1 from pooled samples of *P. clarkii* and related members from order *Picornavirales*. The tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
RT-PCR results for known viruses detected in ten C. quadricarinatus individuals obtained from Indonesian (Java) aquaculture. Three primer pairs were used for amplifying 551 nts long region of Cherax quadricarinatus reovirus (Hayakijkosol et al., 2021), 207 nts long region of athtab bunya-like virus (Nambiar, Owens, and Elliman 2023), and 104 nts long region of chequa iflavirus (Sakuna, Elliman, and Owens 2017).

	C. quadricarinatus (Cq) individuals									
RT-PCR result	Cq1	Cq2	Cq3	Cq4	Cq5	Cq6	Cq7	Cq8	Cq9	Cq10
Cherax quadricarinatus reovirus		X	X		X	X		X		X
Athtab bunya-like virus	X	X	X		X	X	X	X		X
Chequa iflavirus		X			X	X	X	X		X

Indonesian (Java) aquaculture, we detected virus-like contigs corresponding to three known viruses (Cherax quadricarinatus reovirus, athtab bunya-like virus, chequa iflavirus) and three novel viruses (from orders *Hepelivirales*, *Ghabrivirales* and *Elliovirales*). We did not detect any of those viruses in other analyzed crayfish species. The co-occurrence of these three known viruses – Cherax quadricarinatus reovirus, athtab bunya-like virus and chequa iflavirus – has been previously reported in *C. quadricarinatus* populations in aquaculture and associated with high mortality (Jaroenram et al., 2021b; Nambiar et al., 2023). This emphasizes the global spread of these viruses in aquaculture. Athtab bunya-like virus and chequa iflavirus are of particular concern as they can spread vertically via eggs, making them highly resistant to surface sterilization procedures and susceptible to spread in aquaculture (Jaroenram et al., 2021b). In our study, five out of ten *C. quadricarinatus* individuals were RT-PCR positive for all three viruses, three individuals were positive for one or two viruses and two were virus-free (Table 5).

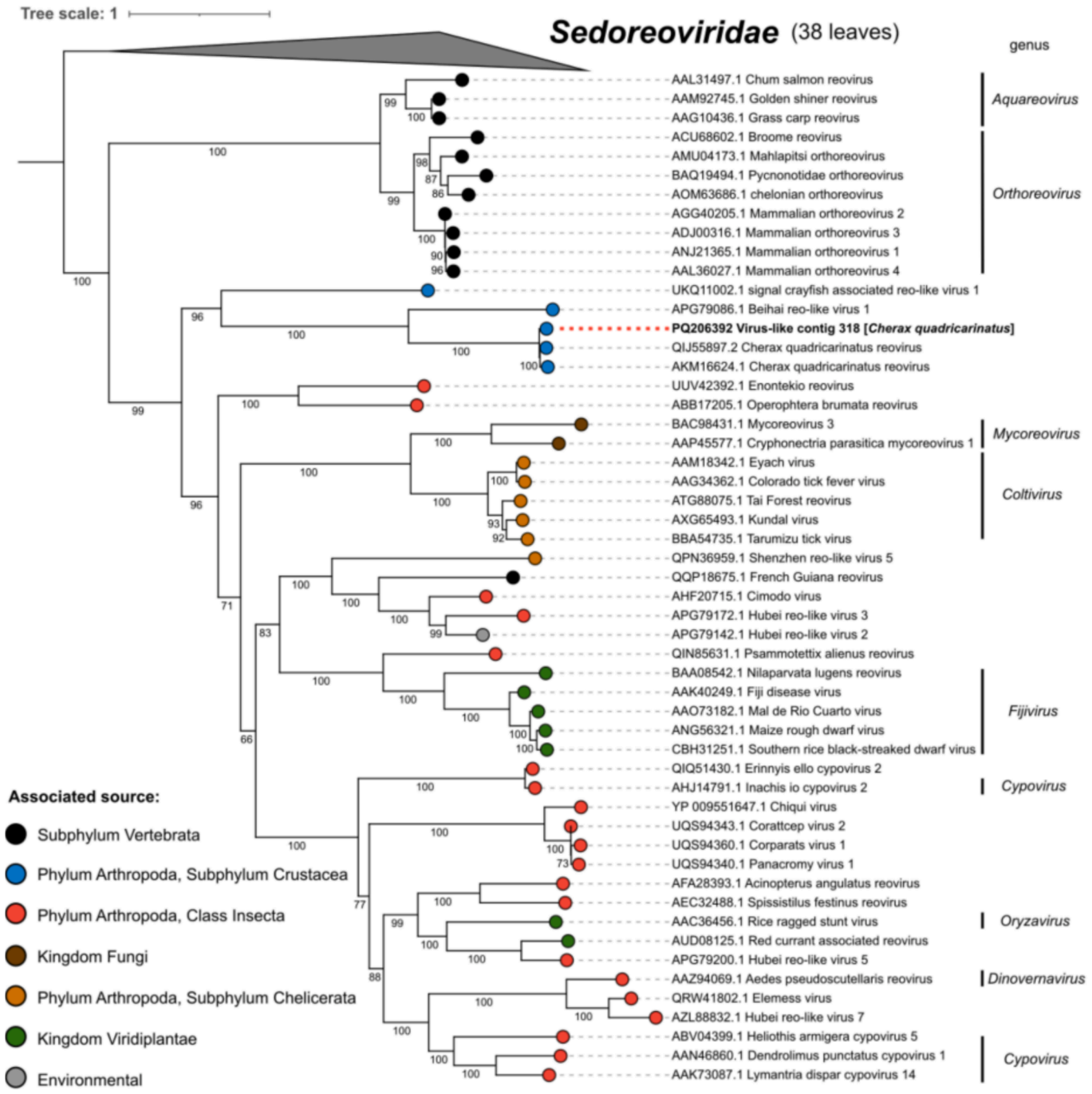

Fig. 4. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences of Cherax quadricarinatus reovirus (Virus-like contig 318) obtained from pooled sample of *C. quadricarinatus* individuals and related members of order *Reovirales*. Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. Members of different genera of the *Spinareoviridae* family are marked on the tree. To reduce the size of the phylogenetic tree, the branch with members of *Sedoreoviridae* family is collapsed. The complete tree is available in Supplementary Fig. S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

This emphasizes the complexity of dealing with viral infections in pet trade and aquaculture, especially given the potential of co-infection to increase mortality.

