



Amphipoda and the *Riboviria*: viral diversity on a global scale[☆]

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ABSTRACT

RNA viruses are the most abundant group of pathogens on the planet; however, little is known about their association with amphipods (Crustacea: Peracarida). Here, we explore RNA virus diversity across invasive and native amphipod species inhabiting freshwater, marine, brackish and terrestrial environments around the globe, and analyse this data in relation to host invasion status, environment type, and between host taxa. We sampled tissue from 8 amphipod populations and screened an additional 152 from NCBI for RNA viruses. In total, 412 viral contigs were recovered, including 134 novel virus species, 41 with complete genomes. Viral contigs were associated with the: *Articulavirales* (n = 35); *Bunyavirales* (n = 38); *Durnavirales* (n = 6); *Ghabrivirales* (n = 28); *Hepelivirales* (n = 43); *Jingchuvirales* (n = 16); *Martellivirales* (n = 4); *Mononegavirales* (n = 139); *Nidovirales* (n = 1); *Nodamuvirales* (n = 2); *Picornavirales* (n = 97); *Wolframvirales* (n = 3).

The alpha diversity of RNA viruses significantly differed between environments (freshwater, marine, and brackish) and amphipod host species. Host invasion status was not a significant driver in viral diversity. β diversity analysis revealed no significant difference in multivariate dispersion between environments, suggesting that freshwater, marine and terrestrial environments are equally rich in amphipod virus diversity; however, environment did have a significant effect on the viral community composition. This study contributes to our view of viral evolution and diversification among the Crustacea and boosts our understanding of RNA virus diversity and host range associated with invasion ecology and disease ecology within benthic communities.

1. Introduction

Our knowledge of the RNA virosphere continues to expand, providing novel insights into the evolutionary history of many viral families, whilst broadening our understanding of RNA virus biodiversity across hosts and environments (Koonin et al., 2006; Wolf et al., 2018). Previously, RNA virus discovery was focused on vertebrates due to their potential impact on public health and global economies (Shi et al., 2018); however, metatranscriptomic studies on the RNA virosphere associated with invertebrates have begun to bridge this knowledge gap

(Shi et al., 2016; Jackson et al., 2022). Knowledge of RNA virus diversity within the taxonomic order Amphipoda remains limited.

Crustaceans host a range of RNA viruses (from families: *Reoviridae* (Vago, 1966), *Bunyavirales* (Bojko et al., 2019b), *Rhabdoviridae* (Jahromi, 1977), *Paramyxoviridae* (Johnson and Farley, 1980), *Dicistroviridae* (Zhang et al., 2011), *Picornaviridae* (Johnson, 1984), *Totiviridae* (Lightner et al., 2004), *Roniviridae* (Chantanachookin et al., 1993) and *Nodaviridae* (Qian et al., 2003), primarily associated with economically important hosts (Bateman and Stentiford, 2017). The virosphere study performed by Shi et al. (2016) screened 21 crustacean libraries,

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Mononegavirales

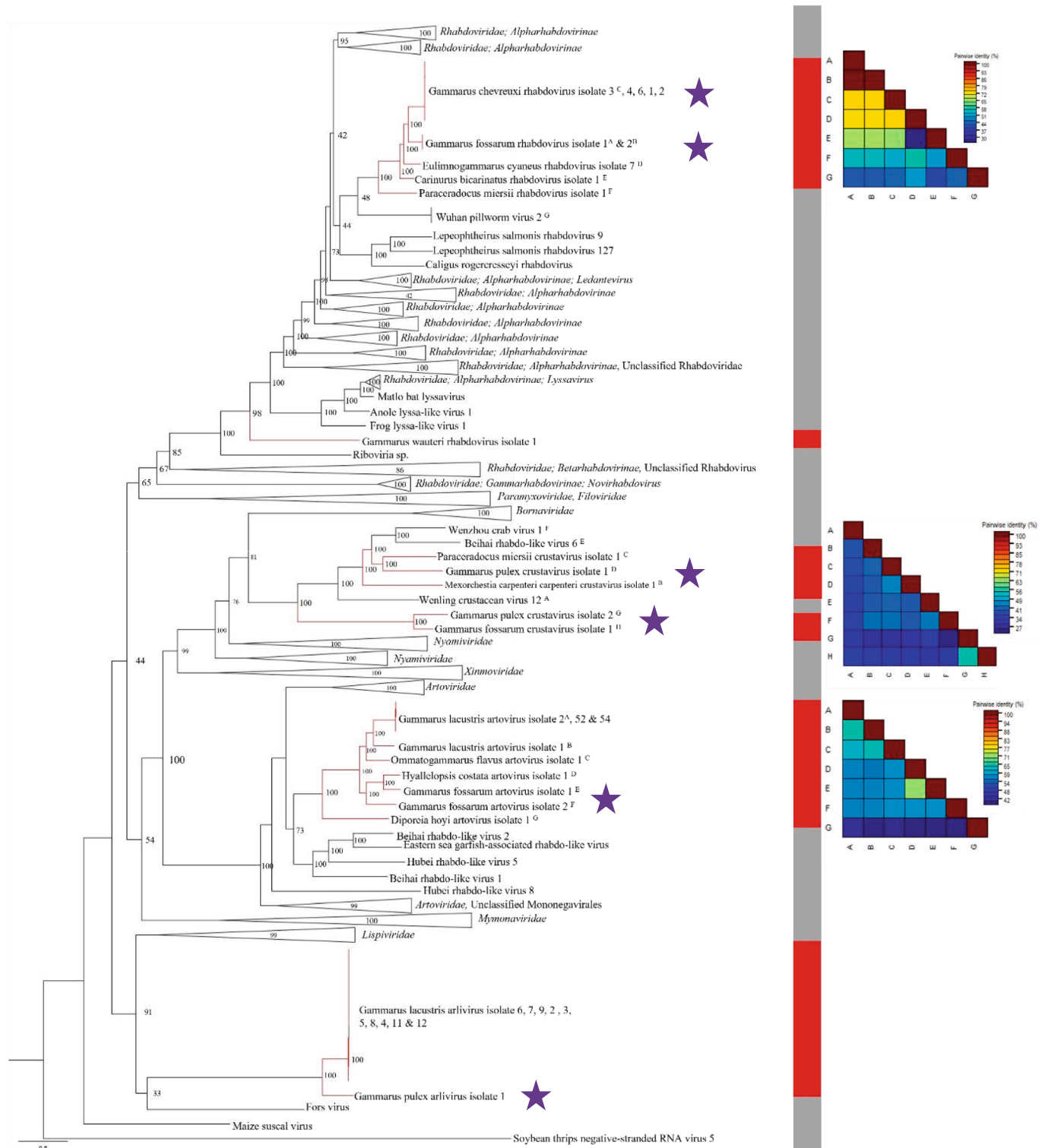


Fig. 1. An RdRp based phylogeny of the *Mononegavirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. Pairwise similarity matrices for three phylogenetic clades, containing novel viruses, are also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

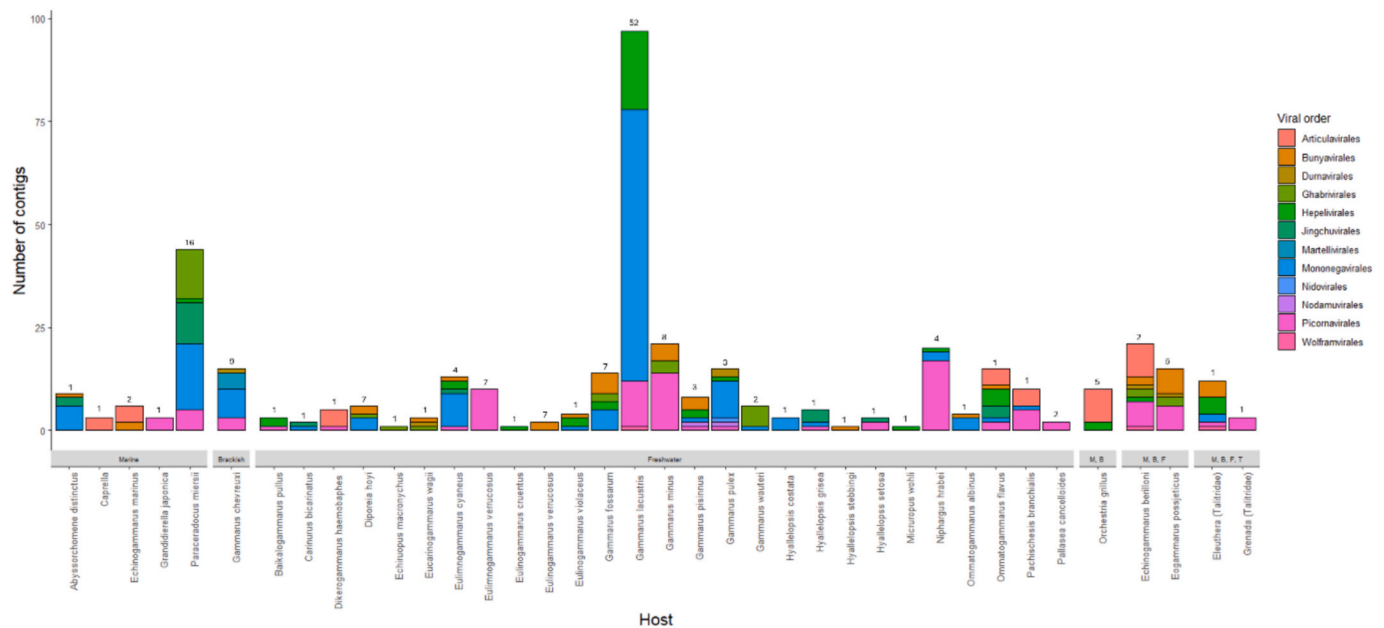


Fig. 2. A representation of the diversity of RNA viral orders discovered in amphipod hosts, by viral contig. Each viral order is displayed as a colour. Number on each bar relates to the number of SRA datasets screened for each host species. Hosts are split by habitat type as listed by the World Register of Marine Species (WoRMS) (M, Marine; B, Brackish; F, Freshwater).

uncovering 76 novel crustacean-infecting RNA viruses. Wild crustacean virus diversity is important to understand possible sources of viruses that emerge in fisheries and aquaculture (Stentiford, 2012; Stentiford et al., 2017; Behringer et al., 2020; Duermit-Moreau et al., 2024) and to improve understanding of viral ecology and pathogenesis across crustacean communities.

