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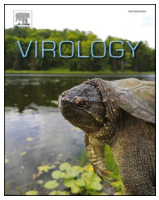

Novel viruses discovered in metatranscriptomic analysis of farmed barramundi in Asia and Australia

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ABSTRACT

Barramundi aquaculture is at risk of severe disease outbreaks and massive production losses. Here we used bioinformatics to screen 84 farmed barramundi transcriptomes to identify novel viruses that could threaten barramundi aquaculture and to establish a barramundi aquaculture virome. We discovered five novel viruses: latid herpesvirus 1 (LatHV-1) from the *Alloherpesviridae* family, barramundi parvovirus 1 (BParV1) from the *Parvoviridae* family, barramundi calicivirus 1 (BCaV1) from the *Caliciviridae* family, and barramundi associated picorna-like virus 1 and 2 (BPicV1 and BPicV2) from the *Picornaviridae* family. LatHV-1, BCaV1, and BParV1 are closely related to pathogenic viruses found in other fish species that can cause mass mortality in farms. To aid in future viral surveillance, we also designed and successfully tested an RT-PCR assay for the detection of BCaV1. Overall, we discovered a range of pathogenic viruses in barramundi aquaculture, paving the way for developing effective detection methods to assist early outbreak management.

Abbreviations

ASCV	Atlantic salmon calicivirus
BCaV1	Barramundi calicivirus 1
BPaV1	Barramundi parvovirus 1
BPicV1	Barramundi picorna-like virus 1
BPicV2	Barramundi picorna-like virus 2
dsDNA	Double stranded DNA
IctHV-1	Ictalurid herpesvirus 1
IctHV-2	Ictalurid herpesvirus 2
ISKNV	Infectious spleen and kidney necrosis virus
LatHV-1	Latid herpesvirus 1
ML	Maximum likelihood
NCBI	National Centre of Biotechnology Information
NNV	Nervous necrosis virus
nt	Nucleotides
ORF	Open reading frame
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
SRA	Sequence read archive
SSDV	Scale drop disease virus
+ssRNA	Positive sense single stranded RNA
USD	United States dollar

1. Introduction

Barramundi, or the Asian sea bass (*Lates calcarifer*), is a food fish with high consumer demand that is both wild caught and farmed. The barramundi aquaculture industry primarily operates in the Southeast Asia region and Australia, and is worth $372 million USD worldwide with Australia constituting 17% of that worth in 2020 (OECD, 2022).

Viral disease in aquaculture causes significant global economic damage, even in countries with strong biosecurity. Disease outbreaks in barramundi aquaculture can be severe, with viruses such as nervous necrosis virus (NNV), scale drop disease virus (SDDV), infectious spleen and kidney necrosis virus (ISKNV), lates calcarifer herpesvirus (LCHV), lates calcarifer birnavirus, and red seabream iridovirus (RSIV) significantly impacting fish production (Chen et al., 2019; de Groof et al., 2015; Hick et al., 2011; Simmelink et al., 2024; Sumithra et al., 2022; Thanasaksiri et al., 2019). NNV from the *Nodaviridae* family has caused mass deaths in barramundi farms in Thailand, Malaysia, and Australia since the 1970s with mortality rates of up to 100% (Hick et al., 2011). Barramundi farms have also been afflicted by viruses of the *Iridoviridae* family such as SDDV, ISKNV, and RSIV resulting in mortality rates ranging from 40 to 50% in Thailand, Malaysia, India, and Vietnam (de Groof et al., 2015; Dong et al., 2017; Nurliyana et al., 2020; Senapin et al., 2019; Sumithra et al., 2022). Thus, viruses are an important cause

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of reduced barramundi aquaculture production, yet there are currently no viral treatments or vaccines for barramundi viruses, screening programs for novel disease-causing viruses, and few detection methods commercially available to mitigate this.

Using computational methods, we can now discover viruses and develop nucleic acid detection methods before they cause irrevocable damage to barramundi populations in fish farms. We have previously used metatranscriptomic analyses of RNA from understudied organisms to uncover novel viruses that in turn can facilitate the pre-emptive discoveries of more distantly related viruses (Harding et al., 2022; Mercer et al., 2022; Russo et al., 2018, 2021).

Using a bioinformatic BLAST-based work-flow, we have recently discovered two caliciviruses and a nidovirus in agnathan fish (Mercer et al., 2022), with potential to be pathogenic. Our methodology was also used for viral discovery in 122 species of reptiles and amphibians which revealed 26 novel viruses from 15 different viral families highlighting the capability of RNA sequencing and subsequent metatranscriptomics for viral discovery (Harding et al., 2022).

The aim of the current study was to conduct a metatranscriptomic bioinformatics analysis of all available RNA sequencing datasets of barramundi to identify and characterise novel viruses in order to expand our current understanding of the barramundi virome. Additionally, we aimed to assess the genetic relationship of these new viruses to other previously characterised related viruses to determine their pathogenic potential. RT-PCR and PCR based diagnostic assays can be developed for rapid viral detection in a variety of fish sample types. Therefore, we also aimed to safeguard barramundi farms from an outbreak of these novel viruses by developing and testing a molecular assay to detect the novel calicivirus, barramundi calicivirus 1.

2. Materials and methods

2.1. Viral discovery in public transcriptomic data

Barramundi (*Lates calcarifer*) RNA sequencing datasets (n = 84; Appendix A; Appendix B) were analysed in this study; Two of which were original datasets from this study and 82 of which were downloaded from the National Centre of Biotechnology Information (NCBI) Sequence Read Archive (SRA) database. The raw reads were assembled using Trinity v.2.8.4, viral homologues were annotated using DIAMOND v.0.9.31 and filtered for viral sequences according to previous methods (Harding et al., 2022; Mercer et al., 2022). A novel virus species is determined by having less than 90% amino acid pairwise identity to its closest viral relative.

2.2. Barramundi sample source and sample preparation

Whole farmed barramundis (n = 6; Appendix B) approximately 700–750 g were purchased whole from the Sydney Fish Markets, packaged separately in plastic bags, and transported on ice for processing within 1 h of purchase. These fish originated from a barramundi farm in Northern Territory, Australia.

Fish muscle was filleted away from the skeleton and organ cavity. Intestinal tracts were excised from surrounding organs by hand. All organs were stored separately at −80 °C until use.

2.3. RNA extraction

From each fish, ~80 mg of intestine was used for each RNA extraction using TRIzol/chloroform extraction (Russo et al., 2018). The tissues were homogenised with 1 mL of TRIzol and 1.4 mm diameter ceramic beads (MP Biomedicals, Sydney, Australia) while maintaining the sample at 4 °C. The homogenate was added to 200 μL of chloroform which was shaken for 15 s and incubated at room temperature for 3 min before centrifugation at 13,000 rpm for 15 min at 4 °C. The upper aqueous phase was extracted, and an equivalent amount of 70% ethanol was added to it. The Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) was then used to further purify the RNA according to manufacturer instructions. The RNA was eluted in 40 μL total RNase-free water. RNA was pooled with three samples per pool at 200 ng/μL and sequenced using the Illumina NovaSeq6000 platform (2x100 bp paired end reads) by the Ramaciotti Centre for Genomics (UNSW Sydney, Australia). Two RNA sequencing datasets (SRA accession nos. SRR28869355, SRR28869354) were generated (~10,000,000 reads per dataset) from pooled gut samples.

