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RESEARCH REPORT

Identification of five picorna-like viruses associated with the endangered cave-dwelling bivalve *Congeria kusceri* (Bole, 1962)

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Abstract

Congeria kusceri is a bivalve mollusk species endemic to the Dinaric Karst, which displays unique adaptations that have allowed its survival in the subterranean environment with small morphological changes compared with its fossil relatives. Anthropic activities have recently impacted the surface flow of the Neretva river, impairing the seasonal water cycle that has characterized the habitat of this species for hundreds of thousands of years. The lack of an adequate water supply, together with pollution from agricultural and farm water runoff, are posing a serious threat to *C. kusceri*, as evidenced by the sharp population decline observed in several locations during the past few decades.

Due to the limited knowledge available about the basic biology of this filter-feeding species, the precise factors that may affect its health status and reproduction and therefore represent a hazard for its conservation are still unclear. Here, through a transcriptomic approach, we describe the nearly-complete genomes of five *C. kusceri*-associated RNA viruses belonging to the Picornaviridae family and phylogenetically related with picorna-like viruses previously described in other Mollusca. Although it is presently unknown whether these viruses may have a detrimental effect on bivalve health, we observed a significant increase of viral load during the summer season.

Key Words: virome; Karst; subterranean; Picornaviridae; transcriptome; RNA-sequencing

Introduction

Congeria kusceri (Bole, 1962) is a freshwater bivalve endemic to the Dinaric Karst, a vast region geologically characterized by the presence of carbonate rocks and located in the Balkan Peninsula, between the Adriatic Sea and the Pannonian Basin (Zupan Hajna, 2019). *C. kusceri* is currently listed as a vulnerable (VU) species in the European Red List of non-marine mollusks (Cuttelod *et al*., 2011) and as a critically endangered (CR) species in the Croatian Red List of subterranean fauna (Bilandžija and Jalžić, 2009). A member of a once widespread genus of freshwater bivalves that inhabited the Paratethys Sea, *C. kusceri* is a living fossil which maintained an apparent morphological stasis since the end of Miocene (approximately 5.3 Mya). The development of several unique adaptations, such as a K-selected reproductive strategy, larval brooding, and a long lifespan (Morton and Puljas, 2013), allowed the survival of this species in the subterranean environment to the present day (Stepien *et al*., 2001; Bilandžija *et al*., 2013). *C. kusceri*, whose current range of distribution includes just eight known sites in caves in to the Neretva river basin in Croatia and Bosnia and Herzegovina, was long thought to be the only member of a single pan-Dinaric genus. However, recent molecular studies have established that two other reproductively isolated congeneric species survive in the Dinaric karst, i.e. *Congeria mulaomerovici* and *Congeria jalzici* (Bilandžija *et al*., 2013).

The adaptation of *C. kusceri* to a highly stable cave environment poses a serious threat for its survival, since this species is not expected to be able to withstand significant long-term environmental alterations. In particular, the annual cycles which typically characterize the waters influx in Karst caves play an important role in regulating shell growth, reproduction and other important aspects of *Congeria*'s life cycle (Morton and Puljas, 2013; Puljas *et al*., 2014; Jovanović Glavaš *et al*., 2016). The building of dams, hydropower plants and channels for agricultural land use over the past few

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decades have undoubtedly modified the water regime of the Neretva river basin, with a severe impact on the amount of water that seasonally floods the cave systems where *C. kusceri* lives. These alterations are now threatening the survival of this species, which has already disappeared from some of the caves where large populations had been historically documented and is quickly declining in others (Bilandžija *et al*., 2021).

Moreover, agriculture and farm runoff can lead to organic and inorganic pollution in the subterranean environment (Sasakova *et al*., 2018). This, together with the progressive salinization of the lower parts of the Neretva basin due to seawater infiltration, poses a further threat for the unique habitat of *Congeria*, which is thought to have remained unchanged for hundreds of thousands of years (Vranješ *et al*., 2013). Such environmental changes could have a significant impact on the cave microbial communities, as well on the *Congeria*-associated microbiota, primarily consisting of chemolithoautotrophic bacteria (Rigonni *et al*., 2021).