Outside of a handful of amphipod-infecting DNA viruses (e.g., *Dikerogammarus haemobaphes nudivirus*, *Dikerogammarus haemobaphes mininucleovirus*, *Dikerogammarus haemobaphes amphinimavirus*, *Gammarus roeselii bacilliform virus*, ‘*Diporeia* CRESS-DNA viruses’); virus diversity is largely unexplored in amphipods (Bistolos et al., 2017; Bojko et al., 2017; Bojko et al., 2019a; Allain et al., 2020; Subramaniam et al., 2020; Warren et al., 2023; Burgess and Bojko, 2024; Warren et al., 2022; Burgess et al., 2026; Petersen et al., 2024). Two flaviviruses have been detected in *Gammarus chevreuxi* and *Gammarus pulex* (Parry and Asgari, 2019). *Diporeia* spp. have been shown to carry viral haemorrhagic septicaemia virus (VHSV) (Hewson et al., 2013). Finally, a tomosvirus was detected from *Diporeia* sp.; however, this is thought to have infected algal matter within the amphipod (Bojko and Ovcharenko, 2019).

The prolific discovery of viruses in (meta)transcriptomic datasets resulted in a great leap in our understanding of viral diversity (Shi et al., 2016); however, this method must be accompanied with pathological and ecological approaches to determine the pathological relevance and impacts of newly discovered viruses on host fitness. Giant river prawns (*Macrobrachium rosenbergii*) that are infected with infectious precocity virus (*Flaviviridae*) show reduced growth, with subsequent economic losses due to yield loss (Zhou et al., 2017; Dong et al., 2021). Wenzhou shark flavivirus in gazami crab (*Portunus trituberculatus*) populations provides evidence for horizontal transmission between crabs and Pacific spadenose sharks, suggesting dual host flaviviruses are not restricted to hematophagous vectors, such as mosquitoes and ticks (Parry and Asgari, 2019; Lensink et al., 2022). Unveiling such intricacies in amphipod virus ecology beyond molecular based discovery will help to guide further development of the field and develop systems to explore the ecological impacts of amphipod viruses.

Biological invasions are occurring more frequently (Seebens et al., 2017). Interdisciplinary approaches must be taken to address the rise in

global connectivity, and subsequent translocation of viruses (Roy et al., 2017; Nunez et al., 2020). Invasive amphipods have been shown to cause significant ecological damage (Wood et al., 2021). DNA viruses in invasive amphipods have been shown to alter their behaviour and/or induce mortality (Bojko et al., 2019a). These findings suggest that viruses can control amphipod populations and offset the impact of a highly invasive host population. *Gammarus pulex* flavivirus is the only RNA virus known to be associated with an invasive amphipod (Parry and Asgari, 2019; Wood et al., 2021), meaning that their relevance to amphipod pathogen diversity, pathology, and ecological impact, remain unknown.

Before the impact and ecological role of amphipod RNA viruses can be explored in detail, there must first be an assessment of RNA virus diversity associated with amphipod species. We investigate RNA virus diversity in both invasive and native amphipod species, using newly sequenced and publicly available transcriptomic datasets. We correlate α and β viral diversity across amphipod hosts, as well as explore this diversity across the environmental habitats of the hosts, to develop the first broad-scale virological diversity screen for this important group of detritivorous and keystone crustaceans.

2. Materials and methods

2.1. Data availability and data mining

A total of 570 paired-end transcriptomes from NCBI's sequence read archive (SRA) were screened using the Serratus Explorer RdRP search, using default parameters (27th January 2022) (Edgar et al., 2022). The SRA datasets screened included 100 species from the Amphipoda. The 100 species belong to 25 amphipod families (Uristidae, Acanthogammaridae, Alicellidae, Crypturopodidae, Baikalogammaridae, Caprellidae, Gammaridae, Pontoporeiidae, Anisogammaridae, Eulimnogammaridae, Micruropodidae, Pontogeneiidae, Aoridae, Hirondelleidae, Pallaseidae, Pardaliscidae, Hyaellidae, Macrohectopidae, Niphargidae, Ommatogammaridae, Talitridae, Pachyschesidae, Ponto-gammaridae, Maeridae, Scopelocheiridae). SRA runs that had viral RNA-dependent RNA polymerase (RdRp) hits determined by Serratus were retrieved using the SRA toolkit. The datasets that has viral hits

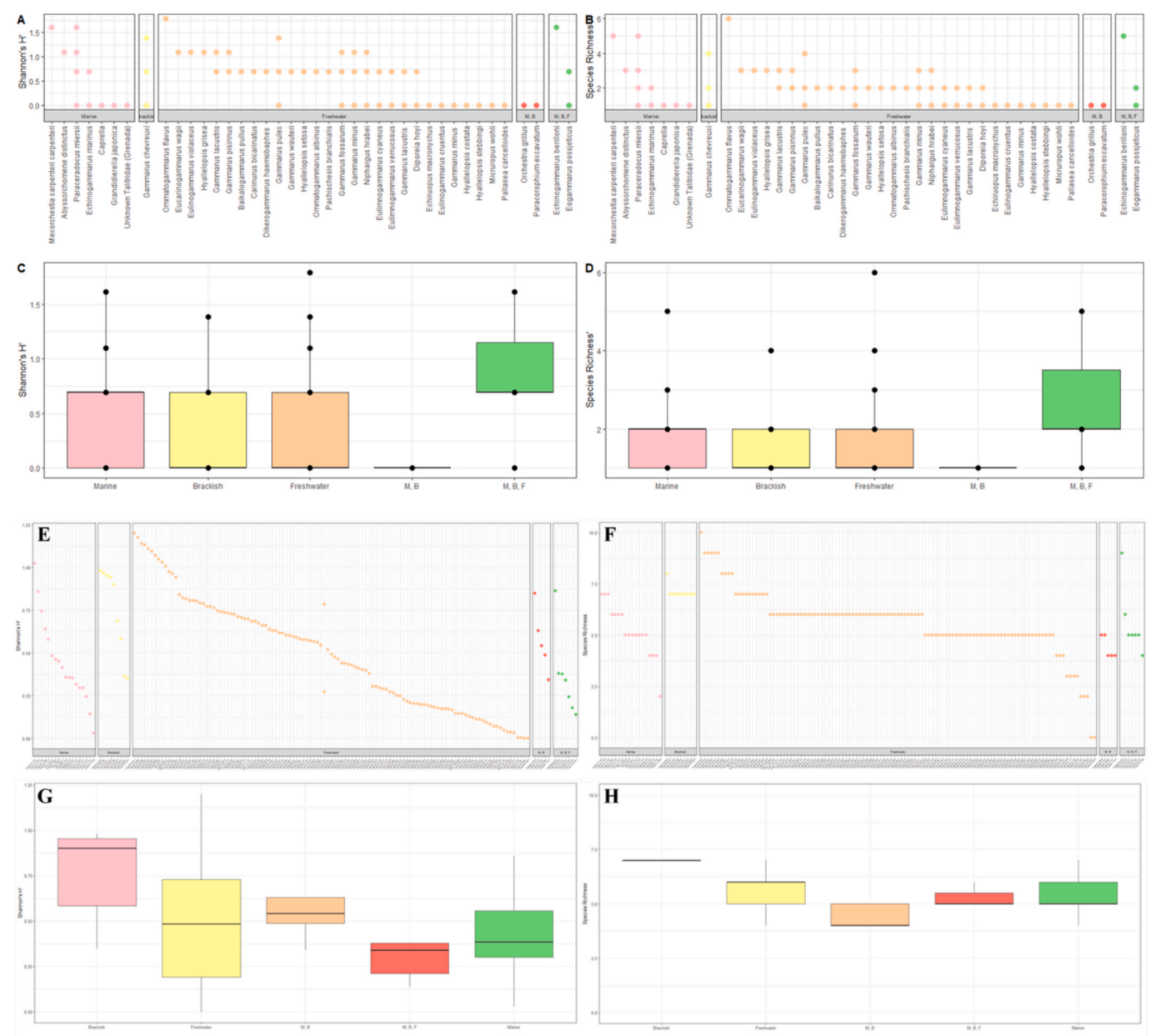


Fig. 3. Alpha (α) diversity analysis of different hosts and environments, based on the RNA virome uncovered within this study. α diversity analysis of viral discovery in different hosts (A, B, E, F) and environments (D, E, G, H). Figures A-D display the diversity indices based on the presence and absence data, whilst figures E-H represent the analysis conducted using relative abundance data. α diversity was measured using Shannons H' index and Species Richness. Each point represents an SRA, some points overlap. Colour indicates the hosts habitat environment, as listed by the World Register of Marine Species.

were considered a “pass” (Supplementary Table 1; Edgar et al., 2022).

2.2. RNA extractions and sequencing

To compliment data obtained from NCBI, RNA was extracted from seven amphipod species (Supplementary Table 2) that had been fixed in 98% ethanol and stored at -20°C . These species did not have datasets present on NCBI and were included to further represent the global diversity of the amphipod RNA virome. RNA was extracted from 15 whole individuals per population using a Trizol extraction method as detailed in Zhao et al. (2020). Extracts were then pooled and sent on dry ice for total RNA and/or mRNA sequencing at Novogene. Details about the specimens extracted and the sequencing undertaken can be seen in Supplementary Table 2.

A pooled sample of hepatopancreas dissected from nine *Dikerogammarus haemobaphes* individuals was sent to Novogene for RNA

extraction and mRNA sequencing. All sequencing carried out at Novogene was performed on an Illumina Novoseq 6000. For mRNA sequencing, library preparation was performed using the NEB Next® Ultra™ RNA Library Prep Kit (E7530). For total RNA sequencing, library preparation was performed using the NEBNext® Ultra™ Directional RNA Library Prep Kit (E7420) and the NEB Next® Multiplex Small RNA Library Prep Set for Illumina® (E7300). Paired end sequencing was performed, generating 150 bp reads. Raw reads were trimmed using Trimmomatic v.0.39 (Bolger et al., 2014). Default Trimmomatic parameters were used (ILLUMINACLIP:TrueSeq3-PE-2.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36). The trimmed reads were assembled using SPAdes v3.15.3 (Bankevich et al., 2012) in default mode, using kmer values for 150 bp reads (21, 33, 55, 77). Quality of the assemblies were assessed using quast v5.2.0 (Gurevich et al., 2013). Results of this quality assessment can be seen in Supplementary Table 4.