3.5.1. *Cherax quadricarinatus reovirus*

Virus-like contig 318 (4174 nts), likely representing a near complete sequence of one of the genome segments of Cherax quadricarinatus reovirus, was detected in pooled sample of *C. quadricarinatus* individuals. BLASTn query showed high identity with both available *C*herax quadricarinatus reovirus sequences deposited under accession numbers KM405245.1 (1,456 nts) and MN308286.2 (1,456 nts) (with 99.72 % and 99.45 % identity on nucleotide level) originating from China and Australia (Hayakijkosol et al., 2021). Phylogenetic analysis of conserved segment of RdRp placed Virus-like contig 318, together with the other two known sequences of Cherax quadricarinatus reovirus within *Spinareoviridae* family of *Reovirales* order, into a distinct cluster of

unclassified viruses associated with crustacean hosts (Fig. 4). This cluster contains also Beihai reo-like virus 1 discovered in mantis shrimp (*Stomatopoda*; Shi et al., 2016) and a recently discovered signal crayfish associated reo-like virus 1 associated with signal crayfish in Croatia (Bačnik et al., 2021). Although the genome of members of *Reovirales* order consists of several segments (Matthijnssens et al., 2022), we were only able to recover the one containing RdRp sequence, which is also the only genome segment available for above mentioned closely related viruses. To our knowledge, this is nonetheless the longest available sequence of Cherax quadricarinatus reovirus deposited in NCBI GenBank, expanding the previously known genome segment for 2718 nts. In our study, Cherax quadricarinatus reovirus was detected in six out of ten *C. quadricarinatus* individuals by RT-PCR (Supplementary Table S1), suggesting that this virus might have relatively high prevalence in traded animals. First report of reovirus-like particles in *C. quadricarinatus* with associated disease symptoms comes from Australia from 1990 s (Edgerton et al., 2000), where it was later also demonstrated that viruses of such morphology can cause necrosis of hepatopancreocytes and inflammatory cells (Hayakijkosol et al., 2021). In those two studies, no sequence data was generated. Later, a partial genomic sequence of Cherax quadricarinatus reovirus was reported from China in 2014 (NCBI GenBank: KM405245) and Australia in 2019 (NCBI GenBank: MN308286). Recently, new diagnostic tests have been proposed for detection of the virus (Hayakijkosol et al., 2021; Jaroenram et al., 2021a) and it was shown that Cherax quadricarinatus reovirus can be transmitted through fecal-oral route, but not via eggs (Jaroenram et al., 2021b). A related virus was discovered in signal crayfish exhibiting hepatopancreatitis in Croatia (Bekavac et al., 2022), although a direct causal link with the disease was not yet established (Bačnik et al., 2022). In summary, available information indicates that this virus might represent an important crayfish pathogen, warranting surveys in aquaculture and the pet trade to assess its prevalence and potential impacts on crayfish health.

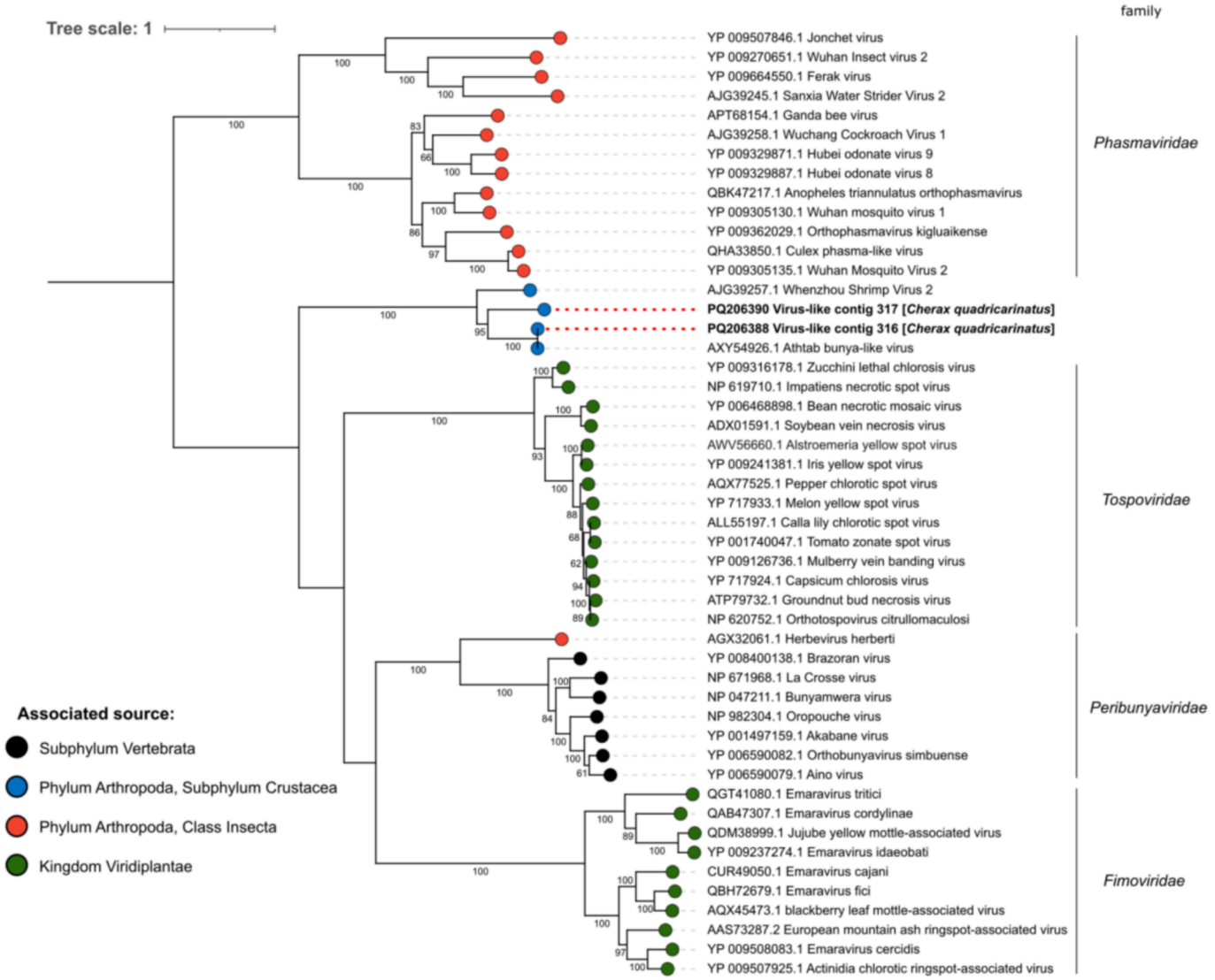

Fig. 5. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequence retrieved from L segment of athtab bunya-like virus (Virus-like contig 316) and Virus-like contig 317 sequences obtained from pooled samples of *C. quadricarinatus* hepatopancreas and members of *Elliovirales* order. Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. Members of different families of the *Elliovirales* order (class *Bunyaviricetes*) are marked on the tree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5.2. Athtab bunya-like virus

Four virus-like contigs were recovered from pooled sample of *C. quadricarinatus* individuals with high similarity to members of the *Elliovirales* order. Results of BLASTn query returned athtab bunya-like virus L segment (NC_040754.1) and M segment (NC_040755.1) obtained from *C. quadricarinatus* (Sakuna et al., 2018) as the best matches for Virus-like contig 316 (6809 nts) and Virus-like contig 321 (3582 nts), with 97.42 (NC_040754.1) and 93.91 % (NC_040755.1) identity scores on nucleotide level, likely representing the same virus. Athtab bunya-like virus sequences were detected in eight out of ten *C. quadricarinatus* individuals by RT-PCR (Supplementary Table S1), indicating its high prevalence in the sampled population.