It is presently unknown whether alterations in the microbiome and in the virome found in Karst subterranean waters can pose a threat for *C. kusceri* survival and no scientific literature is available on this subject. Nevertheless, alterations of the bacterial and viral communities associated with bivalve tissues have been clearly linked with mass mortality events and with a general decline of the health of marine bivalve populations (Barbosa Solomieu *et al*., 2015; Lasa *et al*., 2019). *Picornaviridae* are among the most frequently described viruses associated with bivalve mollusks. Even though their detection is often the result of bioaccumulation from ingested food particles or pathogens of bivalve associated parasites (Crespo-González *et al*., 2008), some of them can infect mollusks and replicate in their tissues (Renault, 2016). In particular, ultrastructural studies have identified on several occasions Picorna-like viral-like particles (VLPs) in bivalves affected with granulocytomas (Rasmussen, 1986; Jones *et al*., 1996) and numerous reports have suggested that Picorna-like viruses may be the main responsible or contributors to mass mortality events observed in different species of marine bivalves (Carballal *et al*., 2003; Renault and Novoa, 2004). It is however still unclear whether these RNA viruses should be just considered as bivalve pathogens or rather as a component of the virome associated with these filter-feeding organisms under normal physiological conditions, considering their frequent detection though metatranscriptomic approaches in absence of detectable disease (Moreira *et al*., 2012; Rosani and Gerdol, 2017; Rosani *et al*., 2019).

The information available about Picornaviridae infections in freshwater bivalves is extremely limited. High viral loads of different Picorna-like viruses have been previously observed in association with mass mortality events of *Actinonaias pectorosa* in the Clinch river (USA), even though they were unlikely to be the cause of the pathology (Richard *et al*., 2020). Although other picornaviruses have been also tentatively indicated as potential pathogens in North American freshwater bivalves (Goldberg *et al*., 2019), on some occasions they have been linked with concurrent infections by parasitic trematodes (Ip and Desser, 1984).

Considering the role potentially played by Picornaviridae as bivalve pathogens and the high susceptibility of cave-dwelling animals to environmental alterations, the detection of these viruses in *C. kusceri* may represent a potential threat for the conservation of this highly endangered species.

Materials and methods

Viral genome identification and characterization

The transcriptome of *Congeria kusceri* was generated through the *de novo* assembly of RNA-sequencing data obtained from multiple tissues (gills, mantle, gonads, digestive gland and adductor muscle). All tissues were carefully dissected with the aid of a scalpel, following the severing of the adductor muscle which allowed valve opening, from a total of ten individuals sampled between July and September 2018. The full methodology is described in detail elsewhere (Scapolatiello *et al*., 2021). This sequence dataset was screened for the presence of contigs encoding proteins containing a RNA-dependent RNA polymerase (RdRp) domain. In brief, the viral RdRp HMM profiles belonging to the clan RdRP (CL0027) were obtained from Pfam (Finn *et al*., 2009) and used as queries for HMMER v.3.3.2 (Finn *et al*., 2011) against the virtually-translated proteome of the species (obtained with TransDecoder, https://github.com/TransDecoder/). Positive matches were identified with an e-value threshold of $1x10^{-5}$. The reliability of the selected contigs was evaluated by the visual inspection of the uniformity of mapped Illumina reads along the full length of the contigs and double checked through the comparison with an alternative de novo assembly obtained with metaSPAdes v.3.10 (Nurk *et al*., 2017), which fully confirmed the sequences obtained. The presence of Open Reading Frames (ORFs) was evaluated with the CLC Genomics Workbench 20 (Qiagen, Hilden, Germany) and the encoded proteins were: (i) used as queries for a BLASTp search (Altschul *et al*., 1990) against the NCBI nr protein database, looking for significant homologies with sequences from previously described viruses; (ii) screened with InterProScan v.5 to detect conserved protein domains (Jones *et al*., 2014); (iii) analyzed with HHPRED, looking for structural similarities not detectable using the two methods described above (Söding *et al*., 2005). This approach allowed the identification of five viral genomes, whose completeness was assessed based on the pairwise comparison with the sequences of the closest available relatives detected in public databases.