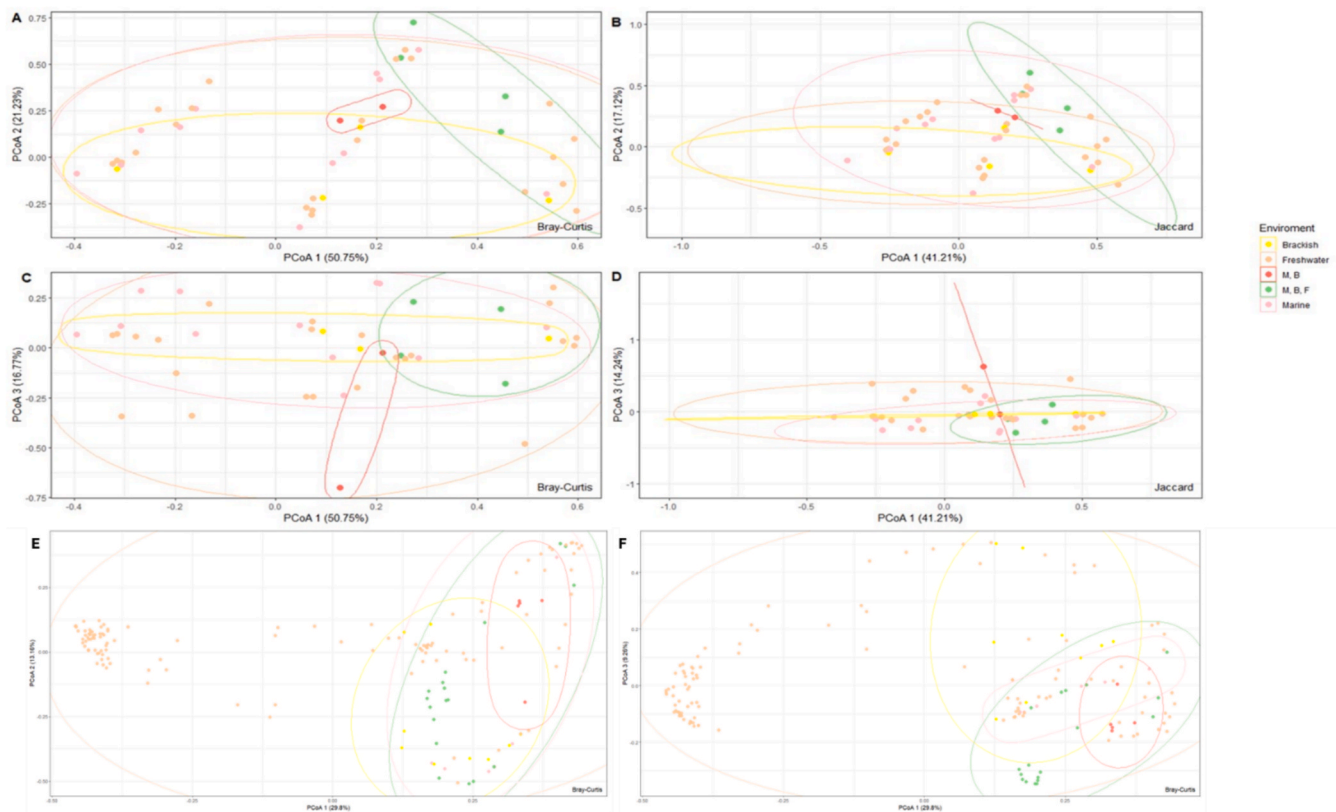


Fig. 4. Beta (β) diversity analysis of different environments, based on the RNA virome uncovered within this study. PCoA plot using Bray-Curtis (A, C, E, F) and Jaccard dissimilarity (B & D) indices of the hosts, screened for novel RNA viruses. β diversity was plotted in both PCoA 1 and 2 (A, B, E), and PCoA 1 and 3 (C, D, F). Each point represents a different host species. Colour indicates the hosts habitat environment, as listed by the World Register of Marine Species. Figures A–D display the diversity indices based on the presence or absence of viruses, figures E and F show the diversity data based on relative abundance. The ellipses in the Bray-Curtis plots (A & C) were created using the `geom_mark_ellipse` function in the `ggforce` package based on the covariance of the data. The Jaccard dissimilarity ellipses (B & D) were created using the `stat_ellipse()` function in the `ggplot2` package based on the confidence interval.

2.3. Host identification

For those specimens where the species assignment was unknown an extra identification step was performed. A protein blast database of amphipod cytochrome oxidase 1 proteins was created using a total of 10,824 sequences, obtained from NCBI (22nd March 2023). Blastx was then used to compare the assembled contigs to the database. Contigs that showed similarity to the custom blast database were then subject to Blastn against the nr database. Results of the Blastn search, and the subsequent taxonomic assignments, can be seen in [Supplementary Table 2](#).

2.4. Viral screening

Blastx (e-value limit: $1e-50$) was ran on command line to compare assembled contigs to the NCBI non-redundant database (nr) (February 2022). Contigs with similarity to viral proteins were incorporated into a reference protein database for a second round of Blastx, to detect more divergent viral species. Contigs with viral hits that had an assembled coverage greater than 5X (based on SPAdes coverage values) and were longer than 500 base pairs were annotated in Prodigal v2.6.3 (Hyatt et al., 2010). Resulting amino acid sequences were queried against an NCBI nr database using default Blastp parameters to verify viral annotation and provide putative gene function. Genome completeness of non-segmented viruses was assessed using CheckV v.0.8.1 database v.1.1 (Nayfach et al., 2021). Genome illustrations were created using gggenes in the R studio interface (Allaire, 2012; Wilkins and Kurtz, 2019). Diversity metrics were calculated using the `vegan` package and visualised using `ggplot2` and `cowplot` (Wickham, 2011; Wilke, 2019). Amphipod

habitat designations were assigned based on the species designation on the World Register of Marine Species (WoRMS). In cases where a species was listed with more than one habitat designation, the combination was included. This resulted in the following habitat designations: “Marine”, “Brackish”, “Freshwater”, “Marine and Brackish”, “Marine and Brackish and Freshwater”. An amphipod was defined as invasive if listed in Wood et al. (2021). The invasive amphipods screened as part of this study were *D. haemobaphes*, *D. villosus*, *Echinogammarus berilloni*, *Gammarus fossarum*, *G. pulex*, *G. chevreuxi* and *Grandidierella japonica*.

2.5. Phylogenetics

RdRP based phylogenies were performed for each of the viral orders available. Species recognised by the International Committee on Taxonomy of Viruses (ICTV) were included, alongside the top 100 Blastp hits for each viral RdRP sequence. Blastp results were filtered to remove bacterial and eukaryotic hits. Duplicate sequences were removed using seqkit v.2.3.1 (Shen et al., 2016). RdRP amino acid sequences were aligned using mafft v.7.515 (Supplementary Table 3), then trimmed using TrimAl v.1.4.1 (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009; Katoh and Standley, 2013). Phylogenies were inferred from trimmed files using IQ-Tree v.2.2.0.3 (Minh et al., 2020). All alignment and phylogenetic parameters can be seen in [Supplementary Table 3](#). Best fit models were determined according to Bayesian Information Criterion (BIC). A maximum likelihood tree was created in each case and used 1000 bootstrap iterations. The resulting phylogenetic tree was visualised and annotated using FigTree v1.4.3 (<https://www.tree.bio.ed.ac.uk/software/figtree/>). Sequence Demarcation Tool (SDT v1.2) was used to produce similarity plots for selected clades. The mafft alignment

Articulavirales

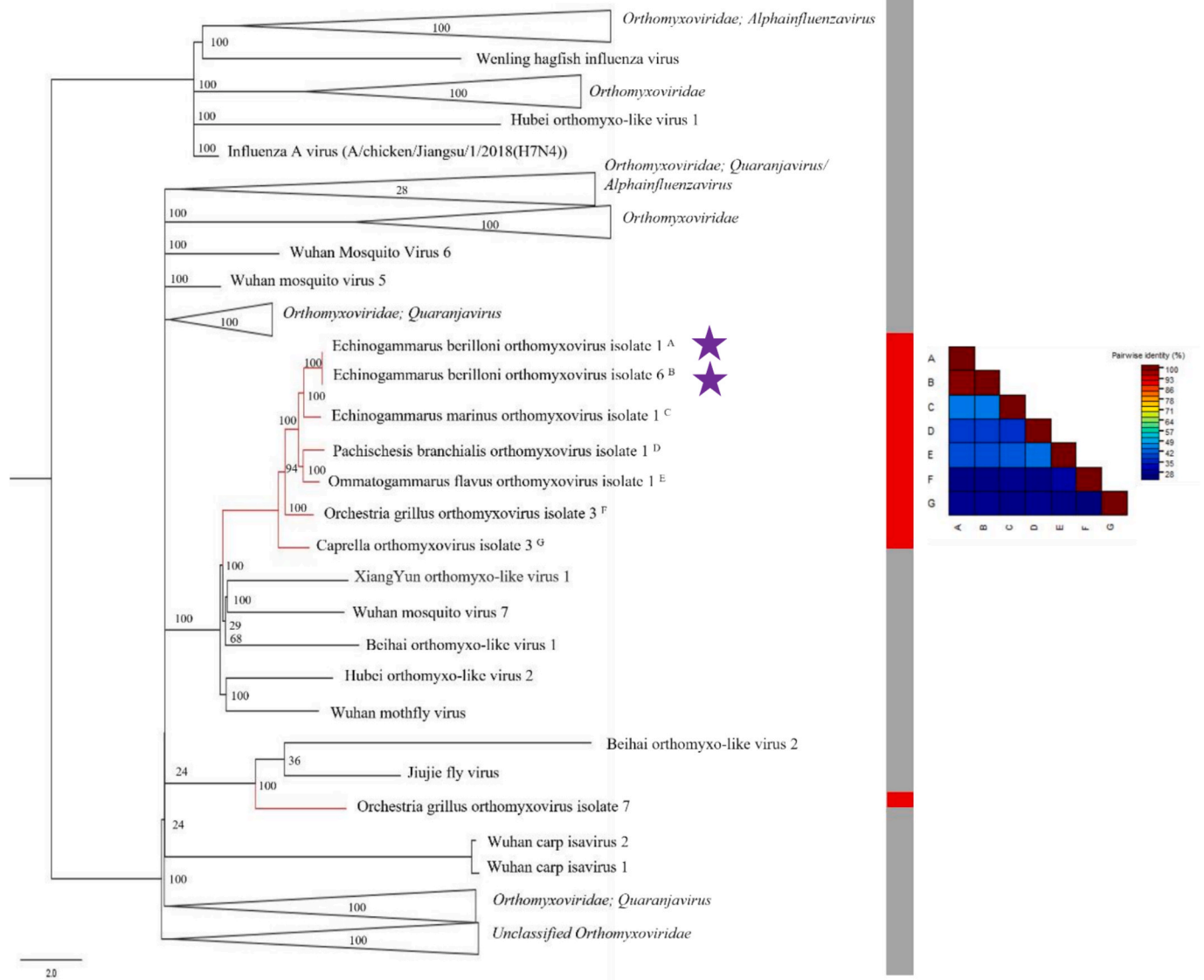


Fig. 5. An RdRp based phylogeny of the *Articulavirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. A pairwise similarity matrix for one phylogenetic clade, containing novel viruses, is also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strategy was used within SDT on the viral isolates.

3. Results

3.1. Viral discovery

In total, 412 contigs were recovered from the 160 datasets subject to Blastx search. These contigs are associated with a potential 134 novel viruses, 41 of which are deemed to be complete, supported by CheckV results (Supplementary Table 5). ORF arrangements for the complete genomes were conserved in each viral order (Supplementary Fig. 1–4; Supplementary Table 6). Viral contig length ranged from 401 nt to 17,118 nt. The mean viral contig length recovered was 5875 ± 4109 nt ($\pm$ standard deviation).