The best matches of Virus-like contig 317 (5157 nts) and Virus-like contig 320 (3621 nts) according to BLASTx were also athtab bunya-like virus L segment sequence (YP_009553307.1) and M segment (YP_009553308.1) with 46.42 and 50.33 % identity on amino acid level, respectively. Although further research is needed and species demarcation criteria are not well defined, we currently assume that Virus-like

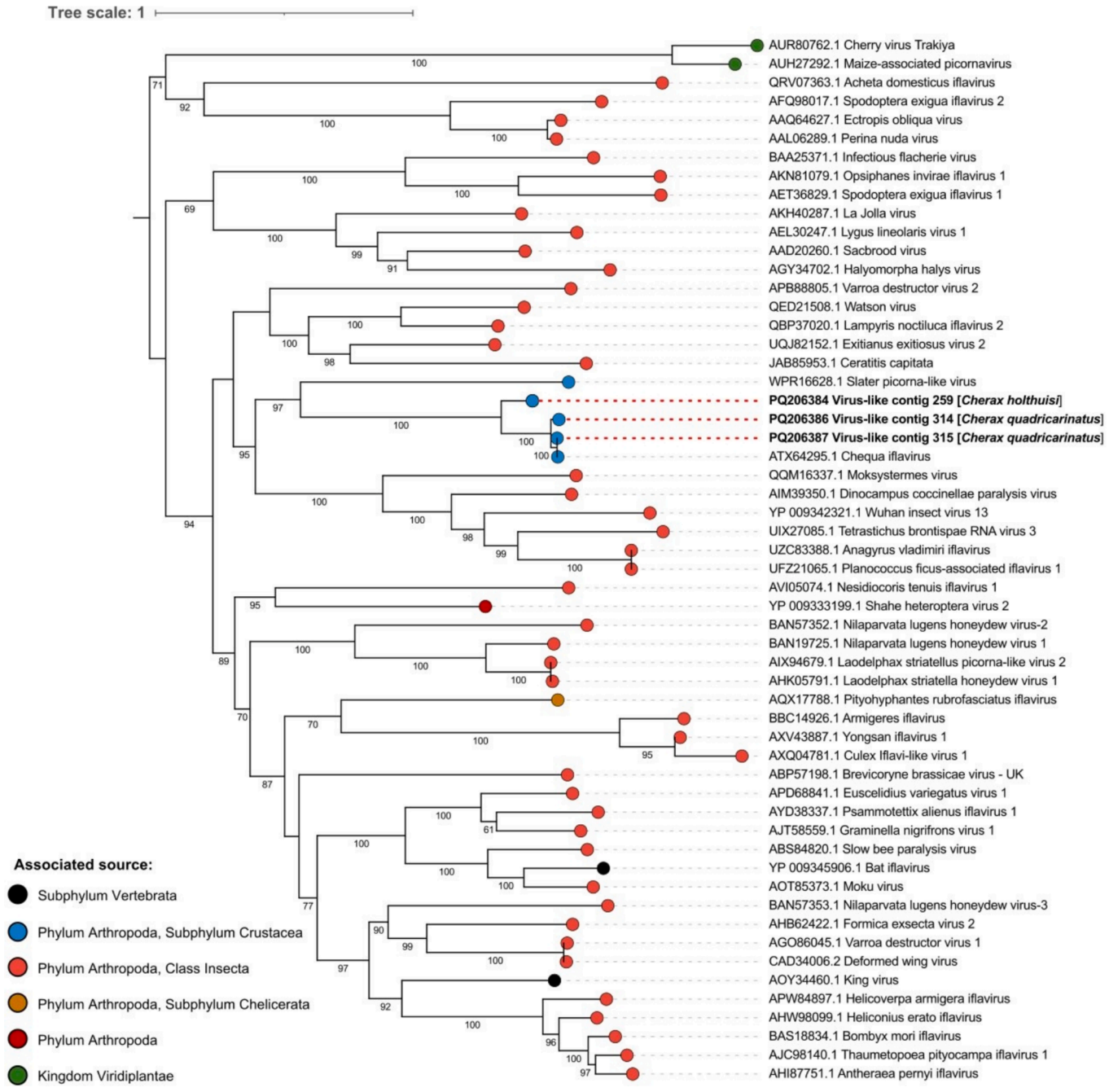

Fig. 6. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences conserved segment of chequa iflavirus (Virus-like contig 314 and Virus-like contig 315) from pooled sample of *C. quadricarinatus* individuals and Virus-like contig 259 obtained from pooled sample of *C. holthuisi* individuals and related members of *Iflaviridae* family. Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

contig 317 and Virus-like contig 320 represent different segments of the same novel bunya-like virus.

Phylogenetic analysis of conserved segments of RdRp sequence from L segment placed virus-like contig 316 and virus-like contig 317 into distinct cluster of unclassified viruses associated with crustacean hosts (Fig. 5) in the *Elliovirales* order (class *Bunyaviricetes*). This cluster seems to be divergent from other known bunya-like viruses, and among known viruses, it seems to be most closely related to members of *Tospoviridae* and *Fimoviridae* families infecting plants and *Peribunyaviridae* family infecting vertebrates. Athtab bunya-like virus was first reported from stressed *C. quadricarinatus* in Australia (Sakuna et al., 2018), where high titer of the virus was found in muscle tissue with lesions. However, a direct link between the virus and disease has not yet been established. In the same study the virus was found in coinfection with chequa iflavirus, as is the case also in our study (see next section). The detection of the same virus in *C. quadricarinatus* in our study, along with the discovery of a novel virus in the same clade, reinforces the association of the virus with this crayfish species. However, further research is needed to explore the connection between the two viruses and their effects on crayfish health.

Other crustacean associated bunya-like viruses from *Phenuiviridae* family (*Hareavirales* order) were also discovered recently; bunya-like brown spot virus was associated with a massive outbreak in a French population of the endangered, white-clawed crayfish (*Austropotamobius pallipes*; Grandjean et al., 2019), and a closely related Astacus bunya-like virus 1, which was not associated with mortality in noble crayfish (Zingre et al., 2023).

3.5.3. *Chequa iflavirus*

Two virus-like contigs with high similarity to chequa iflavirus (Virus-like contig 314, 9324 nts and Virus-like contig 315, 9129 nts) were recovered from the pooled sample of *C. quadricarinatus* individuals. According to BLASTn results, aligned part of Virus-like contig 314 had 95.74 % identity and aligned part of Virus-like contig 315 had 84.44 % identity to the chequa iflavirus (NC_036389), associated with *C. quadricarinatus* (Sakuna et al., 2017). In our study, chequa iflavirus was detected in six out of ten *C. quadricarinatus* individuals by RT-PCR (Supplementary Table S1). In the phylogenetic analysis, both sequences clustered together with a known sequence of chequa iflavirus and with another sequence from *C. holthuisi* recovered in this study (described in the next section). All sequences represented a distinct cluster associated with crustaceans (Fig. 6), while most other known phylogenetically related members of the *Iflaviridae* family are associated with insects (Valles et al., 2017). Given the limited understanding of the phylogenetic relationships in the *Iflaviridae* family, which do not display an obvious host-based evolutionary relationship (Valles et al., 2017), it cannot be ruled out that chequa iflavirus originated from a recent horizontal transfer between insects and freshwater crayfish, as both often share overlapping ecosystems (e.g., water bodies).

Chequa iflavirus was first discovered in *C. quadricarinatus* in Australia (Sakuna et al., 2017), in coinfection with athtab bunyavirus, as mentioned earlier. However, a clear link with a disease has not yet been established. It has also been suggested that the virus is circulating in *C. quadricarinatus* as documented in 1990 s, without causing significant mortalities (Sakuna et al., 2017). Recently, crayfish individuals resistant to chequa iflavirus were discovered (Nabiar et al., 2023).