2.4. Viral gene annotation

Annotation of open reading frames (ORFs) and genomic features was conducted in Geneious Prime software v.2023.2.1 by comparing proteins and conserved protein domains from related viruses. ORFs were identified using the "Find ORFs" function in Geneious Prime with a minimum length of 200 aa. Each ORF was annotated using a BLASTx search (E-value cut-off $1e^{-03}$) to determine the identity of the encoded viral protein based on the closest viral relative. Viral transcripts were also queried against the NCBI conserved domain database (CDD) to facilitate annotation of protein domains and motifs.

2.5. Phylogenetic analysis

Phylogenies were inferred following the alignment of novel viral amino acid (aa) sequences with their closest relatives and outgroup viral sequences. All alignments were conducted using MAFFT (v.7.490) (Katoh and Standley, 2013) with default settings and phylogenetic analysis was conducted using RAxML v.8.2.11 (Stamatakis, 2014). Phylogenetic trees were constructed with 500 bootstrap replicates and rooted at the outgroup.

2.6. Primer design

Primers were designed using NCBI PrimerBLAST (Ye et al., 2012) based on the novel barramundi calicivirus 1 (BCaV1) genome sequence from nucleotide positions 4200 to 5800 which covers the region between the RNA-dependent RNA-polymerase (RdRp) and VP1. The amplicon product size was set to 250–800 nt. Primer specificity was set to exclude eukaryotic sequences from the barramundi genome and other caliciviruses. The resulting primers were the forward primer MML395 (5'-TCGTGAGGGGTACAACAAGC-3') and reverse primer MML396 (5'-GTTGACGACTGGGGTAGGTG-3') which bind to the RdRp and VP1 protein regions of the barramundi calicivirus 1 genome to generate a 316 nt amplicon.

2.7. Detection of barramundi calicivirus 1 using RT-PCR

Barramundi intestinal RNA was reverse transcribed using SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific, Sydney, Australia) according to manufacturer instructions then diluted to ~50 ng/μL. The subsequent PCR was run with cDNA samples using iTaq Universal SYBR Green Supermix (BioRad, Sydney, Australia). Reactions were performed in 20 μL containing 2 μL of cDNA. Forward and reverse primers were included at a final concentration of 500 nM. Thermocycling reaction conditions were: Taq activation at 95 °C for 1 min with 40 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s followed by a final hold step at 4 °C.

Amplicons were stained with Gel Loading Dye, Purple (New England Biolabs, Ipswich, United States) and run on a 2% Tris-acetate-EDTA (TAE) agarose gel with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Sydney, Australia) at 100 V for 60 min. The products were run alongside a 50 nt DNA Ladder (New England Biolabs, Ipswich, United States).

3. Results

3.1. Barramundi RNA reveals new variety of potentially pathogenic viruses

Out of the total of 84 barramundi transcriptomes analysed, seven contained novel viral sequences with identity to viruses within the *Alloherpesviridae*, *Caliciviridae*, *Parvoviridae*, and *Picornaviridae* families (Table 1).

3.2. Herpesvirus discovered in the transcriptomes of laboratory-reared barramundi

We identified a herpesvirus which we have provisionally named latid herpesvirus 1 (LatHV-1; GenBank accession no. BK064844; Fig. 1) in the gill transcriptomes from three RNA datasets of laboratory reared barramundi (which were biological replicates i.e., the gill tissues of three separate fish) from Hainan, China (GenBank accession nos. SRR14003371, SRR14003380, SRR14003386) (Fu et al., 2021). The combined LatHV-1 near-complete transcriptome comprised of 149 transcripts totalling 106,484 nt, consisting of a 94,948 nt unique region and a 5768 nt flanking terminal repeat region at the 5' and 3' ends of the entire genome (Fig. 1). The number of transcripts present in each transcriptome were 25, 50, and 74 respectively with contig lengths ranging from 216–11,876 nt. These transcripts were assembled into a single genome using the *de novo* assembly (Fig. 1).

Overall, this virus shared 98.4% nucleotide pairwise identity with lates calcarifer herpesvirus (LCHV) (Genbank accession no. PP098466.1) (Simmelink et al., 2024). LatHV-1 and LCHV are likely different strains of the same virus, but this virus will be henceforth referred to as LatHV-1 unless it is specified that the LCHV sequence is being discussed. Compared to other species in the *Alloherpesviridae* family, this virus shared 51% nucleotide identity with the catfish ictalurid herpesvirus 1 (IctHV-1; GenBank accession no. NC_001393) (Davison, 1992) over 106,484 nt, 38% pairwise identity with the sturgeon acipenserid herpesvirus 2 (AciHV-2; GenBank accession no. NC_043042.1) over 100,806 nt, and 34% pairwise identity over 146,197 nt with another catfish virus, silurid herpesvirus 1 (SilHV-1; GenBank accession no. MH048901.1).

The closest relative, IctHV-1, was originally discovered in channel catfish (*Ictalurus punctatus*) in Alabama, USA. Relative to IctHV-1 (134,226 nt), we have discovered around 80% of the LatHV-1 genome. Unmapped sequences in LatHV-1 likely include ORF2-ORF12 which lie in the terminal repeat regions and also gaps between ORFs, which are not transcribed. In total, we identified 61 ORFs in the unique region and three ORFs in the two terminal repeat regions. Most of the ORFs were complete and ranged from 225–4935 nt in length (Fig. 1) and all 12 core genes conserved in *Alloherpesviridae* were identified in the barramundi virus (Fig. 1 in bold) (Borzák et al., 2018).

Compared to the IctHV-1 genome, the current LatHV-1 genome assembly is missing ORFs 16–18 and ORFs 77–79 from the 5' and 3' ends of the unique region, respectively. In LatHV-1, there is an additional ORF73 between ORF24 and ORF25 that is not present in IctHV-1 but is homologous to ORF73 present in AciHV-2 (*Acipenser transmontanus*) (Doszpoly et al., 2011).

Our genomic analyses revealed that in the LatHV-1 genome, some regions showed a different organisation compared to other viruses of the same family. Most notably, ORF57 and ORF58 in alloherpesviruses such as SilHV-1, AciHV-2, and the Auburn 1 strain of IctHV-1 are two separate ORFs. However, in LatHV-1 and the S98-675 strain of IctHV-1, ORF57 + 58 comprises one ORF.

Phylogenetic analysis of LatHV-1 together with other viruses in the *Herpesvirales* order shows that LatHV-1 clusters with viruses in the *Alloherpesivirdae* family that infect silurid and ictalurid catfish (Fig. 2) and is the first virus of this family known to infect barramundi.

3.3. Novel barramundi parvovirus 1 capsid sequence

Barramundi parvovirus 1 (BParV1; GenBank accession no. BK064846; Fig. 3A) represents a partial genome sequence from the *Parvoviridae* family that was discovered in the liver transcriptome of a farmed barramundi from Singapore (GenBank accession no. SRR13950764) (Domingos et al., 2021). One transcript was discovered which encoded partial ORFs of VP1 and VP2 that were 345 aa and 190 aa in length, respectively. Compared to other related parvovirus genomes, the BParV1 transcript accounted for around 23% (1136/4944 nt) of a complete genome. Noting that there are no fish-associated members of the *Parvovirinae* subfamily, its closest relative using BLASTx was the gorilla bocavirus (Kapoor et al., 2010) with 51.8% pairwise identity across the 1136 nt transcript of VP1 and VP2. Phylogenetic analysis (Fig. 3B) showed that although BParV1 clustered within the *Parvovirinae* subfamily, it did not group within other genera, which mostly consist of viruses associated with mammals such as primates, bovines, and mice (Fig. 3B).