Over half of the viral contigs were predicted to encode a viral RdRp (235/412). The majority of viral contigs were associated with the

Mononegavirales (n = 139). The *Mononegavirales* were distributed among a variety of families, with the largest number related to members of the *Artoviridae* family, followed by *Lispiviridae* and *Rhabdoviridae* (Fig. 1). Discoveries in the *Picornavirales* order (n = 97) held the highest number of novel complete viruses discovered in this study (n = 17). The remainder of viral contigs were spread among several viral orders: *Articulavirales* (n = 35), *Bunyavirales* (n = 38), *Durnavirales* (n = 6), *Ghabrivirales* (n = 28), *Hepelivirales* (n = 43), *Jingchuvirales* (n = 16), *Martellivirales* (n = 4), *Mononegavirales* (n = 139), *Nidovirales* (n = 1), *Nodamuvirales* (n = 2), *Wolframvirales* (n = 3) (Fig. 2). Complete genes were found associated with *Durnavirales* (n = 2), *Ghabrivirales* (n = 1), *Hepelivirales* (n = 8), *Jingchuvirales* (n = 1), *Mononegavirales* (n = 10), *Picornavirales* (n = 17), *Wolframvirales* (n = 2).

Bunyavirales

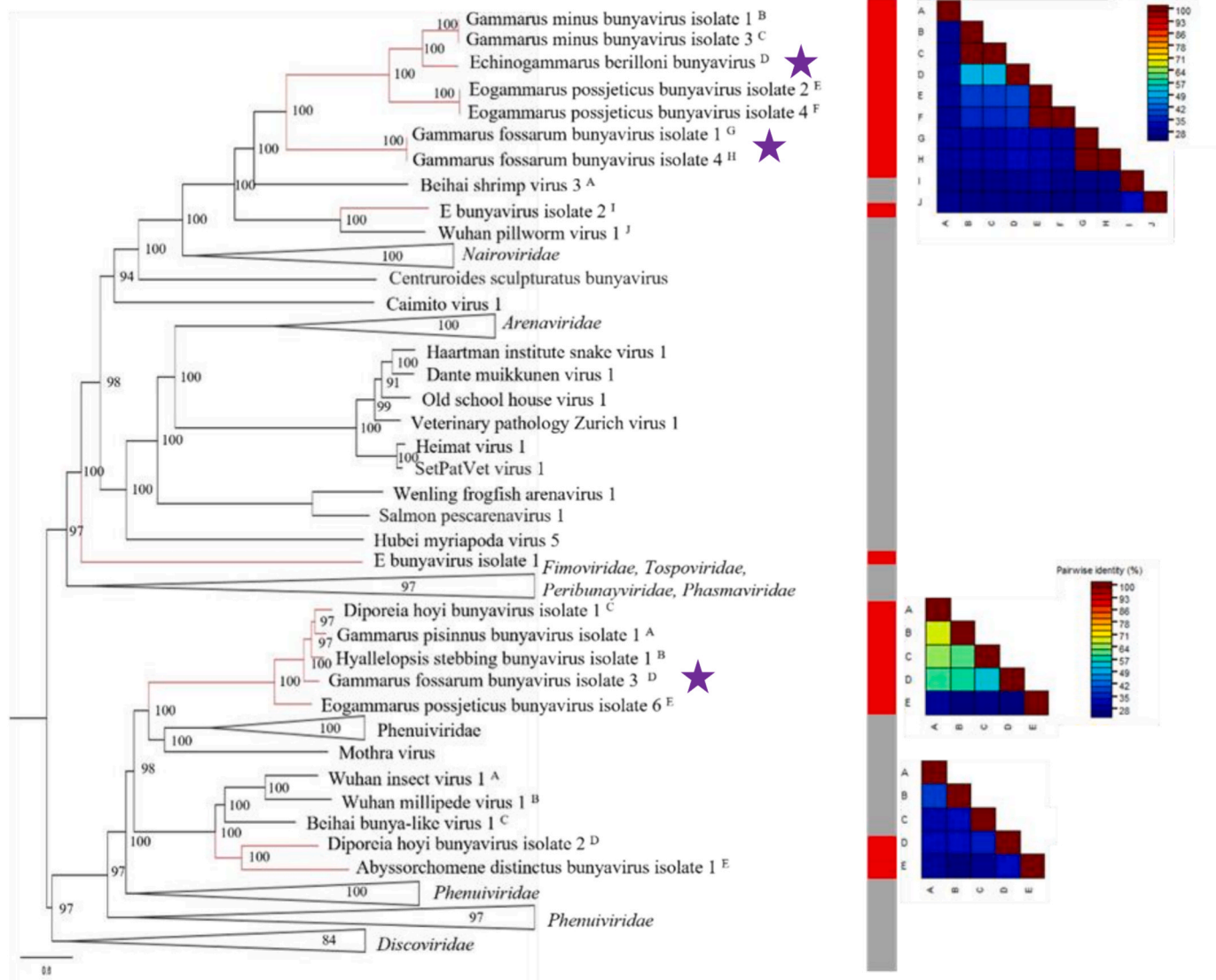


Fig. 6. An RdRp based phylogeny of the *Bunyavirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. Pairwise similarity matrices for three phylogenetic clades, containing novel viruses, are also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Phylogenetics

Most amphipod viruses formed monophyletic clades within each viral order (Fig. 1, 5–15). In some cases, novel clades or isolates share a paraphyletic relationship: *Articulavirales* (Fig. 5); *Bunyavirales* (Fig. 6); *Jingchuvirales* (Fig. 10); *Picornavirales* (Fig. 13); *Wolframvirales* (Fig. 14). It is difficult to discern whether these paraphyletic groups are related to the environment of the host due to incomplete sampling data. Largely, the monophyletic groups were composed exclusively of amphipod RNA viruses discovered in this study. Fewer clades were interspersed with previously described viruses: *Ghabrivirales* (Fig. 8); *Mononegavirales* (Fig. 1); and *Nyamiviridae* (Fig. 1). Close relatives of the novel amphipod viruses are mainly unclassified. Statistical support across the phylogenies was generally high. *Bunyavirales* and *Jingchuvirales* had universally strong support across each tree, with bootstrap values >84 and >74, respectively (Figs. 6 & 9). Other phylogenies, such as the picornavirus

phylogeny (Fig. 13, have some lower bootstrap support (>10 in some cases).

3.3. Diversity of the amphipod virome based on presence and absence of viral species

Viral sequences were recovered from 40 amphipod species, five species had no viruses (*Oxyacanthus sowinskii*, *Micruropus parvulus*, *D. villosus*, *Melita awa*, *Neoserica* sp.). However, sample sizes for these species was low (*Oxyacanthus sowinskii* = SRR3467039, *Micruropus parvulus* = SRR3467081, *D. villosus* = pooled sample of 20, *Melita awa* = pooled sample of 15, *Neoserica* sp. = pooled sample of 15). Often, more than one virus of the same viral order was recovered. This was common for *Picornavirales*, where five amphipod species contained more than one (*Niphargus hrabei*, *Gammarus lacustris*, *Ommatogammarus flavus*, *Gammarus minus*, *E. berilloni*). Only the datasets from *G. lacustris* were

Durnavirales

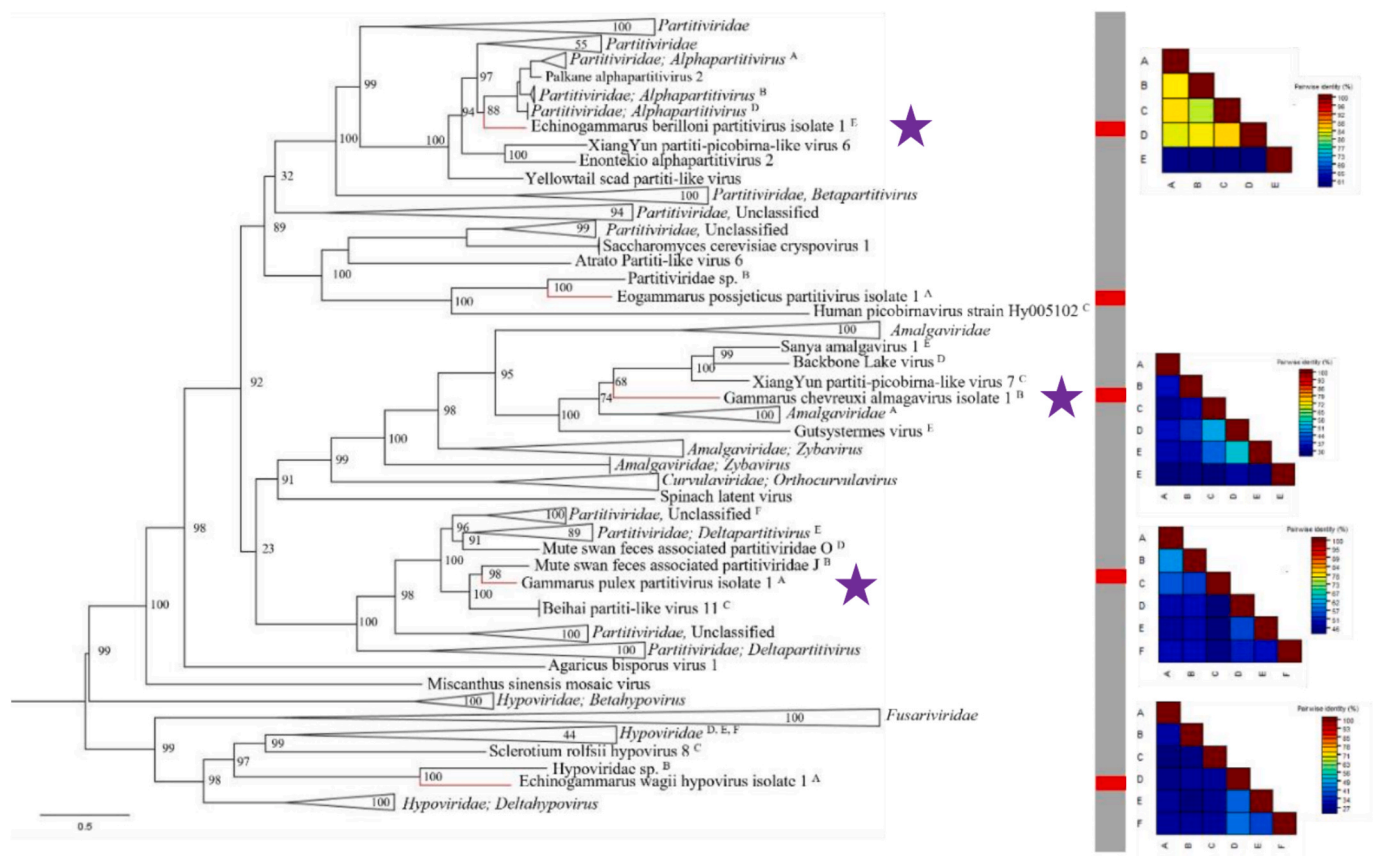


Fig. 7. An RdRp based phylogeny of the *Durnavirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. Pairwise similarity matrices for four phylogenetic clades, containing novel viruses, are also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ghabrivirales