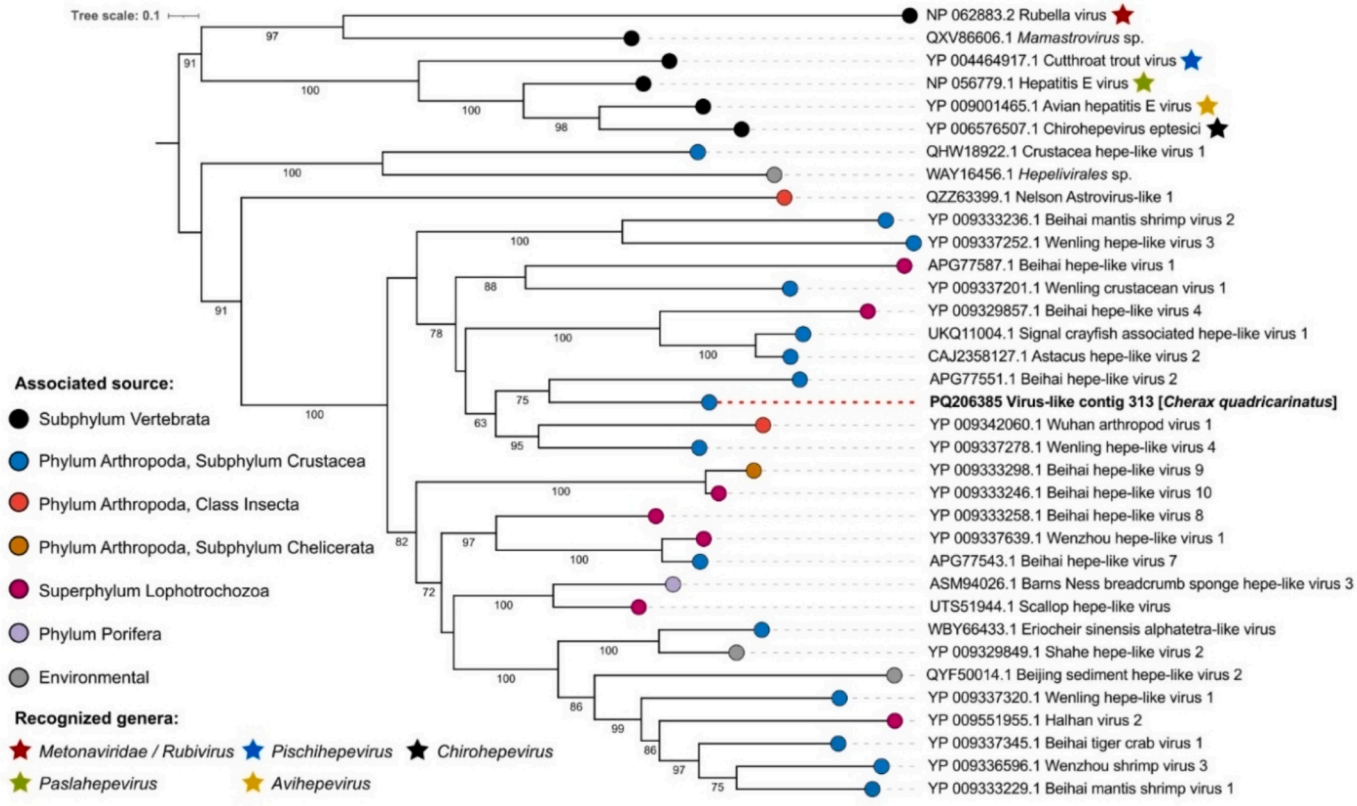

Fig. 7. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences of the Virus-like contig 313 obtained from pooled sample of *C. quadricarinatus* individuals and related members of *Hepelivirales* order. Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5.4. Novel hepe-like virus

Virus-like contig 313 (10866 nts), likely representing a near complete genome of a novel virus from *Hepelivirales* order, was detected in pooled sample of *C. quadricarinatus* individuals. BLASTx query returned Wenling hepe-like virus 4 (YP_009337278.1) isolated from crustacean host in China (Shi et al., 2016) as the highest scoring match with 34.44 % sequence identity on amino acid level. Phylogenetic analysis placed Virus-like contig 313 into a wider clade of virus-like sequences belonging to the *Hepelivirales* order, predominantly associated with crustacean hosts or other organisms associated with aquatic environments (Fig. 7). Assessing the significance of presence of this virus in crayfish is challenging, as closely related species and members of the extended clade (Shi et al., 2016; Bačnik et al., 2022; Zingre et al., 2023) were primarily identified through metagenomic studies. In addition, except for crustacean hepe-like virus 1 isolated from *Macrobrachium rosenbergii* exhibiting growth retardation (Dong et al., 2020), there are no reports of viruses belonging to *Hepelivirales* order being associated with pathological symptoms of crustaceans. In summary, further research is needed to experimentally confirm the association between several recently discovered hepe-like viruses (Shi et al., 2016, Bačnik et al., 2022; Zingre et al., 2023; Guo et al., 2025) and freshwater crayfish hosts and gain understanding of virus transmission and the potential impact on crayfish.

3.5.5. Novel virus from Ghabrivirales order

Virus-like contig 338 (5844 nts), likely representing a near complete sequence of genome segment of a novel virus from the *Ghabrivirales* order, was detected in pooled sample obtained from *C. quadricarinatus* individuals. BLASTx query returned Beihai Sea slater virus 3 (YP_00933402.1) isolated from wharf roach (*Ligia exotica*) in China (Shi et al., 2016) as the highest scoring match with 31 % sequence identity on amino acid level. Phylogenetic analysis placed virus-like contig 338 into a clade of sequences from *Ghabrivirales* order associated with arthropod hosts, with closest related viruses being associated with crustacean hosts (Fig. 8). Assessing the significance of host status for closely related viruses is difficult, as the closest relatives were identified through metagenomic studies (Shi et al., 2016). However, a few viruses from the *Ghabrivirales* order have been reported to cause disease in crayfish, including Cherax giardiavirus-like virus (Edgerton et al., 1994) and infectious myonecrosis virus, which causes a notifiable disease, infectious myonecrosis, in penaeid shrimp, as recognized by WOAH and FAO (Prasad et al., 2017). Additionally, Callinectes sapidus toti-like virus 1 and 2 have more recently been associated with pathological changes in Atlantic blue crab species (*Callinectes sapidus;* Zhao et al., 2022).