Table 1
Novel barramundi viruses discovered in this study.

Assigned name	Genbank accession no.	Transcriptome(s) of origin	Viral family	Total length (bp)	% of genome completed	Closest viral relative BLASTx	% Identity to closest BLASTx hit	Isolation source of closest viral relative
Latid herpesvirus 1 (LatHV-1)	BK064844	SRR14003371, SRR14003380, SRR14003386	*Alloherpesviridae*	106,484	81%	Ictalurid herpesvirus 1 strain Auburn 1	**51.0%**	Channel catfish
Barramundi calicivirus 1 (BCaV1)[a]	PP767962	SRR28869354	*Caliciviridae*	7233	100%	Beihai conger calicivirus	**53.6%**	Conger eel
Barramundi parvovirus 1 (BParV1)	BK064846	SRR13950764	*Parvoviridae*	1136	23%	Bocavirus gorilla/GBoV1/2009	**51.8%**	Gorilla
Barramundi associated picorna-like virus 1 (BPicV1)[b]	BK064845	SRR13950759	*Picornaviridae/ Dicistroviridae*	3561	50%	Mute swan faeces associated picorna-like virus 12	**28.6%**	Mute swan faeces
Barramundi associated picorna-like virus 2 (BPicV2)[a, b]	PP767961	SRR28869355, SRR28869354	*Picornaviridae/ Dicistroviridae*	8499	94%	Beihai mollusks virus 1	**57.4%**	Marine invertebrates

[a] Viruses discovered in original RNA sequencing dataset.
[b] Virus likely infects invertebrate hosts.

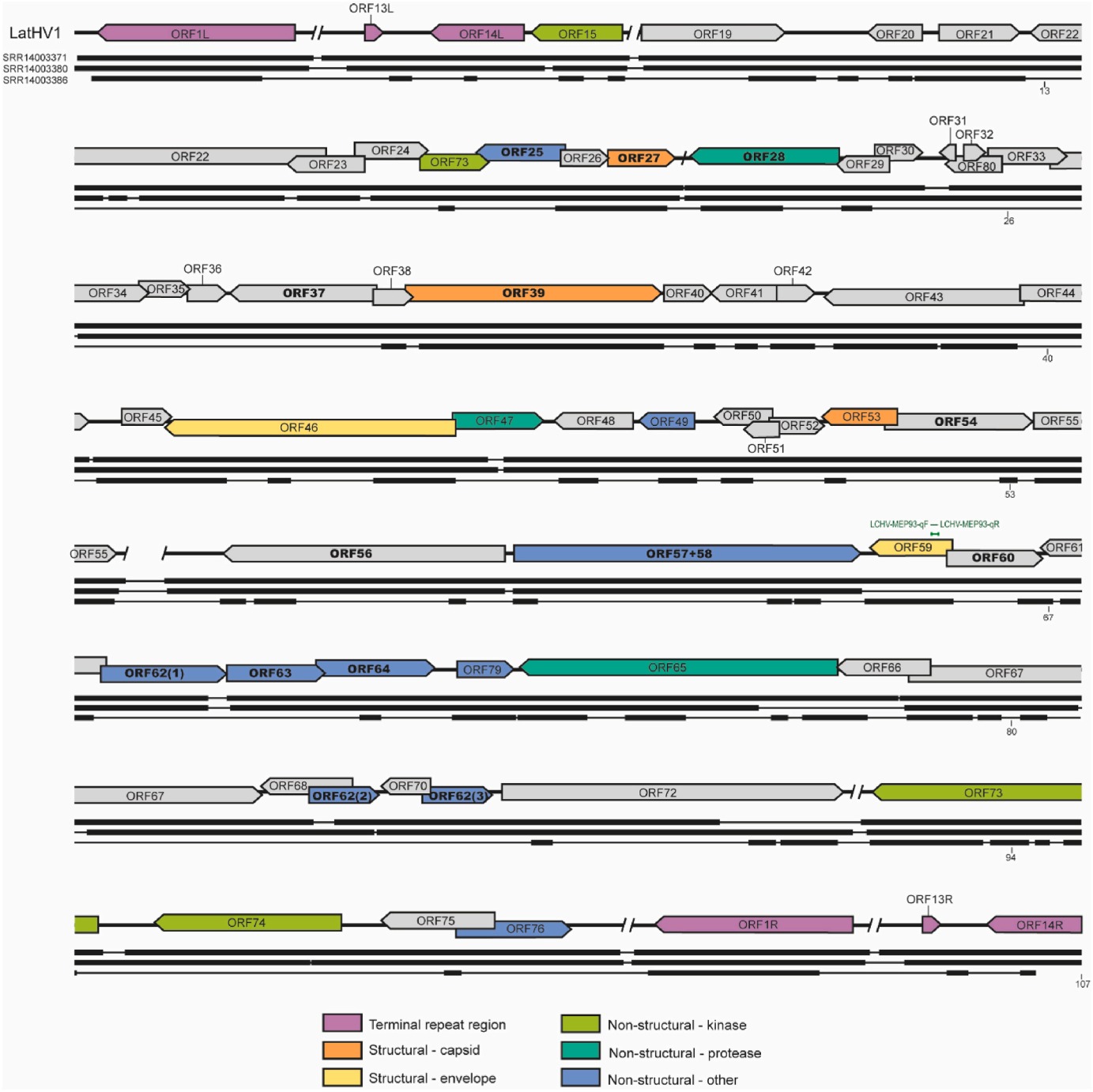

Fig. 1. Genome structure and alignment of latid herpesvirus 1 from three barramundi transcriptomes
Genome organisation of latid herpesvirus 1 (LatHV-1). Contigs from barramundi gill RNA sequencing datasets (GenBank accession nos. SRR14003371, SRR14003380, SRR14003386) are represented by black bars which were mapped to and annotated according to ictalurid herpesvirus 1, strain Auburn 1 which is not shown (GenBank accession no. NC_001493). An annotated to-scale consensus sequence of LatHV-1 was generated from this alignment. Bolded labels indicate core *Alloherpesviridae* genes. Coloured blocks indicate ORFS (purple = terminal repeat regions, orange = capsid proteins, yellow = envelope proteins, green = kinases, teal = proteases, blue = other non-structural proteins, grey = undefined). Numbers at the ends of each row indicate kilobases and broken lines indicate missing sequences in that region. Sequence breaks are not to scale. The 3' repeat region is shown. The green bar above ORF59 shows the region amplified by primer set LCHV-MEP93-qF and LCHV-MEP93-qR (Meemetta et al., 2020).

3.4. Australian barramundi RNA datasets a reveal novel calicivirus in aquaculture

To identify novel viruses in order to expand the barramundi virome in aquaculture, six barramundi intestine RNA samples sourced from the Sydney Fish Markets were sequenced in two pools (n = 3 samples per pool). Novel viral sequences with identity to viruses within the *Caliciviridae* and *Picornaviridae* families were present in both of the pooled barramundi gut sequencing data (Table 1).