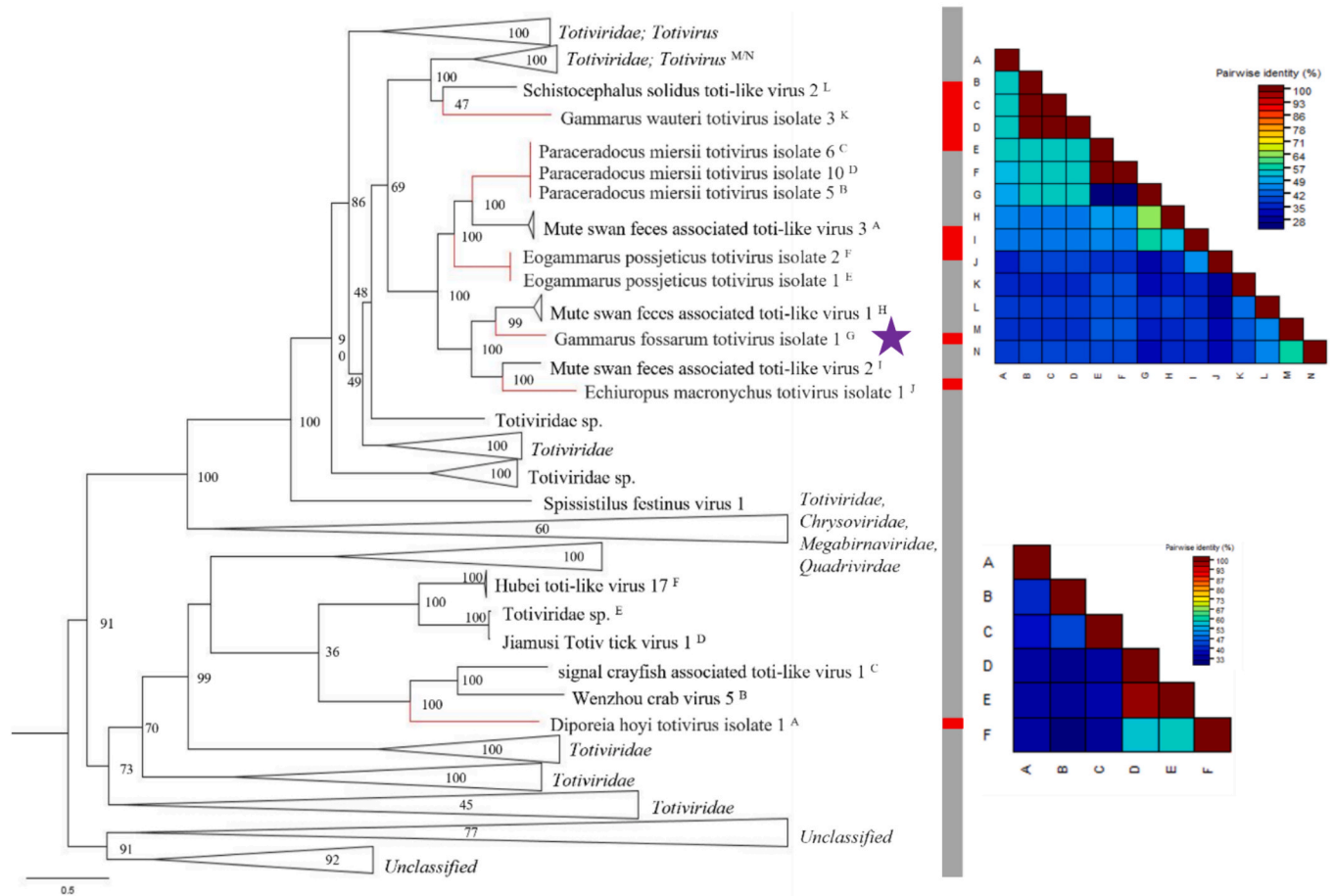


Fig. 8. An RdRp based phylogeny of the *Ghabrivirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. Pairwise similarity matrices for two phylogenetic clades, containing novel viruses, are also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

generated using pooled specimen extracts.

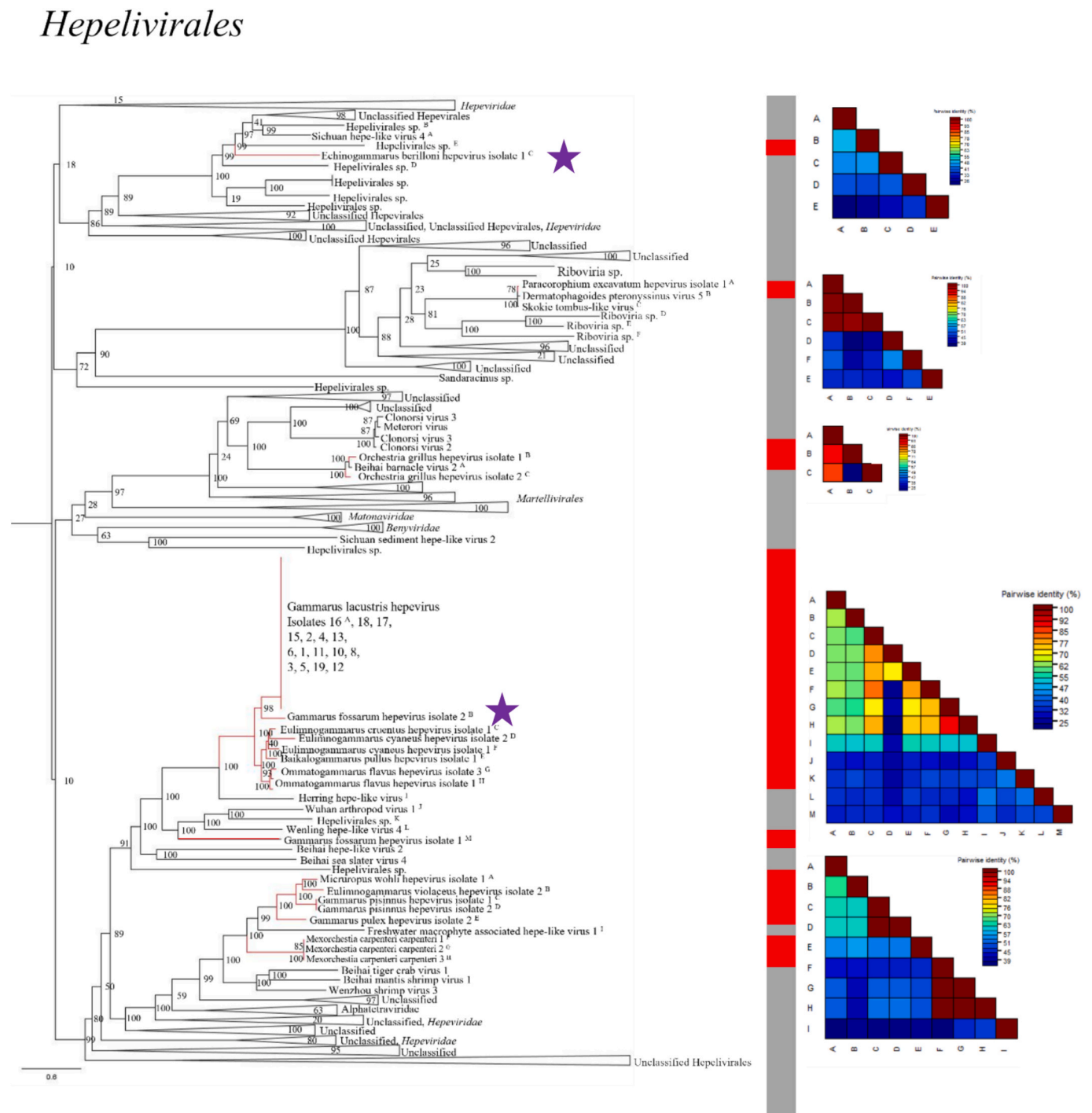
Alpha (α) diversity (Shannon H' index) ranged from 0 to 1.79 (Fig. 3), species richness ranged from 1 to 6 (Fig. 3A). The greatest host α diversity was associated with *Ommatogammarus flavus* (Fig. 3A and B). Significant differences between the different environments (Kruskal Wallis, $\chi^2_{df=4} = 9.9327$, $p = 0.04158$; Poisson distributed generalised linear model (GLM), dispersion parameter = 1, $z = -2.404$, $df = 151$, $p = 0.0162$) and hosts (Kruskal Wallis $\chi^2_{df=39} = 72.439$, $p = 0.00091$) on the α diversity of the RNA viruses discovered was observed. Despite the overall significance of the effect of environment and host on RNA viral diversity, pairwise comparison was not statistically significant in either case (Dunn test with Holm adjustment, Table 1). Beta (β) diversity was measured using the Bray-Curtis (Fig. 4A & C) and the Jaccard (Fig. 4B–D) dissimilarity indices. Multivariate dispersion (β dispersion and permutation test) and the differences in community composition (ADONIS) were tested using the Bray-Curtis dissimilarity index as the input. There is no significant difference in multivariate dispersion between the habitat environments (F -statistic = 0.497, $p = 0.757$). However, environment had a significant effect on viral community composition (F -statistic = 6.1266, residual- $df = 151$, $p = 0.001$), meaning that there was a significant difference in viral community composition between at least two of the defined environmental groups. There is also a significant difference in multivariate dispersion ($p =$

0.044) and community composition ($p = 0.001$) between the host species.

The difference in viral diversity between invasive (7 species, 16 datasets) and non-invasive (38 species, 145 datasets) host species was also tested but, the Shannon H' index did not significantly differ between amphipods that are defined as invasive or native (Mann-Whitney U test, $p = 0.1194$). Multivariate dispersion indicates that there is homogeneous dispersion amongst invasive and non-invasive species (F -statistic = 0.1965, $p = 0.6450$).

3.4. Diversity of the amphipod virome based on relative viral read abundance

No significant differences between environments (Kruskal Wallis, $\chi^2_{df=4} = 7.8142$, $p = 0.09863$; Poisson distributed generalised linear model (GLM), dispersion parameter = 1, $z = -0.755$, $df = 154$, $p = 0.450$) and hosts (Kruskal Wallis $\chi^2_{df=153} = 152.49$, $p = 0.4964$) on the α diversity of the RNA viruses discovered was observed (post hoc analysis presented in Table 1). Beta (β) diversity was measured using the Bray-Curtis (Fig. 4E & F) dissimilarity index. Multivariate dispersion (β dispersion and permutation test) and the differences in community composition (ADONIS) were tested using the Bray-Curtis dissimilarity index as the input. There is a significant difference in multivariate



dispersion between the habitat environments (F -statistic = 2.3918, p = 0.042). However, environment had a significant effect on viral community composition (F -statistic = 7.08, residual-df = 148, p = 0.001), meaning that there was a significant difference in viral community composition between at least two of the defined environmental groups. The Shannon H' index did not significantly differ between amphipods that are defined as invasive or native (Mann-Whitney U test, p = 0.5466).