Phylogenetic analyses

Since the five picorna-like viruses identified in *C. kusceri* displayed a different genome architecture and were all incomplete, the most conserved protein domain in Picornaviridae, i.e. the RdRp, was extracted to create a multiple sequence alignment (MSA) with MUSCLE v.3.8.31 (Edgar, 2004). The regions corresponding to the RdRp domain were similarly extracted from known viruses displaying a

significant homology with the five genomes under scrutiny, based on the detection of a significant BLASTp and pairwise identity at the amino acid level, higher than 40%. The previously published transcriptome of the closely related species *Dreissena rostriformis bugensis* (Péden *et al*., 2019), deposited in the NCBI Transcript Shotgun Assembly database under the accession ID GHIX00000000.1, was similarly screened to detect other uncharacterized viruses. Redundant sequences (i.e. those displaying a pairwise similarity higher than 90%) were removed with CD-HIT (Li and Godzik, 2006).

The MSA was trimmed with Gblocks v.0.91b in order to remove highly divergent and phylogenetically uninformative regions (Talavera and Castresana, 2007). Then, this sequence set was analyzed with modeltest-ng v.0.1.3 (Darriba *et al*., 2019) to identify the most appropriate model of molecular evolution according with the corrected Akaike Information Criterion (Cavanaugh, 1997), which was found to be a LG model (Le and Gascuel, 2008), with a proportion of invariable sites and a gamma-distributed rate of variation among sites. Phylogenetic inference was carried out with MrBayes v.3.2.7a (Huelsenbeck and Ronquist, 2001), using two independent Monte Carlo Markov Chain (MCMC) analyses in parallel running for 250,000 generations each, sampling trees each 1,000 generations. The first 25% the obtained trees were discarded during the burn-in process and the convergence of all the estimated parameters of the model was evaluated with Tracer v.1.7.1 (Rambaut *et al*., 2018).

Quantification of viral abundance

The trimmed Illumina reads obtained from the 10 samples available were mapped against the five reference viral genomes with the CLC Genomics Workbench v.20, using stringent parameters (*length fraction* = 0.75, *similarity fraction* = 0.98) to avoid non-specific matches. Based on the number of reads mapped on each viral genome, the average read coverage per base was calculated for each virus and each sample, obtaining a normalized measure of abundance that could allow a direct comparison among samples.

In detail, the five sampled tissues were gills, mantle, gonad, digestive gland and adductor muscle, which were taken from individuals collected in the Pit in Predolac in the Neretva river basin (Croatia) in July and September 2018, i.e. at the beginning and the end of summer, two critical time points for the life cycle of *C. kusceri*, as well as for the annual water cycle of the Karst subterranean environment. Each sample derived from a pool of five different individuals. The sequencing data analyzed in this study have been deposited in the Sequenced Read Archive (SRA) under the BioProject PRJNA704250 (SRR13767952-SRR13767961).

Results and discussion

Viral genome organization

We identified five RNA viruses characterized by the presence of a complete ORF encoding a protein with a RNA-directed RNA polymerase (RdRp) domain. Upon further scrutiny, and based on the homology detected with other known Picornaviridae, all five genomes were found to be partial, as they were missing a region of variable length at the 5'-end. Since the library preparation protocol used a poly-A selection step, this result was expected, due to the enrichment of Illumina reads towards the 3'-end of poly-adenylated mRNAs (Love *et al*., 2016), including those of viral origin (Rosani *et al*., 2019). The five viruses were named "*Congeria* picorna-like-virus", followed by a progressive number (from 1 to 5), thereafter abbreviated as Cplv1, Cplv2, Cplv3, Cplv4 and Cplv5.

Overall, while the genomes of all five viruses encoded, as expected, the presence of a RNA-directed RNA polymerase (RdRp), a picornain 3C peptidase and a number of structural proteins, their organization largely varied. In line with previous reports about other Picornaviridae isolated from invertebrates (Shi *et al*., 2016), structural and non-structural proteins were organized in polyproteins. These could be either encoded by a single or by two distinct ORFs and they could be interchangeably encoded either at the 5' or the 3' ends of the viral genome. However, the regions encoding the picornain and RdRp domains were always detected in pair (with the peptidase located in a 3' position compared with RdRp).

In detail, the sequence of *Congeria* picorna-like virus 1 (Cplv1) was 6,133 bp long and harbored two ORFs. Based on the comparison with its known close relatives, the assembled viral genome was expected to lack approximately 3 Kb at its 5' end, corresponding to the N-terminal region of the replicative polyprotein, which contained the picornain and RdRp domains. The second complete ORF encoded the structural polyprotein (Fig. 1).