3.4.1. Novel full length barramundi calicivirus

Caliciviridae viruses are small positive sense single stranded RNA (+ssRNA) viruses that have been found to infect a wide range of vertebrates (Vinjé et al., 2019). A full-length novel calicivirus of 7322 nt,

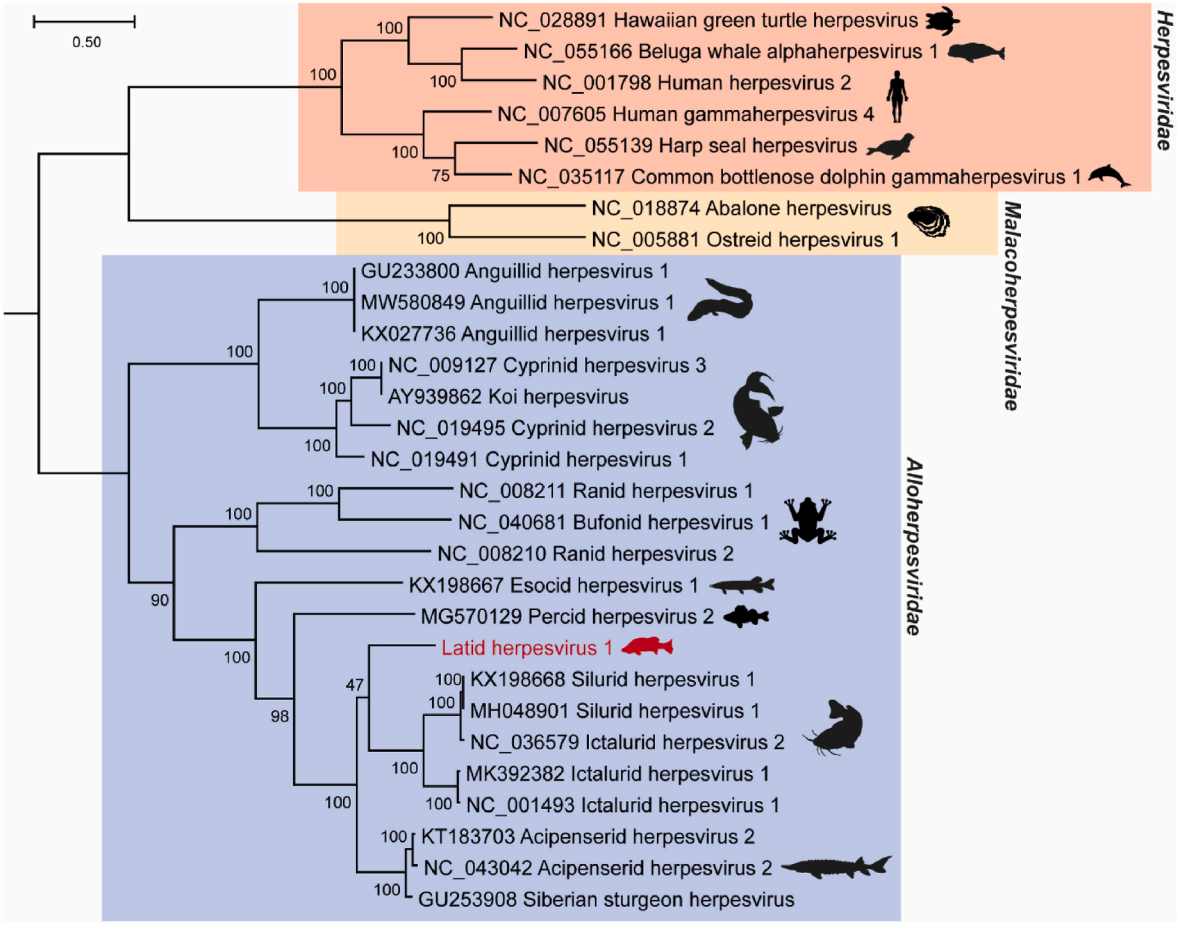

Fig. 2. Phylogenetic analysis of latid herpesvirus 1 DNA polymerase/ORF57 + 58
Maximum Likelihood phylogeny of DNA dependent DNA polymerase (2601 aa positions) of 29 *Herpesvirales* sequences including the novel LatHV1. Alignments were created with MAFFT v.7.450, trimmed manually, and phylogenies inferred with RAxML v.8.2.11. Shaded colour represents viral family (red = *Herpesviridae*; yellow = *Malacoherpesviridae*; blue = *Alloherpesviridae*); images indicate host type. GenBank accessions prefix the labels of each sequence used. Node labels indicate bootstrap support from 500 replicates (%). Scale bar represents aa substitutions per site.

denoted barramundi calicivirus 1 (BCaV1; GenBank accession no. PP767962; Fig. 4A), was discovered in gut transcriptome of farmed barramundi from Northern Territory, Australia. BCaV1 was assembled from a single 7233 nt transcript from one gut transcriptome containing three gut samples from different fish.

BCaV1 is composed of two ORFs: ORF1 encodes a 2222 aa long non-structural polyprotein and the viral capsid, VP1; and ORF2, which encodes a 184 aa long putative accessory structural protein. Overall, this virus shares ~54% pairwise identity with Beihai conger calicivirus (GenBank accession no. MG599956) over its entire genome which was originally discovered in Beihai, China and is 6981 nt in length (Shi et al., 2018).

Phylogenetic analyses of the RdRp and VP1 amino acid sequences of BCaV1 demonstrated that it clustered with viruses from the *Caliciviridae* family that have been found in both fish and amphibians (Fig. 4B and. C).

3.4.2. Development of an RT-PCR detection method for barramundi calicivirus 1

An RT-PCR detection method was developed to detect BCaV1 RNA for monitoring the virus in barramundi samples. Primer set MML395-MML396 was designed to amplify a 316 nt region at the 3' end of the RdRp and the 5' end of VP1 (Fig. 4A) which produced a single distinct band and no nonspecific binding (data not shown). RT-PCR amplification was performed on the RNA isolated from six barramundi intestine samples, which demonstrated that BCaV1 was present in 1/6 of the samples, consistent with the RNA sequencing results. The positive sample belonged to the RNA sequencing dataset in which BCaV1 was discovered. The RT-PCR was also performed on six liver samples from the same fish, which were all negative for BCaV1.

3.5. The barramundi aquaculture virome

3.5.1. Barramundi associated picorna-like virus 1

Picornavirales is an order of positive sense single stranded RNA (+ssRNA) viruses that encompasses many viral families that infect a wide range of vertebrate and invertebrate hosts (Le Gall et al., 2008). The partial genome of a picorna-like virus, denoted barramundi associated picorna-like virus 1 (BPicV1; GenBank accession no. BK064845; Fig. 5A) was discovered as a single transcript in the muscle transcriptome of a farmed barramundi from Singapore (GenBank accession no. SRR13950759; Fig. 5A). The total length of the sequence was 3561 nt which is approximately 50% of the predicted genome length when compared to Mute swan faeces associated picorna-like virus 12 (Hill et al., 2023).