4. Discussion

The RNA virome of our study based on 100 species of Amphipoda included 12 of the 30 known RNA viral orders. Viruses were present in 93% of amphipod species screened, highlighting their commonality as viral hosts. *Mononegavirales* were the most prevalent viral order in the amphipod RNA virosphere. Other virus groups (e.g. *Negevirus*) recently identified in peracarids (e.g. isopods) are absent, based on our survey

Jingchuvirales

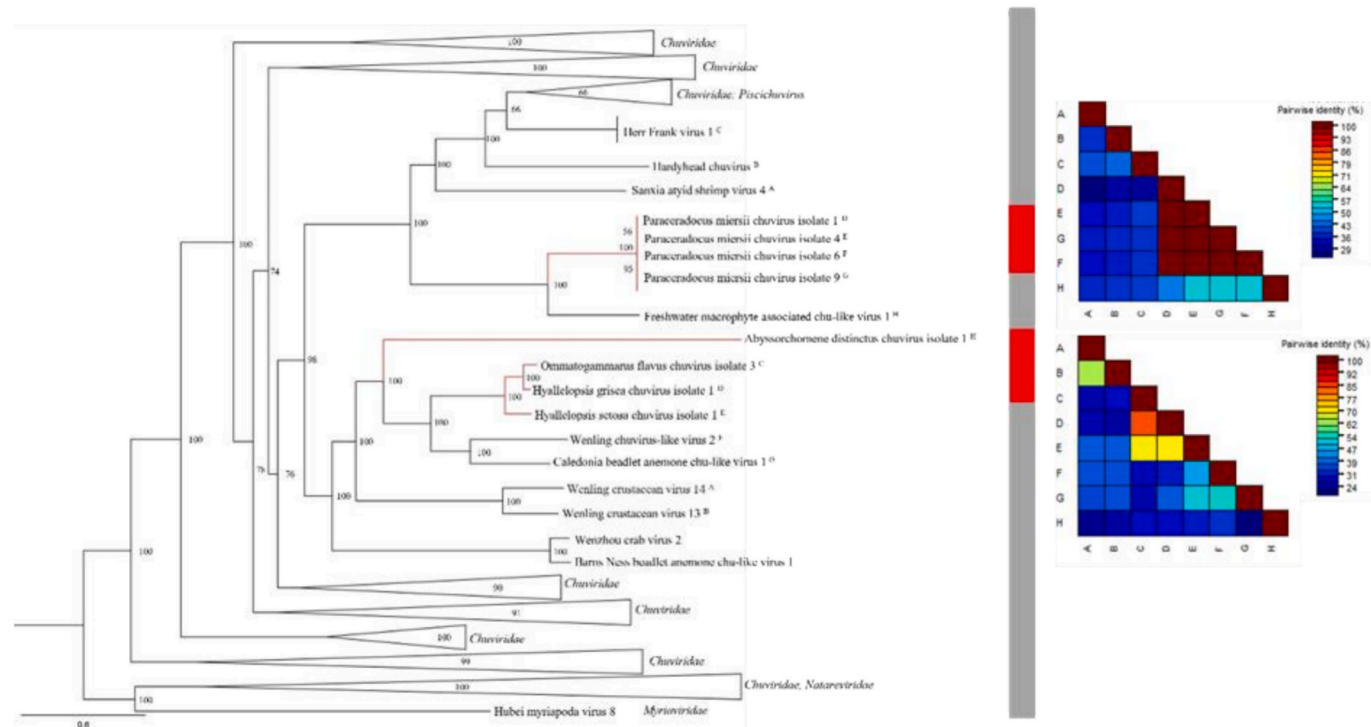


Fig. 10. An RdRp based phylogeny of the *Jingchuvirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. Pairwise similarity matrices for two phylogenetic clades, containing novel viruses, are also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Martellivirales

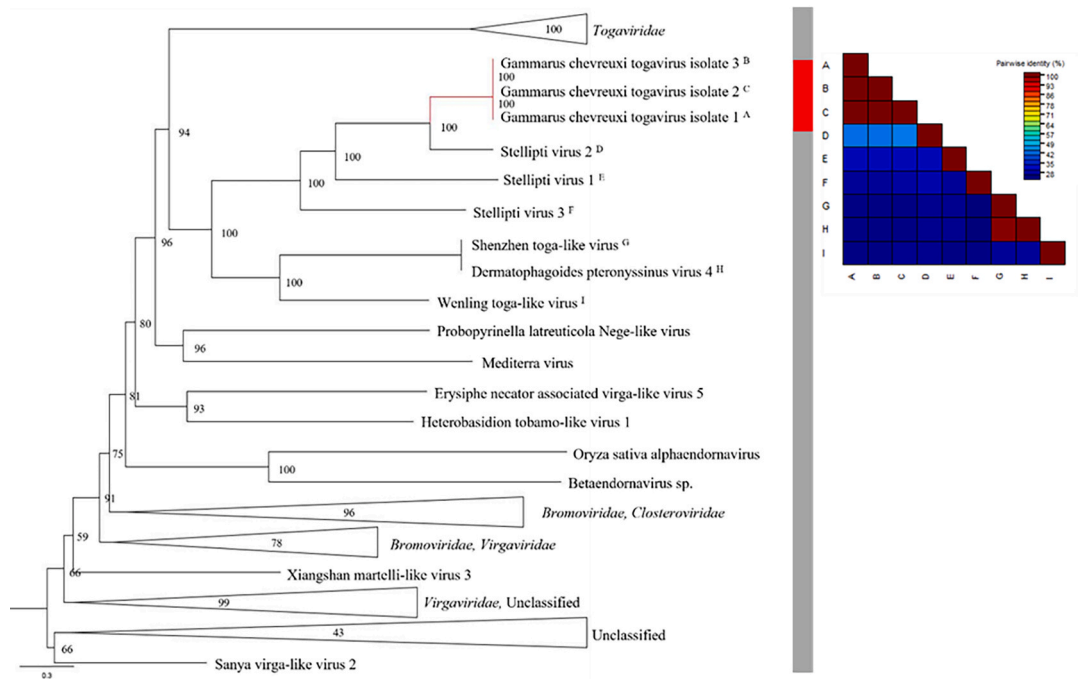


Fig. 11. An RdRp based phylogeny of the *Martellivirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. A pairwise similarity matrix for one phylogenetic clades, containing novel viruses, is also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nidovirales

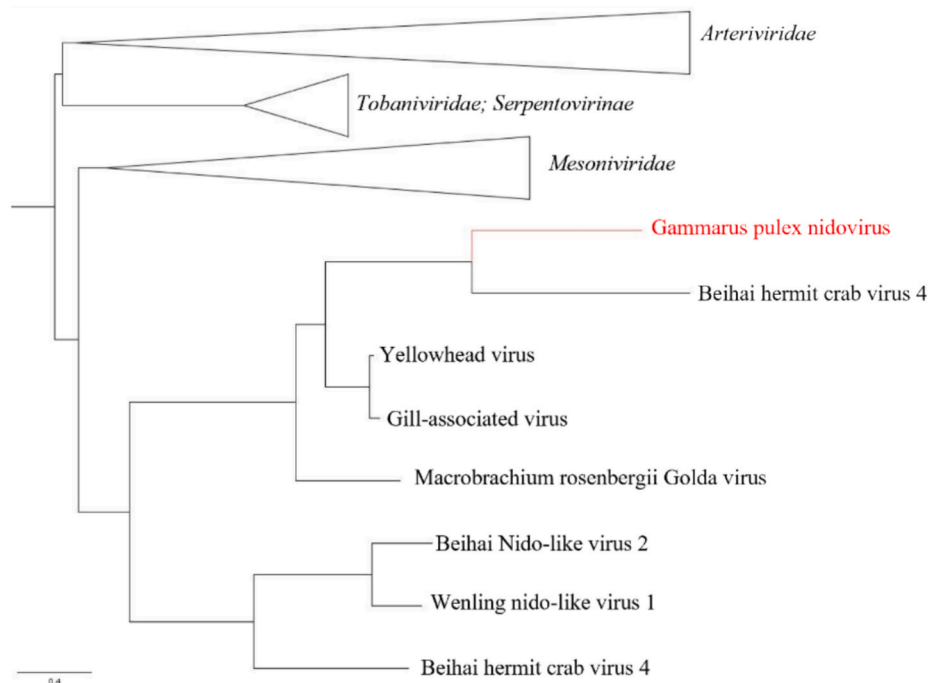


Fig. 12. An RdRp based phylogeny of the *Nidovirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Bojko et al., 2020). Significant differences in diversity were observed between environments, but not between those amphipod hosts that are invasive and those that are native. A huge diversity of viruses has been identified, which may be influential in amphipod population dynamics, ecology, and disease ecology. These viruses may cause host behavioural change, or result in more rapid host mortality, as seen with DNA viruses in amphipods (Bojko et al., 2019b).

4.1. The RNA virosphere of the Amphipoda

The RNA virosphere of the Amphipoda is diverse. It contains novel members from all five phyla in the *Orthornavirinae* (*Lenarviricota*, *Pisuviricota*, *Kitrinoviricota*, *Duplornaviricota*, *Negarnaviricota*) (Kolundžija et al., 2022). The most prevalent RNA viruses in amphipod transcriptomes were mononegaviruses (Kuhn et al., 2021), containing 11 families and 123 genera (Amarasinghe et al., 2019). The amphipod-associated mononegaviruses grouped into the *Rhabdoviridae*, *Nyamiviridae*, *Arteriviridae* and *Lispiviridae*. These families are ecologically diverse with members infecting plants or animals from a range of environments (Dietzgen et al., 2017a, b, 2019). In some cases, the viruses we detected represent novel viral clades. In the case of the viruses related to the *Nyamiviridae*, the novel viral isolates cluster with the established invertebrate-infecting *Crustavirus* genus (Fig. 1).

Other screening studies of animal and environmental samples, particularly from marine habitats, have found picornaviruses to be the most abundant (Ng et al., 2012; Shi et al., 2016; Sadeghi, Tomaru and Ahola, 2021; Jackson et al., 2022; Zayed et al., 2022; Scro et al., 2023). The abundance of picornaviruses may be because of the sequencing

chemistry commonly used in historic or host-focal studies, which includes polyA capture methods during library preparation, predisposing + ssRNA virus detection (Visser et al., 2016; Gann et al., 2021; Jackson et al., 2022).

The *Picornavirales* is composed of eight families and 102 genera. Invertebrate-infecting picornaviruses are currently assigned to two accepted families, the *Dicistroviridae* and *Iflaviridae*. The RNA virosphere elucidated here reveals picornaviruses to be the second most prevalent viral order infecting the Amphipoda and includes the most complete genomes identified in this study.