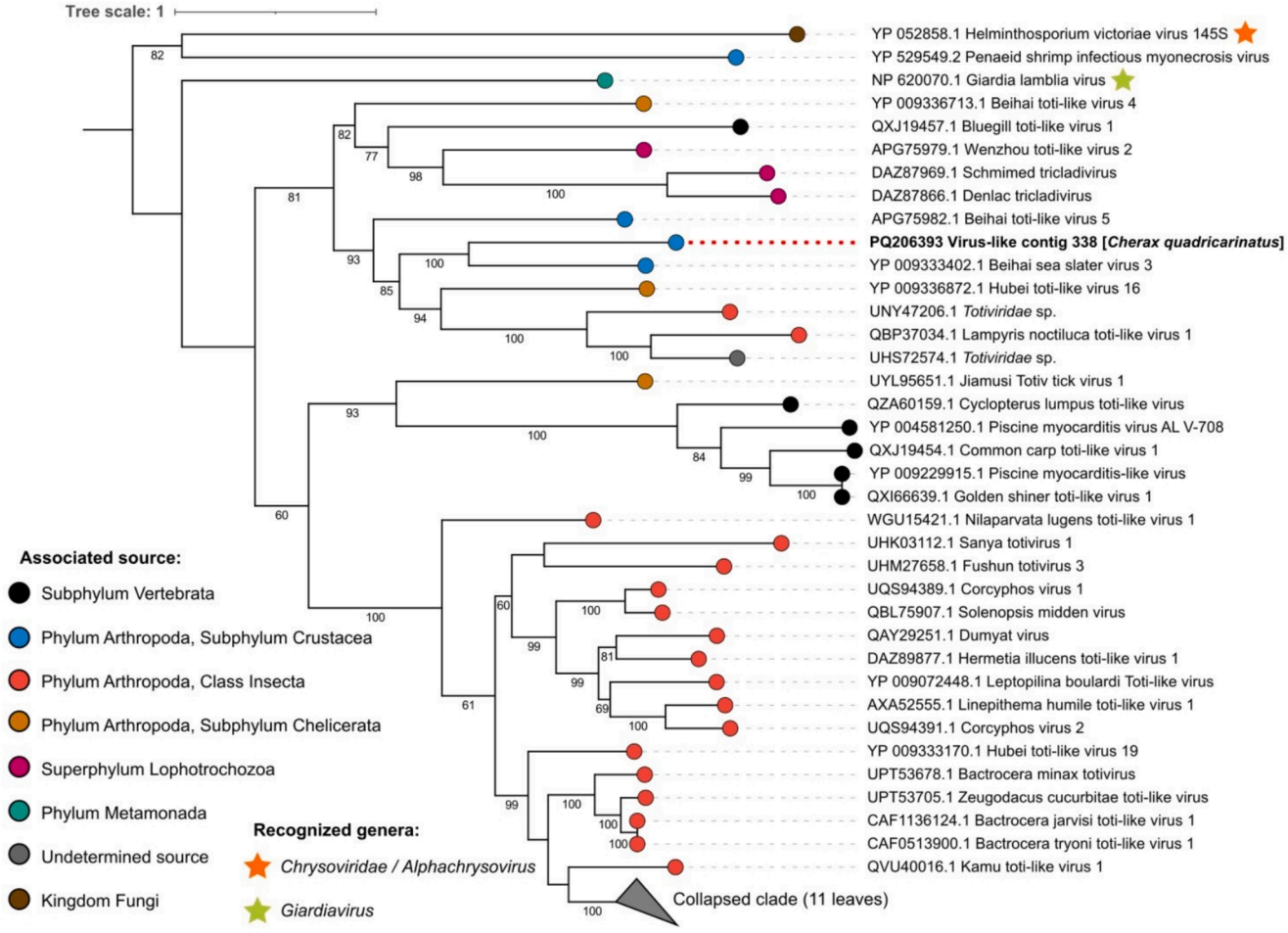

Fig. 8. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences of Virus-like contig 338 obtained from pooled samples of *C. quadricarinatus* and related members of *Ghabrivirales* order. Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. To reduce the size of the phylogenetic tree, the bottom clade containing the members from the same geographical source (China) associated with host organisms from phylum Arthropoda, class Insecta is collapsed. The complete tree is available in Supplementary Fig. S4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Viruses detected in Cherax holthuisi

We recovered diverse set of virus-like contigs from the dataset obtained from pooled sample of *C. holthuisi* individuals, which is an endemic crayfish from the Aitinjo Lake in Indonesian New Guinea. As mentioned above, short contigs of a known Shahe isopoda virus 5 were recovered from this sample and the presence of the virus was confirmed by RT-PCR in two out of ten sampled individuals. The detection of Shahe isopoda virus 5 in *C. holthuisi* hepatopancreas samples, also detected in *P. clarkii* and *P. allenii*, could be due to environmental contamination or incidental contact with this virus during rearing and packaging in the pet trade chain. Previous studies have shown that host's microbial community may become altered under specific stressful conditions often encountered during transport of organisms (i.e., long transport of multiple species, shared water during transport or rearing; Emmenegger et al., 2024). Otherwise, this crayfish species had the most distinct virome composition and was associated with the highest number of previously unknown viruses, possibly a consequence of its isolated native distribution. Studies have shown that isolation often leads to a distinct virome due to restricted gene flow, environmental pressure and evolutionary dynamics (Ter Horst et al., 2023; Robinson et al., 2024). Contigs corresponding to five previously unknown viruses (one from family *Iflaviridae*, one from *Polycipiviridae* family, two from *Picornavirales* order and one from *Orthornavirae* kingdom, which seem to be related to members of *Permutotetraviridae* family) were further analyzed.

3.6.1. Hubei tetragnatha maxillosa virus 1

Virus-like contig 256 (6700 nts), representing a partial genome sequence of a virus belonging to the *Picornavirales* order, was recovered from pooled sample of *C. holthuisi*. The highest scoring BLASTx and BLASTn matches of Virus-like contig 256 showed 88 % sequence identity on amino acid level (YP_009336621.1) and 83 % sequence identity on nucleotide level (KX883296.1) with Hubei tetragnatha maxillosa virus 1 isolated from arthropod mix in China (Shi et al., 2016). Phylogenetic analysis placed Virus-like contig 256 among unclassified members of *Picornavirales* order belonging to family-level clade f.0055 according to the RVMT global RNA virus phylogenetic analysis (Fig. 9, Supplementary Fig. S5; Neri et al., 2022). The genus or species demarcation criteria have not yet been established for related viruses, which makes it difficult to determine whether the obtained sequence could be classified as potentially novel given the relatively high identity with a Hubei tetragnatha maxillosa virus 1 (Shi et al., 2016). According to obtained