In BPicV1, there are two ORFs: the non-structural ORF1 and a putative capsid encoding ORF2 (Fig. 5A). The amino acid sequence encoded by ORF1 showed 29% pairwise identity with its closest BLASTx hit Mute swan faeces associated picorna-like virus 12 (Hill et al., 2023) across 502 aa. Mute swan faeces associated picorna-like virus 12 was originally discovered in the UK and is 7132 nt in length, and likely demonstrates that BPicV1 is not a fish virus. A BLAST search of the

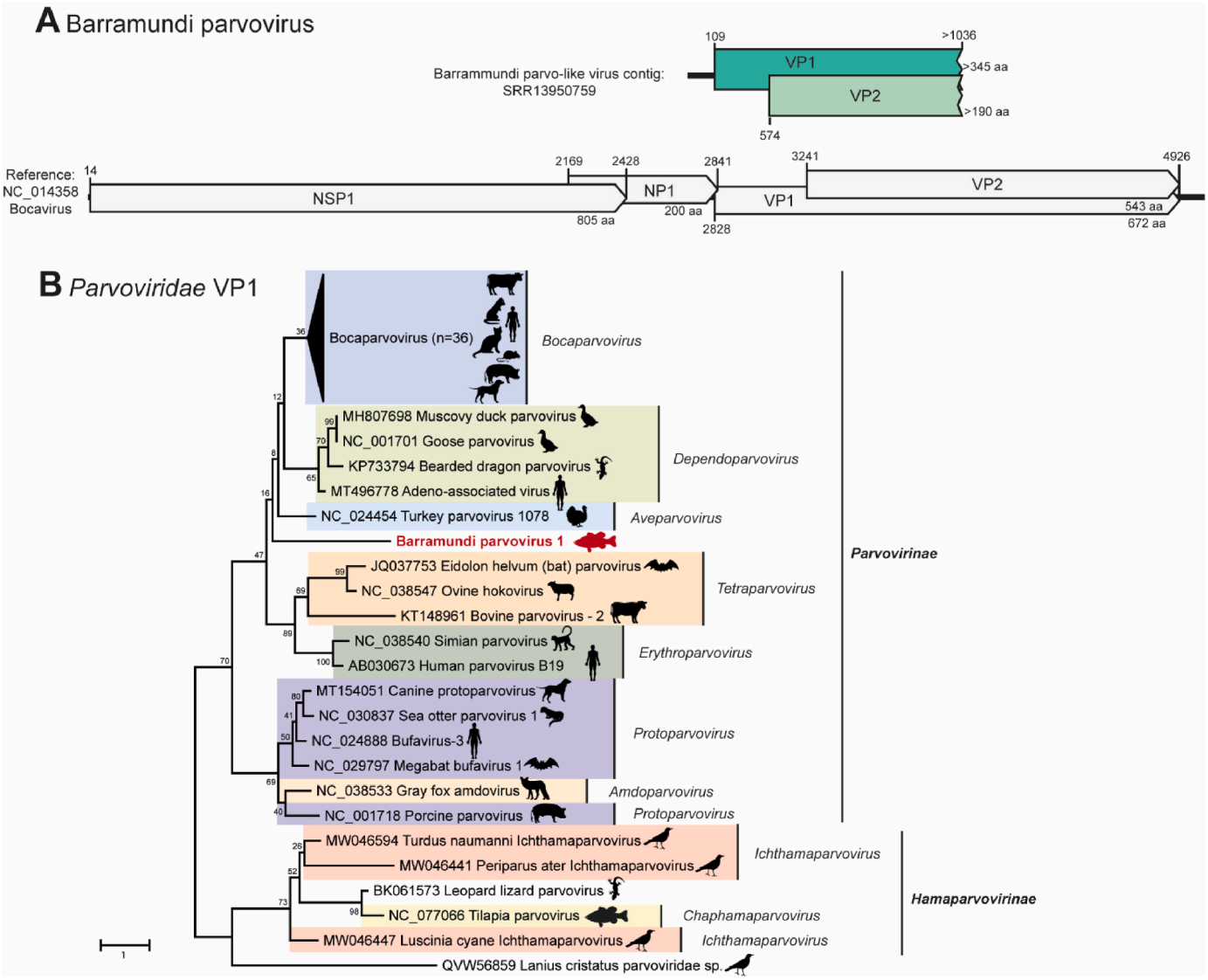

Fig. 3. Barramundi parvovirus 1 phylogeny and genome map
(A) Genome organisation of barramundi parvovirus 1 (BParV1) aligned to its reference genome gorilla bocavirus (GenBank accession no. NC_014358). Putative ORFs were annotated using the "Find ORFs" function on Geneious. Broken blocks signify incomplete ORFs. (B) Maximum Likelihood phylogenies of VP1 proteins (147 aa positions) of 59 *Parvoviridae* sequences including the BParV1 sequence. Alignments were created with MAFFT v.7.490, trimmed manually, and phylogenies inferred with RAxML v.8.2.11. Shaded colour represents subfamilies of *Parvoviridae*; images show host type. GenBank accessions prefix the labels of each sequence used. Node labels indicate bootstrap support from 500 replicates (%). Scale bar represents aa substitutions per site.

amino acid sequence encoding ORF2 did not find any related sequences but its length and position in the genome indicates that it is likely a capsid gene. Overall, BPicV1 does not cluster within any known viral families in *Picornavirales* and is likely to be part of a novel viral family (Fig. 5C and. D). As a result, the host of BPicV1 is inconclusive, but likely comprises part of the barramundi farm virome.

3.5.2. *Barramundi associated picorna-like virus 2*

Using Australian barramundi RNA sequencing data generated from the current study, another picorna-like virus was discovered. Barramundi associated picorna-like virus 2 (BPicV2; GenBank accession no. PP767961; Fig. 5B) is a full-length virus of 8388 nt that was discovered in the two pooled barramundi gut transcriptomes from a farm in Northern Territory, Australia. In total there were 10 transcripts ranging from 232–1696 nt. Similar to BPicV1, there are two ORFs in the viral genome, a non-structural ORF1 and a structural ORF2. The closest relative to BPicV2 was Beihai mollusks virus 1 which shared 57.4% pairwise identity over its full nucleotide length and was originally discovered in Beihai, China in marine gastropods (Shi et al., 2016). Phylogenetic analyses of ORF1 and ORF2 show that BPicV2 does not cluster within any known families within the *Picornavirales* order (Fig. 5C and. D). Similar to BPicV1, the host of BPicV2 is also inconclusive and likely does not directly infect barramundi.

4. Discussion

4.1. *Discovery of five new viruses expands the barramundi virome*

In this study, bioinformatics screening revealed three new viruses with disease potential: the herpesvirus LatHV-1, the parvovirus BParV1, and the calicivirus BCaV1 from China, Singapore, and Australia respectively. LatHV-1 and BCaV1 in particular share around 50% pairwise identity to fish viruses that have recorded high mortality rates or major losses to the aquaculture industry, and therefore should be included in biosecurity screening of barramundi to prevent disease outbreaks occurring in the future.