The *Secoviridae* and *Marnaviridae* infect plants and algae, respectively (Lang et al., 2021; Fuchs et al., 2022). Both groups were associated with amphipods ('*Gammarus lacustris* picornavirus isolate 3' and '*Echinogammarus berilloni* picornavirus isolate 1a') and may be contamination from infected plant or algal matter that was present in/on the specimen at the time of sampling, as suggested previously (Bojko and Ovcharenko, 2019). Marnaviruses can cause disease in crustacean hosts (Xu et al., 2023). The two isolates here cluster more closely with the plant infecting members of this group. The host range of these two families may be broader than currently thought, since some RNA virus families have a host range that extends across several branches of the tree of life, such as the *Reovirales* (Matthijnssens et al., 2022a; Matthijnssens et al., 2022b).

Viruses from the viral orders: *Jingchuvirales*, *Durnavirales*, *Martellivirales*, *Wolfamvirales*, *Nodamuvirales* and *Nidovirales* were infrequent across the amphipod virome. Many of these viruses clustered with unclassified invertebrate-infecting viruses. The novel togavirus (*Martellivirales*) detected in *G. chevreuxi* is most closely related with Stellipti virus, found in a species of flatworm (Platyhelmintha) (Dheilly et al.,

Nodamuvirales

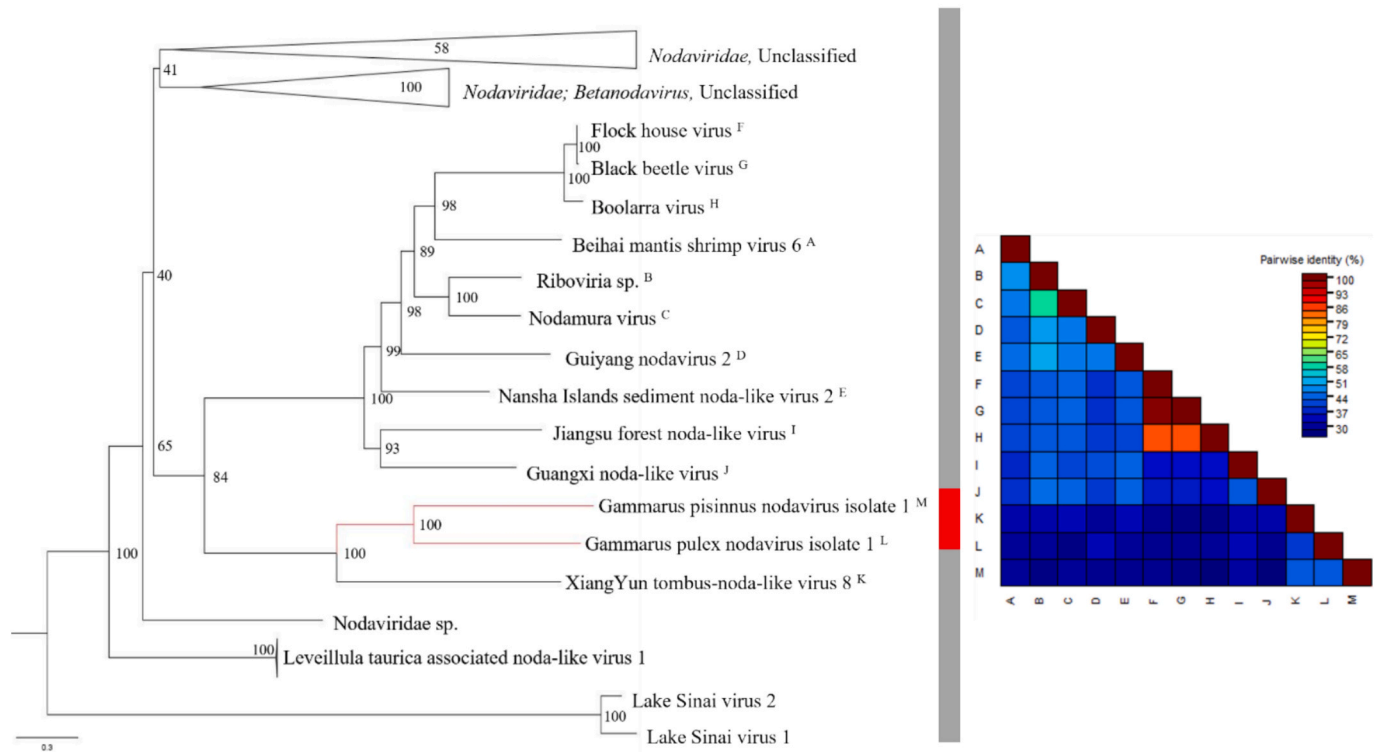


Fig. 13. An RdRp based phylogeny of the *Nodamuvirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. A pairwise similarity matrix for one phylogenetic clade, containing novel viruses, is also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2022). *Nodamuvirales* isolates from *Gammarus* spp. share the closest relationship with 'Xiang Yun tombus-noda-like virus 8', a virus detected in the metagenome of a mosquito (*Culex tritaeniorhynchus*) (NCBI Accession: OL700187).

The unprecedented rate at which viruses have been discovered has resulted in a shift in the understanding of their ecological role. Like bacteria, viruses are now considered to share mutualistic and parasitic relationships with their host(s) (Roossinck, 2015). Unfortunately, discovering viruses via next generation sequencing technologies does not account for pathological detail. There is the possibility that some of the RNA viruses discovered here are not pathogenic. For example, 'Pteromalus puparum negative-strand RNA virus 1' (PpNSRV-1; *Mononegavirales: Artoviridae*) infects a species of parasitoid wasp, *Pteromalus puparum*, and is not pathogenic to the host but instead increases longevity (Wang et al., 2017). As the knowledge on amphipod RNA virus diversity expands, studies can begin such exploration to understand RNA virus relationships in amphipods. Infection by numerous RNA viruses at once, seen in 22 eligible hosts (45 individual SRA datasets), indicates that co-infection is not rare in Amphipoda, and is akin to that observed in other invertebrates (Shi et al., 2016). Co-infection may indicate the role these RNA viruses are playing as mutualistic or possibly commensal symbionts, with no impacts on host fitness or disease (Zhang et al., 2019).

4.2. Amphipod virus distribution among environments

One of the most important factors that governs community structure is environmental heterogeneity (Yang et al., 2015). Viruses frequently face heterogeneous environments both on an intra-host (tissue) scale and

the larger intra-species (genotype) scale (Morley et al., 2016). Previous studies have shown that the marine/freshwater boundary exists with clear separation between microbe lineages in each of the environments (Logares et al., 2009). Studies have focused on freshwater and marine cyanophages, and phycodnaviruses, which infect phytoplankton (Breitbart et al., 2004; Chenard and Suttle, 2008). The significant differences seen in our community composition from both binomial and quantitative perspectives support similar findings that show environment is an important factor in viral diversity, showing that this pattern may extend to RNA viruses that infect amphipods. This indicates evolutionary specialism across viral species in hosts that occupy different environments (Shi et al., 2019; Wu et al., 2020). For example, novel members of the *Artoviridae* were all associated with freshwater amphipod hosts in our study, suggesting that this group may be missing from marine amphipods. It is difficult to discern whether any viral groups are truly dominant in a particular environment due to a paucity of sample metadata and the overlapping environments many amphipod species can inhabit (e.g. marine, brackish and freshwater).

4.3. Amphipod virus distribution among invasive amphipods

Several amphipod species (n = 46) are classed as invaders, with representatives in aquatic environments (Wood et al., 2021). Invasion studies often focus on the invading host; however, this focus is now extending to include co-invasive parasites (Bojko et al., 2021; Bojko et al., 2023a; Bojko et al., 2023b). Many amphipod invaders lack a symbiont screen and comprehensive screening of invasive amphipod species enables monitoring to study parasite release, host range shifts, and viral community changes (Wood et al., 2021). For RNA viruses, a

Picornavirales

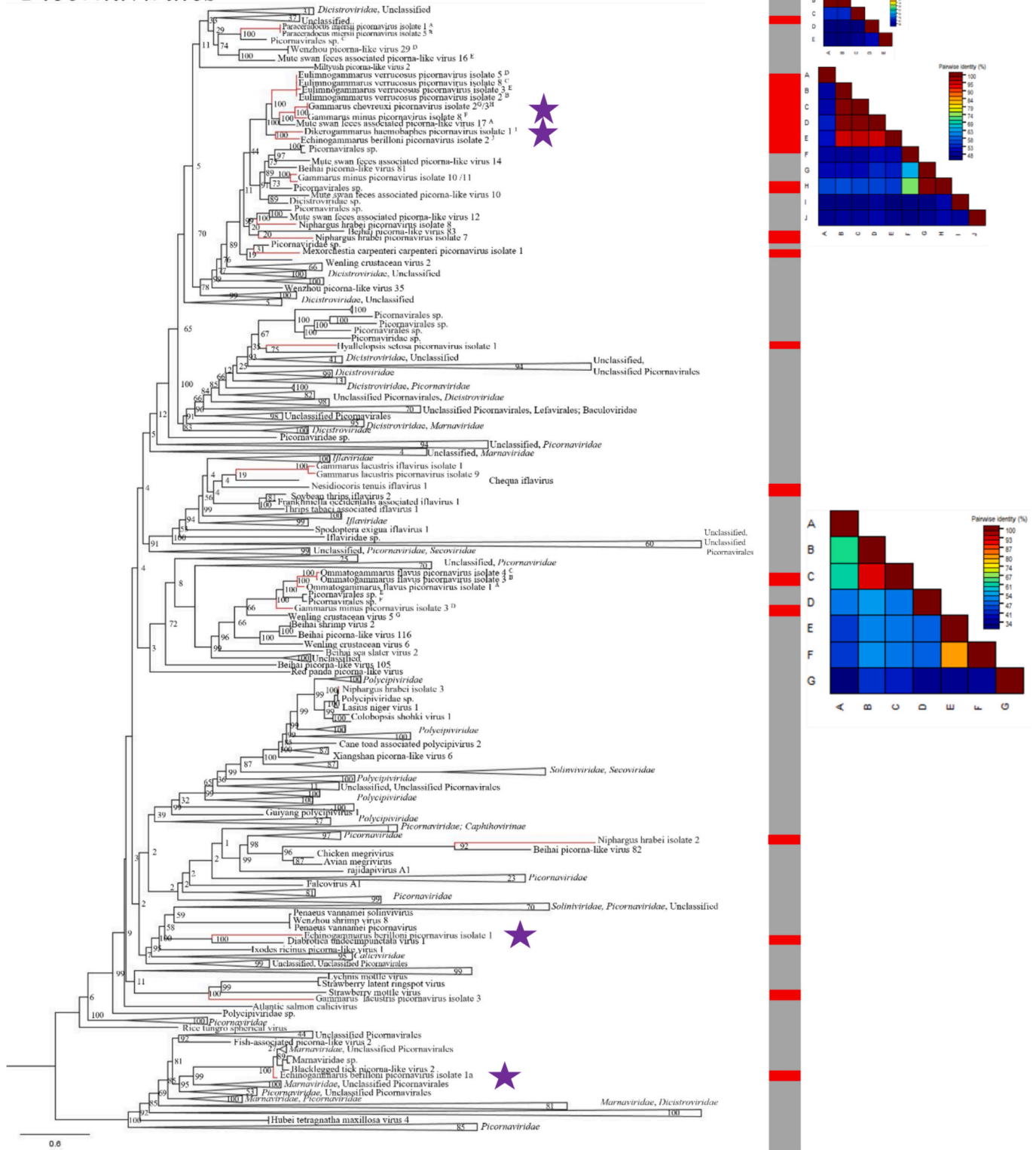


Fig. 14. An RdRp based phylogeny of the *Picornavirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. Pairwise similarity matrices for three phylogenetic clades, containing novel viruses, are also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Wolframvirales