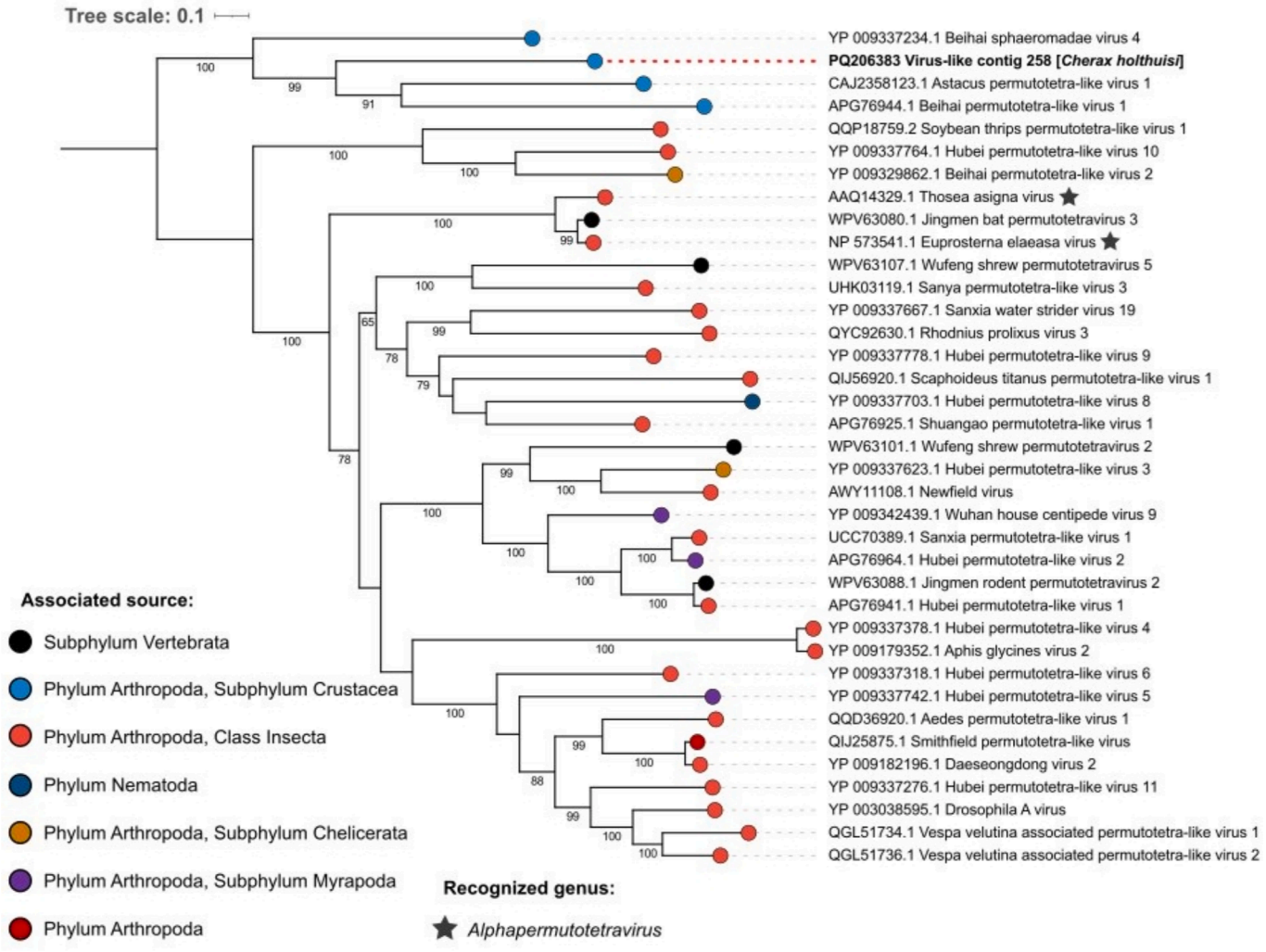

Fig. 9. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences of the virus-like sequence from *Orthornavirae* kingdom related to the members of *Permutotetraviridae* family (Virus-like contig 258) obtained from pooled sample of *C. holthuisi* individuals and *Permutotetraviridae* related members of *Orthornavirae* kingdom. Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

metadata, other related viruses from the same clade were isolated from either vertebrate or insect hosts. However, it must be noted that most viral sequences associated with vertebrate hosts were obtained from mammalian feces except for closely related fisavirus 1 (YP_009111312.1), which was obtained from intestinal content of freshwater carp (Reuter et al., 2015).

3.6.2. Novel viruses in C. Holthuisi

BLASTx query of Virus-like contig 259 (4611 nts) recovered from pooled sample of *C. holthuisi* individuals returned chequa iflavirus (YP_009444707.1) as the best match with 62.99 % sequence identity on amino acid level. Phylogenetic analysis clustered the recovered sequence, together with sequences of chequa iflavirus from this and other studies, in a larger clade alongside viruses recovered from source organisms associated with aquatic environments (Fig. 6). Due to the low sequence identity and the species demarcation criteria of *Iflaviridae* family (Valles et al., 2017), Virus-like contig 259 represents a novel virus with moderate sequence identity to chequa iflavirus, adding data to this little-studied lineage of iflaviruses associated with freshwater crayfish hosts.

Virus-like contig 258 (5161 nts) likely representing a partial genome sequence of a novel virus belonging to the *Orthornavirae* kingdom related to members of *Permutotetraviridae* family was detected only in pooled sample of *C. holhuisi*. BLASTx yielded Astacus permutotetra-like virus 1 (CAJ2358123.1) originating from *Astacus astacus* in Switzerland (Zingre et al., 2023) and Beihai permutotera-like virus 1 (APG76944.1) originating from sesarmid crab (Sesarmidae) in China (Shi et al., 2016) as the best matches with 37.47 and 35.44 % amino acid identity, respectively. Although *Permutotetraviridae* family is currently monotypic (contains only one genus), with only two species confirmed, several studies identified virus-like sequences closely resembling that family based on RdRp sequence similarity (Chen et al., 2022; Shi et al., 2016; Zingre et al., 2023). Due to absence of order, class and phylum taxonomic ranks of *Permutotetraviridae*, the Virus-like contig 258 can be currently placed only in *Orthornavirae* kingdom. According to phylogenetic analysis, viruses related to the members of the *Permutoteraviridae* family are most frequently associated with insects (King et al., 2012) and other invertebrate hosts. As can be seen in Fig. 10, viral *Permutoteraviridae* sequences associated with crustaceans, including Virus-like contig 258, form a distinct clade, suggesting that members of this branch could potentially represent a new family pending further characterization and taxonomic validation.

Virus-like contig 255 (10934 nts) representing a near complete genome sequence of a novel virus from *Polycipiviridae* family was detected in pooled sample obtained from *C. holthuisi* individuals. BLASTx query returned *Riboviria* sp. (WKV34499.1) partial sequence obtained from the bird metagenome in China as the highest scoring match with 36 % identity on amino acid level (not included in the phylogenetic analysis due to its short length). Phylogenetic analysis placed Virus-like contig 255 in the cluster with the members of *Polycipiviridae* family (Fig. 11) together with most closely related arachnid associated members of *Chipolycivirus* genus. A broader cluster contains also ant-specific members of *Sopolycivirus* and insect- and crustacean-associated members of *Hupolycivirus* genera (Olendraite et al., 2019, Temmam et al., 2019), the latter containing only one confirmed species obtained in a metagenomic survey both from Odonata (Arthropoda, Insecta) and crayfish (Shi et al., 2016).

Another partial genomic sequence (Virus-like contig 257, 6100 nts) of a novel virus belonging to *Picornavirales* order was recovered from pooled hepatopancreas sample of *C. holthuisi*. The highest scoring BLASTx match for virus-like contig 257 was Macrobrachium rosenbergii virus 1 (UUV42128.1) with 25 % identity on amino acid level. This virus was abundantly detected both in growth retarded and healthy giant freshwater prawn (*M. rosenbergii*) individuals in China (Zhou et al., 2022). Phylogenetic analysis placed Virus-like contig 257 in a distinct branch of other unclassified viruses from *Picornavirales* order detected in a metagenomic study from crustacean sources (Shi et al., 2016), related to the members of *Solinviviridae* family (Fig. 12). There are currently