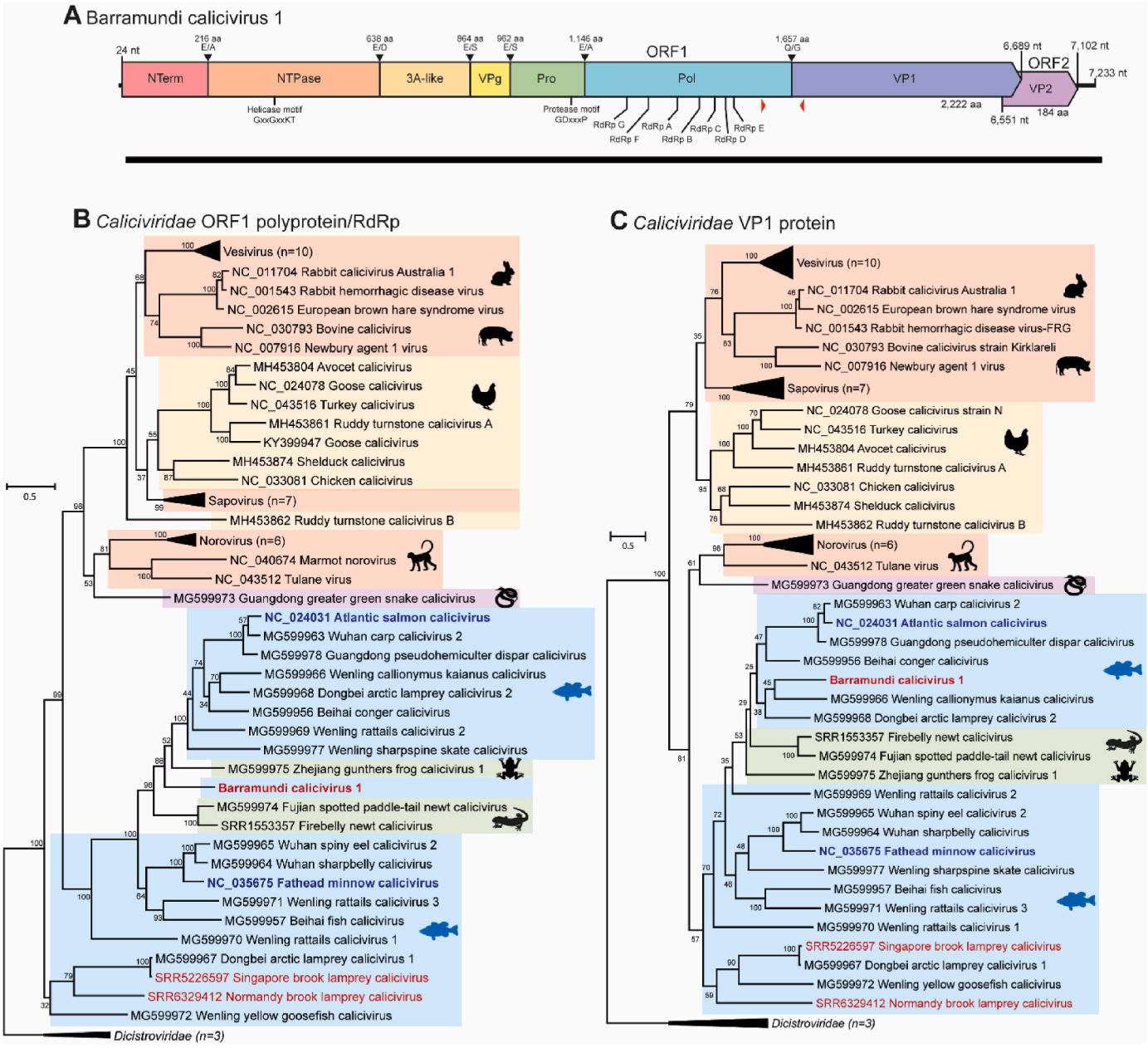

Fig. 4. Barramundi calicivirus 1 genome and phylogeny
(A) Genome organisation of barramundi calicivirus 1 (BCaV1). Contigs from two barramundi gut RNA sequencing datasets are represented by black bars. An annotated to-scale consensus sequence of the BCaV1 genome was generated by aligning these contigs. Conserved enzymatic motifs, putative protease cleavage sites, nucleotide positions, and amino acid length of each encoded protein are indicated on the genome. The binding sites of forward primer MML395 and reverse primer MML396 are shown by forward and reverse red triangles, respectively. (B) Maximum likelihood (ML) phylogeny of the ORF1 encoded non-structural polyprotein (1133 aa positions) of BCaV1 (shown in bold red) with 60 other caliciviruses, and three dicistroviruses (used as an outgroup). (C) ML phylogeny of VP1 capsid protein (841 aa positions) of BCaV1 with 58 other caliciviruses, and three dicistroviruses used as an outgroup. Alignments were created with MAFFT v.7.490, trimmed manually, and phylogenies inferred with RAxML v.8.2.11 using the GAMMA BLOSUM62 protein substitution model. Shaded colour represents host range (yellow = birds; orange = mammals; purple = reptiles; blue = fish; green = amphibians); images indicate host type. GenBank accessions prefix the labels of each sequence used. Labels in blue are piscine caliciviruses that have been associated with clinical symptoms. Labels in red are piscine caliciviruses previously discovered by our group (Mercer et al., 2022). Node labels indicate bootstrap support from 500 replicates (%). Scale bar represents aa substitutions per site.

In addition, two picorna-like viruses were discovered. The picornaviruses were uniquely divergent from known viruses of the *Picornavirales* order.

4.2. *Alloherpesviridae is a major enemy in commercial fish rearing*

Alloherpesviridae is a family of large double-stranded DNA (dsDNA) viruses ranging from 134 to 295 kb which infect and cause disease in fish and amphibians (Davison et al., 2009). Alloherpesviruses pose a major threat to commercial aquaculture and have previously caused major losses through severe disease outbreaks and high mortality of affected fish as reviewed in (Hanson et al., 2011). For example, IctHV-1 was the cause of disease outbreaks in young fish in channel catfish farms in the USA (Fijan et al., 1970), and ictalurid herpesvirus 2 (IctHV-2) caused high fish mortality rates of 80–90% in black bullhead catfish farms in Italy (Hedrick et al., 2003).