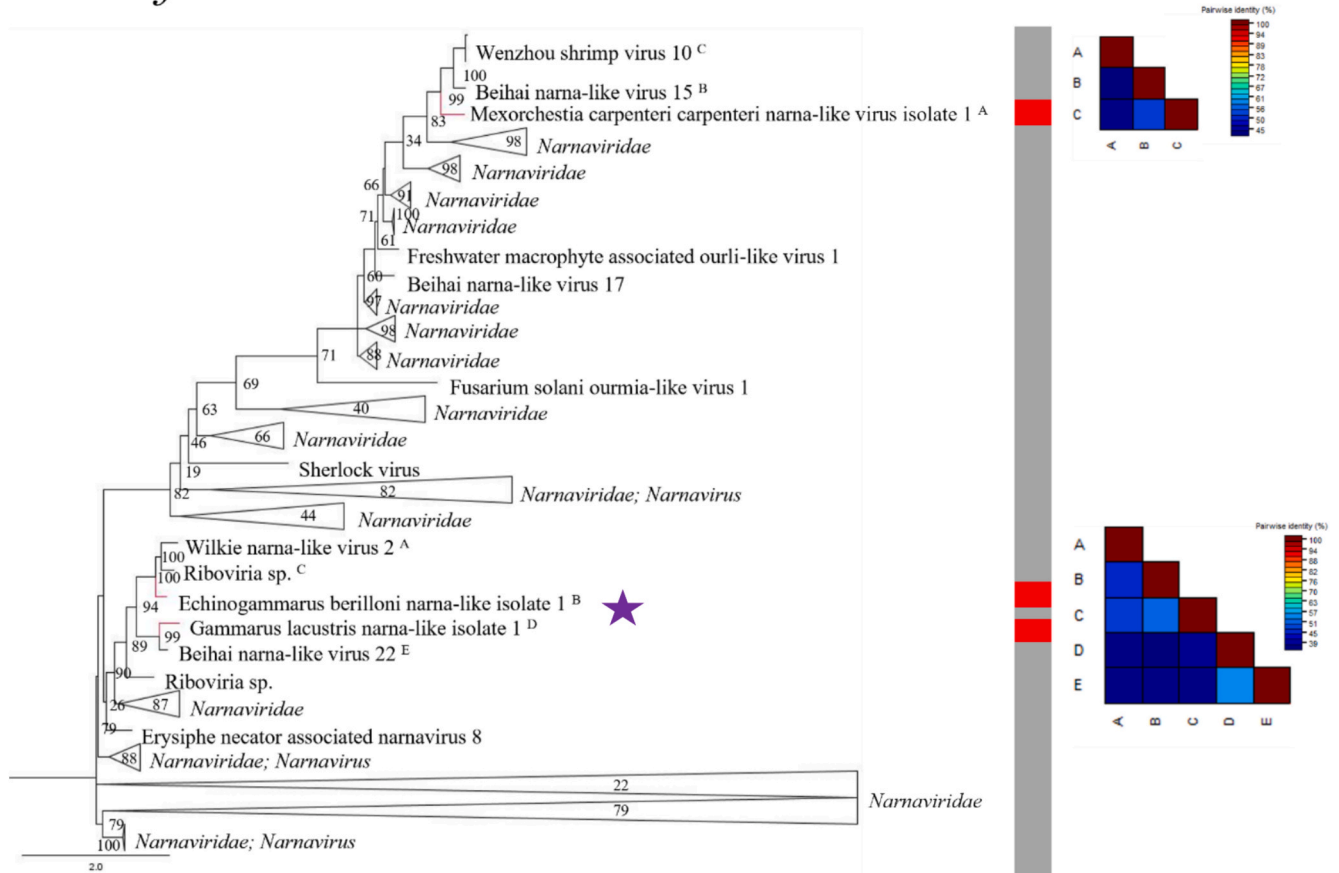


Fig. 15. An RdRp based phylogeny of the *Wolframvirales* viral order, containing novel RdRp sequences identified in this study. Node labels indicate bootstrap support. Within each tree, novel virus branches are coloured red. Novel viruses are also highlighted by the red bars to the right of the tree, where grey indicates previously described viruses. Viruses detected in invasive amphipod species are highlighted with a purple star. Pairwise similarity matrices for two phylogenetic clades, containing novel viruses, are also provided. Sequences included in a pairwise similarity matrix are evidenced by superscript letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rapid evolutionary rate makes them ideally suited to exploit the novel opportunities presented to them when a host translocation event occurs (Faillace et al., 2017). Previously, only one RNA virus was known to be associated with an invasive amphipod (*Gammarus pulex* flavivirus) (Parry and Asgari, 2019; Wood et al., 2021). Screening conducted as part of this study reveals RNA diversity in this host group, spanning 12 viral orders.

To improve and develop control methods for invasive alien species, research on the biology and life history of the species is required (Wood et al., 2021), and improved understanding of the co-evolutionary history of viral lineages and host species would enable deeper insight into biocontrol potential (Faillace et al., 2017). The use of biocontrol agents in invasive and pest populations of insects and plants is common and RNA viruses have been shown to function as biocontrol agents in ants, as one example (Abd-Alla et al., 2020; Valles et al., 2022). To date, no biological control method has been developed for use in invasive amphipods, despite extensive discussion of the subject (Bojko, 2017; Bojko et al., 2019a; Wood et al., 2021; Bojko et al., 2018). Discovering novel RNA viruses associated with amphipods provides several new potential candidates.

Although results from this study suggest that there is no significant difference in the viral diversity between invasive and non-invasive species, this may not be reflective of true differences between the groups. Environmental and population differences along a hosts' invasion range can influence the diversity and abundance of RNA viral

diversity, as observed in signal crayfish (Bacnik et al., 2021). There is often a paucity of environmental data recorded when a species is collected for metagenomic/metatranscriptomic sequencing, which prevents insight into the true distribution of the host and makes it difficult to differentiate between native and truly non-native viruses. This calls for more dedicated experimental exploration using the diversity data we provide. Improved recording of sample metadata would allow the spatial structure and dynamics of viruses and their transport vector hosts to be untangled (Havel et al., 2015).

Six of the species screened in this study are listed as invasive non-native species (*D. haemobaphes*, *D. villosus*, *E. berilloni*, *G. fossarum*, *G. pulex*, *G. japonica*). The killer shrimp (*D. villosus*) is a well-studied invader, with a high impact in its invasive range (Soto et al., 2023; Dodd et al., 2014). In this study, no RNA viruses were discovered in this species, based on samples collected in the invasive range. The demon shrimp (*D. haemobaphes*) is a close relative of the killer shrimp that disrupts food webs and decreases biodiversity in the non-native range, but overall is considered to have a lower direct impact (Mamos et al., 2021). The demon shrimp in this study harboured two RNA viruses in its most northern non-native range (Burgess and Bojko, 2022). This invader has commonly been associated with pathogens, across its entire invasive range (Bojko et al., 2019a; Hatcher et al., 2019). The well documented invasion event, alongside previously conducted parasite screening and behavioural study coupled with new RNA virus discovery, provides a wealth of information that establishes a foundation for continued

Table 1

Results of the pairwise comparison on host environment, calculated using the Dunn test with holm adjustment. M, Marine; B, Brackish; F, Freshwater as defined by the World Register of Marine Species.

Data type	Comparison	Z	P, unadjusted	P, adjusted with Holm
Presence and absence of viral species	Brackish – Freshwater	0.06756912	0.946128635	0.94612864
	Brackish – M, B	1.42143898	0.155189181	0.62075672
	Freshwater – M, B	1.63236862	0.102601856	0.61561114
	Brackish – M, B, F	−1.75694499	0.078927185	0.55249030
	Freshwater – M, B, F	−2.33214064	0.019693293	0.17723964
	M, B – M, B, F	−2.77543440	0.005512802	0.05512802
	Brackish – Marine	−0.68332502	0.494401489	0.98880298
	Freshwater – Marine	−1.30566286	0.191667232	0.57500170
	M, B – Marine	−2.07943040	0.037577808	0.30062247
	M, B, F – Marine	1.44939654	0.147226874	0.73613437
Relative viral abundance	Brackish – Freshwater	2.4131794	0.01581403	0.1581403
	Brackish – M, B	0.8866619	0.37526097	1.0000000
	Freshwater – M, B	−0.7453940	0.45603357	1.0000000
	Brackish – M, B, F	2.4123395	0.01585051	0.1426546
	Freshwater – M, B, F	0.9781299	0.32801007	1.0000000
	M, B – M, B, F	1.2315955	0.21810023	1.0000000
	Brackish – Marine	2.2403217	0.02507005	0.2005604
	Freshwater – Marine	0.3141840	0.75338133	0.7533813
	M, B – Marine	0.8309205	0.40601852	1.0000000
	M, B, F – Marine	−0.6759619	0.49906486	0.9981297

parasite study and modelling in this host.

4.4. Conclusion

Overall, the RNA virosphere of the Amphipoda has been expanded through the work conducted here to include 41 complete novel RNA viruses and 134 putative RNA viruses. These viruses appear to be primarily novel, with few associated with other host groups. Amphipods are an important organism in ecological resilience, providing a highly nutritious prey item for predators, as well as contributing to nutrient cycling through detritivorous behaviour. This new perspective on amphipod viruses will help us to provide a basis for exploring ecological services that can be disrupted by viral infections. We must now explore what all these viruses are doing in amphipod hosts, and how their presence or absence alter their host’s behaviour, survival, and ecological niche, especially in situations where the host is an invasive species.

CRediT authorship contribution statement

Amy L. Burgess: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Donald C. Behringer:** Writing – review & editing. **Louisa E. Wood:** Writing – review & editing, Resources. **Eunji Park:** Writing – review &

editing, Resources, Conceptualization. **Randi Rotjan:** Writing – review & editing, Resources. **Karina Scavo-Lord:** Writing – review & editing, Resources. **Rebecca Branconi:** Writing – review & editing, Resources. **Jamie Bojko:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jip.2026.108601>.

Data availability

While submissions are updated to include novel viruses, we provide all sequence and protein data used in the phylogenies as [Supplementary Table 6](#).

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