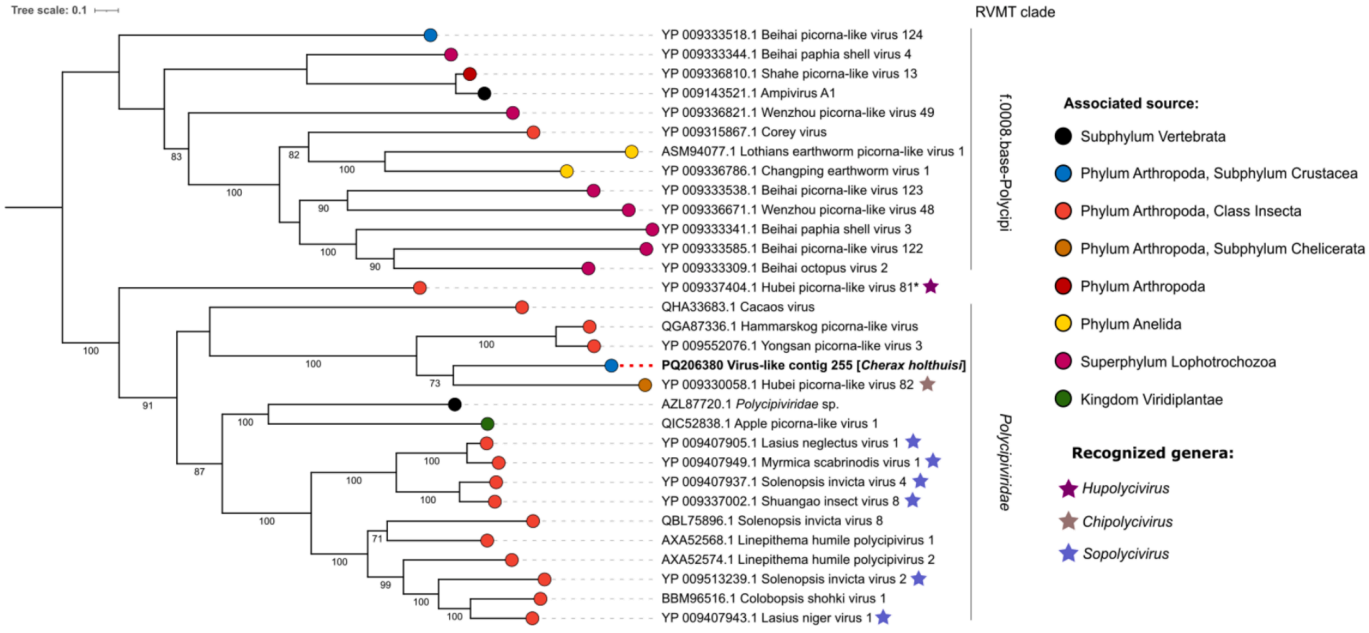

Fig. 10. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences of Virus-like contig 255 from pooled samples of *C. holthuisi* and related members of *Polycipiviridae* family. Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. Virus marked with asterisk (*) was also associated with crayfish source. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

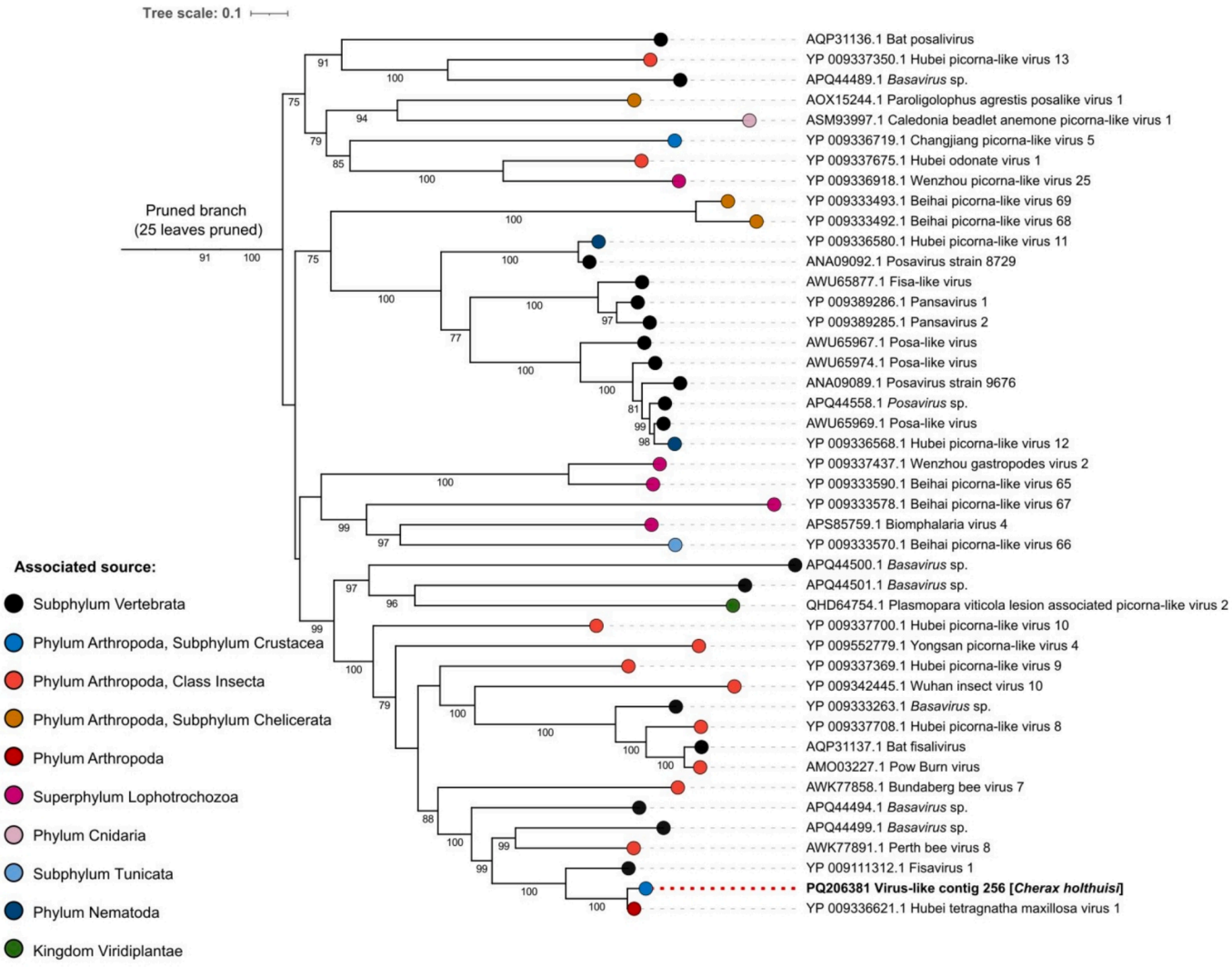

Fig. 11. P Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences of Virus-like contig 256 from pooled samples of C. holthuisi and related members of Picornavirales f.0035 clade (Neri et al., 2022). Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. To reduce the size of the phylogenetic tree, only a part of a pruned tree is presented. The complete tree is available in the Supplementary Fig. S5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

only two classified species in two genera that infect ants in *Solinvivirviridae* family, however many related unclassified virus sequences were obtained from variety of arthropods (Sõmera et al., 2021), including crustaceans (Cruz-Flores et al., 2022).