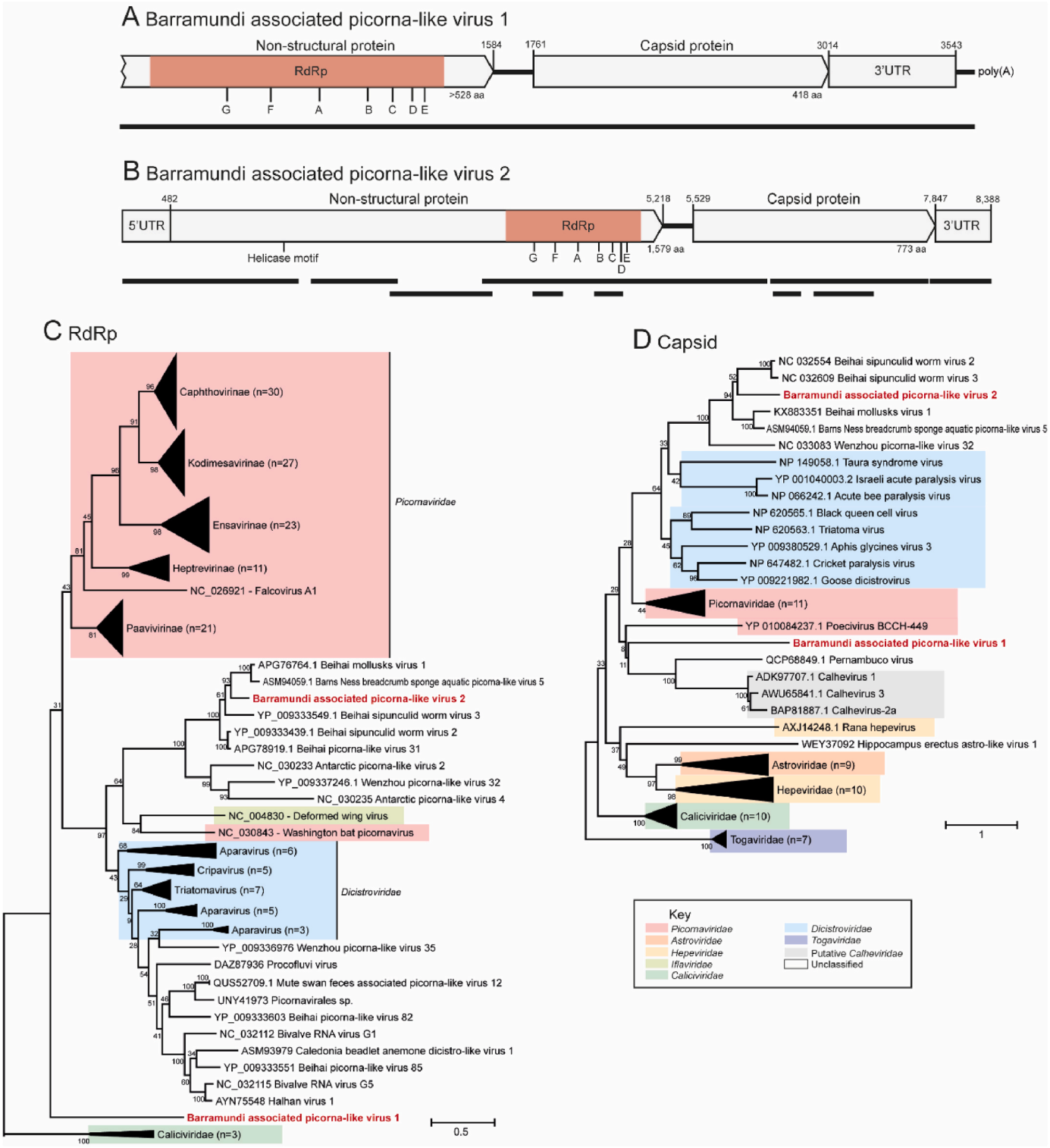

Fig. 5. Phylogeny and genome map of Barramundi associated picorna-like virus 1 and 2 RdRp and capsid regions
Genome organisation of (A) barramundi associated picorna-like virus 1 and (B) barramundi picorna-like 2. Contigs from RNA sequencing datasets are represented by black bars. Annotated to-scale consensus sequences of the novel viruses was generated by aligning these contigs. Conserved enzymatic motifs, nucleotide positions, and amino acid length of each polyprotein are indicated on the genome. Putative ORFs were annotated using the Find ORFs function on Geneious and are indicated with coloured blocks. Broken blocks indicate incomplete ORFs. Maximum likelihood (ML) phylogenies of the (C) RNA-dependent RNA-polymerase proteins (548 aa positions) of 168 *Picornavirales* sequences, and (D) capsid proteins (1223 aa positions) of 69 *Picornavirales* sequences. Alignments were created with MAFFT v.7.490 trimmed manually, and phylogenies inferred with RAxML v.8.2.11. GenBank accessions prefix the labels of each sequence used. Node labels indicate bootstrap support from 500 replicates (%). Scale bar represents aa substitutions per site.

LatHV-1 was identified in the present study independently of LCHV which was first reported in 2015 and the LCHV genome was elucidated in 2024 (Simmelink et al., 2024). LCHV had been responsible for outbreaks in barramundi in Southeast Asian countries resulting in mortality rates of 60–70% (Dang et al., 2023). These numbers are similar to those of outbreaks caused by closely related pathogenic alloherpesviruses such as Ictalurid herpesviruses 1–2 (IctHV-1-2) and Cyprinid herpesviruses 1–3 (CypHV-1-3; Fig. 2). Under experimental conditions, these viruses have mortality rates ranging from 70 to 100% in the fry of their respective hosts (Fijan et al., 1970; Hedrick et al., 2003; Jung and Miyazaki, 1995; Plant et al., 2005; Wang et al., 2012).

Latent infections, which involve the viral genome persisting as multicopy episomes in the nuclei of cells (Grinde, 2013), also pose a threat to the farm by maintaining the virus in the population. This virus has potential to re-emerge through reactivation, which is characteristic of the *Herpesvirales* order. For example, In the *Alloherpesviridae* family, latency in CypHV-3 infections have been observed in both wild carp (*Cyprinus carpio carpio*) and domesticated koi carp (*Cyprinus carpio koi*). Fish with CypHV-3 infections in the latent phase express no clinical symptoms (Xu et al., 2013), but herpesvirus reactivations in survivors could lead to recurring herpesvirus outbreaks resulting in multiple production losses within the same farm.

Currently there have not been any known barramundi herpesvirus outbreaks in Australia, however, this does not preclude importing of the virus through stock movement, or importation from another country. It is prudent to monitor for viruses in fish populations to prevent viral outbreaks. This can be achieved with rapid and flexible detection methods such as PCR, so that appropriate action can be taken. For example, PCR assays for *Alloherpesviridae* usually target the DNA polymerase gene or ORF57 + 58 and have been developed for CypHV-2 (Goodwin et al., 2006) and IctHV-2 (Goodwin and Marecaux, 2010). In addition, a PCR assay has previously been developed for LatHV-1 (LCHV) in barramundi (Meemetta et al., 2020). The primers LCHV-MEP93-qF and LCHV-MEP93-qR (Meemetta et al., 2020) mapped with 100% identity to LatHV-1 sequence and amplifies a 93 nt product from ORF59, which encodes an envelope protein (Fig. 1). As we have elucidated the complete LatHV-1 genome, we can design highly specific PCR primers for the detection of LatHV-1. Thus, monitoring for potential pathogens such as LatHV-1 is an important strategy to implement to prevent costly outbreaks and identify any incursion rapidly.

Vaccination is also crucial for the prevention of viral outbreaks in fish farms. Currently there are no commercial treatments or vaccines against alloherpesviruses, however, some are in development: for example, an inactivated CypHV-2 vaccine has been developed which results in a reported disease prevention efficacy of 71–74% (Dharmaratnam et al., 2022; Zhang et al., 2016).

4.3. Expansion of the piscine clade within the Caliciviridae family

Caliciviruses are a well characterised family of viruses in vertebrates and are known to infect a range of terrestrial vertebrates including humans. Caliciviruses are monopartite single stranded positive sense RNA (+ssRNA) viruses of around 6.4–8.5 kb that are non-enveloped and icosahedral (Vinjé et al., 2019).

Recently, a metatranscriptomic study discovered 19 new fish caliciviruses in China, demonstrating that there is likely a new clade of undiscovered fish caliciviruses (Shi et al., 2018). We have also previously discovered two caliciviruses in agnathan fish transcriptomic data (Mercer et al., 2022) that clustered in this group of fish caliciviruses (Fig. 4B and. C). BCaV1 was discovered in fish originating from Northern Territory, Australia which also clusters within this clade (Fig. 4B and. C). As a result, the discovery of BCaV1 has expanded the host range of *Caliciviridae*, particularly this clade of fish caliciviruses, to include barramundi.