3.7. Biosecurity implications related to viromes of crayfish in pet-trade

This study uncovered a high diversity of viruses in pet-traded crayfish species, including both known crayfish pathogens and eight novel viruses. These findings align with other crayfish virome studies (Bačnik et al., 2022; Zingre et al., 2023; Dong et al., 2024; Guo et al., 2025) and highlight just how understudied viruses in crayfish and other aquatic species are (Zhang et al., 2022). The high diversity of viruses observed in analyzed crayfish species along with findings from other studies (Longhaw 2012; Mrugała et al., 2015; Gippet and Bertelsmeier, 2021; Sasson et al., 2024) underscores that pet trade is highly likely an important introduction pathway of known aquatic pathogens and diseases, as well as a potential source of many unknown and potentially harmful microbes. Our results emphasize the risk of viral disease outbreaks due to intentional or unintentional release of pet-traded crayfish species into the environment (Vodovsky et al., 2017; Haubrock et al., 2021). These viruses might spread to native crayfish species – a mechanism known as pathogen spillover (Prenter et al., 2004), potentially causing outbreaks or declines in endangered populations. The import of prohibited invasive species (*P. clarkii*), which was misidentified and sold under different name, infected with WSSV, serves as an alarming example of risks associated with crayfish pet trade. Additionally, the introduction of new viruses into ecosystems can disrupt the existing microbial dynamics, potentially leading to unpredictable long-term effects on biodiversity (Jones et al., 2015) and ecosystem health (French and Holmes, 2020; French et al., 2025). This risk is particularly evident in the trade of wild-caught and endemic species like *C. holthuisi* in our case, which exhibited the most distinct virome of all analyzed species, nearly entirely composed of novel viruses.

These findings can have important implications for disease management in both crayfish aquaculture and the pet trade. Currently, preventive and control measures in aquaculture primarily focus on well-known pathogens, while disease control in the pet trade is largely

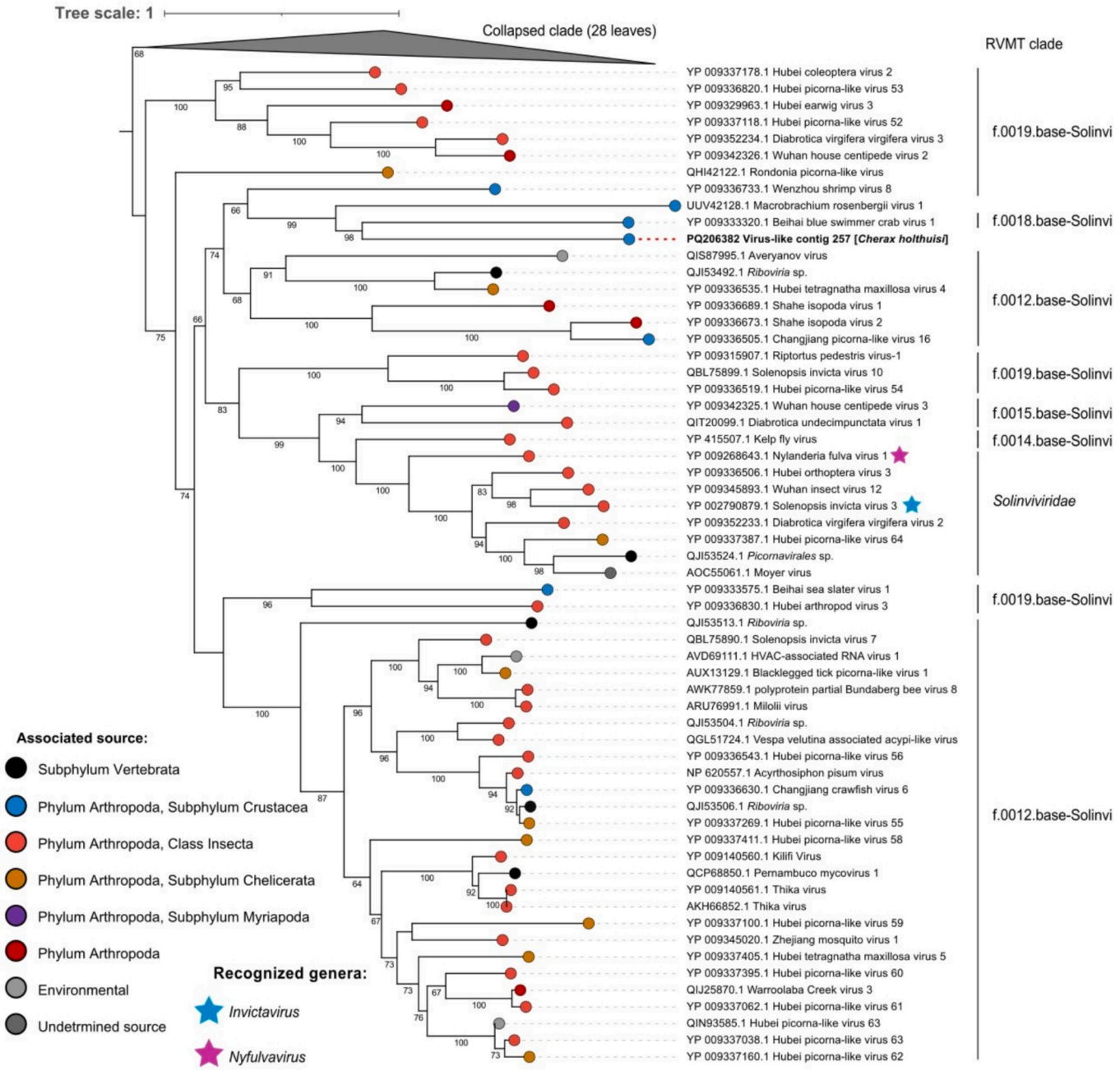

Fig. 12. Maximum likelihood phylogenetic analysis of the RdRp amino acid sequences of Virus-like contig 257 from pooled samples of C. holthuisi and members of Solinviviridae family (Neri et al., 2022). Tree is rooted at mid-point. Values next to the branches represent bootstrap (%) support values (those higher than 60 % are shown), while the branch length represents the average number of amino acid substitutions per site. Tip labels are color coded according to the associated source samples as shown in the legend. The dotted line connecting the leaf corresponding to viral sequence from this study and its label is shown in red. In labels, NCBI Genbank accessions are given in front of contig or virus names. To reduce the size of the phylogenetic tree, a part of the tree is collapsed. The complete tree is available in the Supplementary Fig. S6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

insufficient (Kotovska et al., 2016). However, viruses detected in our study (including WSSV) along with other research (Dong et al., 2024) highlight the need for more comprehensive biosecurity surveillance in the pet trade, including the monitoring of viruses. This remains a challenging task due to the highly diverse and largely unexplored viromes of freshwater crayfish, the limited understanding of their ecology, etiology and the associated risks as well as logistical challenges, insufficient regulatory frameworks, lack of infrastructure and high associated costs.

Data Availability Statement

All sequencing data that support the findings of this study are linked under the accession number PRJNA1142220 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/sra, accessed on 31 January 2025). The sequences of selected viral contigs identified in this study have been deposited in the GenBank under the accession numbers PQ206375.1 – PQ206401.1.

CRediT authorship contribution statement

Katarina Bačnik: Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis, Data curation. **Luka Kranjc:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation. **Leticia Botella:** Writing – review & editing, Funding acquisition, Formal analysis. **Ivana Maguire:** Writing – review & editing, Conceptualization. **Dora Pavić:** Writing – review & editing, Formal analysis. **Jiří Patoka:** Writing – review & editing, Conceptualization. **Paula Dragičević:** Writing – review & editing. **Martin Bláha:** Writing – review & editing. **Ana Bielen:** Writing – review & editing, Supervision, Conceptualization. **Antonín Kouba:** Writing – review & editing, Project administration, Conceptualization. **Denis Kutnjak:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition. **Sandra Hudina:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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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.2025.108345.

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