In this study, we developed an RT-PCR detection method for BCaV1 that was successfully used to screen 6 farmed barramundi. The primer set MML395-MML396 serves as a reliable tool for identifying BCaV1 which offers an efficient diagnostic approach for monitoring BCaV1 in trout farming environments.

Future work can involve developing an antiviral or vaccine for BCaV1 to prevent potential outbreaks. Currently, the only vaccine that has been developed for a fish calicivirus was a formalin inactivated vaccine for ASCV, which significantly reduced the viral load in vaccinated Atlantic salmon (Mikalsen et al., 2014). Therefore, vaccination could greatly improve the health of fish within calicivirus endemic regions and prevent the spread of calicivirus via exports.

4.3.1. Piscine caliciviruses could cause malabsorption in the host

Among piscine caliciviruses, most were discovered using metatranscriptomics and we have no reports or available data of clinical symptoms in the fish where the samples were sourced. The only piscine calicivirus whose pathogenicity had been studied in depth is the Atlantic salmon calicivirus (ASCV), which was first discovered in Atlantic salmon (*Salmo salar*) farms in Norway. ASCV has been reported to cause malabsorption of fats which may be indicated by floating faeces (Mikalsen et al., 2014). This is significant because in Atlantic salmon, lipid malabsorption can lead to stunted growth due to reduced lipid utilisation, and reduced protein utilisation (Duan et al., 2012; Hansen et al., 2020). Therefore, it is possible that fish infected with BCaV1 may also exhibit similar symptoms which can be monitored for as a sign of disease outbreak.

4.4. The second novel aquatic parvovirus expands Parvoviridae host range

Currently there is one fish parvovirus associated with disease, tilapia parvovirus (Liu et al., 2020). Parvoviruses are a family of small single-stranded non-enveloped icosahedral DNA viruses. Recently, the host range of parvoviruses has been extended from mammals to include birds, reptiles, and fish (De Souza et al., 2018; Harding et al., 2022; Liu et al., 2020).

BParV1 was not related to the first fish parvovirus identified, tilapia parvovirus which was discovered in 2019 in farmed Nile tilapia *(Oreochromis niloticus*) in China. Tilapia parvovirus was isolated from an epidemic in 2015 that resulted in a fish mortality rate of 60–70% (Liu et al., 2020). Tilapia parvovirus has also been found as a co-infection with tilapia lake virus from the *Amnoonviridae* family in tilapia farms in India and Thailand (Mishra et al., 2017; Yamkasem et al., 2021). BParV1 clusters within a separate subfamily to tilapia parvovirus which represents the genus *Chaphamaparvovirus* in the *Hamaparvovirinae* subfamily (Fig. 3B).

Our study provides evidence of another parvovirus genus that exists in fish, BParV1 (Fig. 3), which was discovered in the liver tissue of farmed barramundi from Singapore. BParV1 was found to cluster in the *Parvovirinae* family near the *Dependoparvovirus* and *Aveparvovirus* genera (Fig. 3B). Despite the phylogenetic distance, both BParV1 and tilapia parvovirus cluster near reptilian and avian genera within their respective subfamilies (Fig. 3B). The discovery of the parvovirus indicates the presence of another clade of undiscovered fish viruses in the *Parvovirinae* subfamily and expands the known host range of the *Parvoviridae* family among vertebrates.

4.5. Picornaviruses in the fish farm virome

Two picorna-like viruses were discovered in this study within the barramundi farm ecosystem: barramundi associated picorna-like virus 1 and barramundi associated picorna-like virus 2.

Unclassified picorna-like viruses are frequently discovered through metatranscriptomic analysis (Culley et al., 2003; Geoghegan et al., 2018; Zhu et al., 2022). Most of these viruses often share nucleotide and protein pairwise identity with viruses from the *Picornaviridae* family. BPicV1 and BPicV2 are genetically unrelated from each other and are

highly divergent from known families in the *Picornavirales* order (Fig. 5C and. D), sharing only 29% and 57% identity with their closest relatives, respectively. This illuminates the immense diversity of picorna-like viruses in aquatic environments.

The novel picorna-like viruses can provide more details of the viromic environments of barramundi aquaculture. The closest relatives of BPicV1 and BPicV2 are likely invertebrate-infecting and part of a wider aquatic virome. These viruses, whilst unlikely to directly infect vertebrate hosts, indicate the presence of a wide range of other host-virus interactions in the environment in which barramundi are reared.

4.6. Novel viral discovery succeeds with dataset size and tailored sequencing methods

The success of viral discovery using metatranscriptomics has represented a fundamental turning point for virus discovery research (Cobbin et al., 2021) and can be influenced by the size of the dataset used for the study. Using methods similar to the present study, a large-scale metatranscriptomics study conducted by (Shi et al., 2018) screened over 120 species of ray-finned fish as well as other vertebrates, generating a total of ~806 billion reads. Analysis of the RNA datasets revealed at least 100 novel viruses including but not limited to viruses from the *Hepeviridae*, *Astroviridae*, *Caliciviridae*, *Paramyxoviridae*, *Hantaviridae*, and *Picornaviridae* families that infect ray-finned fish out of the 214 novel viruses detected overall. Another study screened four species of wild caught fish (~376 million reads analysed) which resulted in the discovery of 12 novel viruses from the viral families *Astroviridae*, *Flaviviridae*, *Hepadnaviridae*, and *Picornaviridae* (Geoghegan et al., 2018). These studies demonstrate the power of metatranscriptomics in viral discovery and underscore the correlation between dataset size and viral discovery: the larger the dataset, the greater the potential for discovering novel viruses.

Dataset construction is also a crucial factor in viral discovery. In our study, we analysed a total of 3.13 billion reads from NCBI (range: 12–84 million reads per dataset) and generated an additional ~20 million reads from fish sampled in this study. Within these datasets, we discovered three novel viruses in NCBI data, and two from RNA sequencing datasets generated. This shows that not all NCBI datasets are suitable or curated for viral discovery, resulting in less effective viral identification compared to datasets designed for this purpose. While screening publicly available RNA sequencing datasets for novel viruses can be a pivotal initial step, tailoring RNA sequencing methods for viral discovery can maximize results as demonstrated here.

5. Conclusion

As Australia and neighbouring Southeast Asian countries are among the world's largest producers of barramundi, this study therefore confirms the need for monitoring and preparation for large scale viral outbreaks of viruses that can disrupt the barramundi aquaculture industry. The barramundi virome is not well explored despite barramundi being prolifically reared in aquaculture. Through next generation sequencing, metatranscriptomics and bioinformatics, novel viruses that may cause mortality, impact growth, or influence the ecosystem dynamics have been discovered in this study. Molecular diagnostic methods can be developed to monitor and prevent viral outbreaks and develop outbreak management systems.

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CRediT authorship contribution statement

Lewis K. Mercer: Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Formal analysis, Conceptualization. **Emma F. Harding:** Writing – review & editing, Visualization, Methodology. **Tanu Sridhar:** Writing – review & editing, Investigation. **Peter A. White:** Writing – review & editing, Supervision, Resources, Project administration, 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

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