Unlike Cplv1, the 5,614 bp genome of *Congeria* picorna-like virus 2 (Cplv2) included a single partial ORF, which was predicted to lack about 3 Kb of coding sequence. The architecture of the encoded polyprotein included a picornain/RdRp domain pair in the N-terminal part and the structural proteins of the capsid at the C-terminal end (Fig. 1).

The genome of *Congeria* picorna-like virus 3 (Cplv3) was 7,585 bp long and was predicted to lack approximately 1 Kb of sequence at its 5' end, likely making it the most complete out of the five viral genomes identified in the present work. The first incomplete ORF encoded the structural polyprotein, whereas the second complete ORF encoded a polyprotein which included the picornain/RdRp domain pair at the C-terminal end (Fig. 1). In addition, a portion of this polyprotein showed structural homology with the 2C non-structural protein of other Picornaviridae, which bears ATPase and RNA helicase activities (Cheng *et al*., 2013) and is thought to be involved in the inhibition of the TNF-α-mediated activation of NF-κB in the host (Li *et al*., 2016).

The assembled genome of *Congeria* picorna-like virus 4 (Cplv4) was the shortest out of the five genomes identified in this study, with a length of 3,817 bp. The only detectable ORF encoded the picornain/RdRp domain pair in its C-terminal part. Similar to Cplv3, Cplv4 had a region with structural homology with the 2C non-structural protein (Fig. 1). It is presently unknown whether the capsid proteins

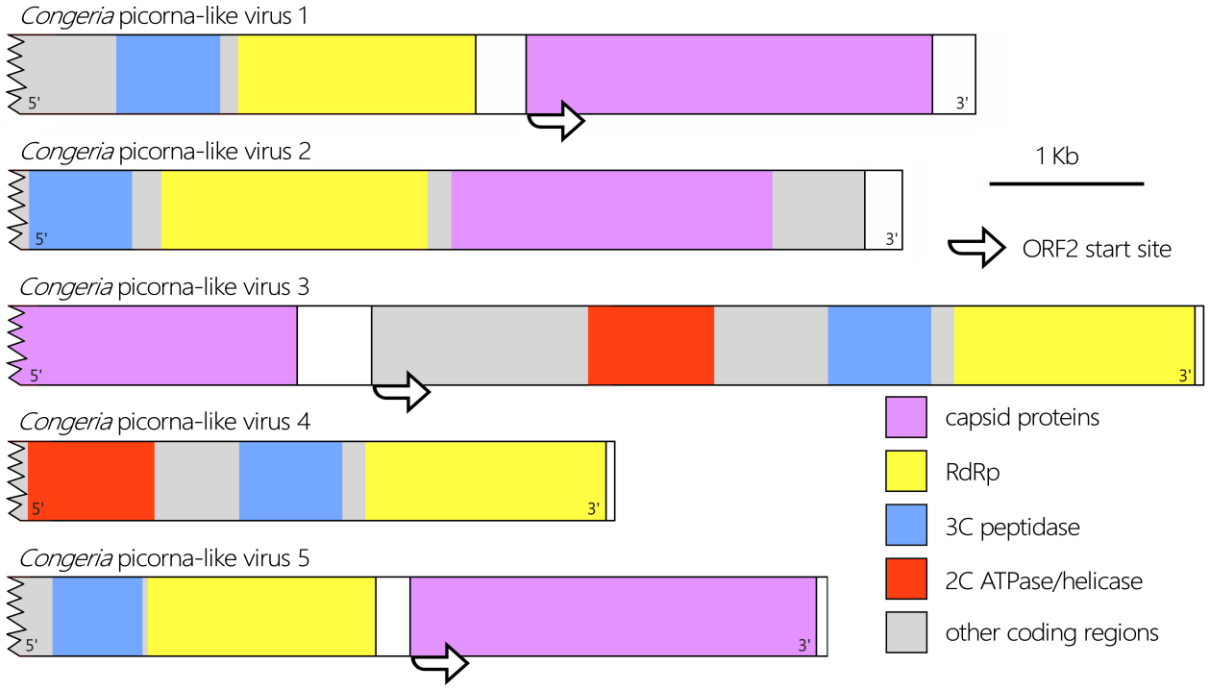

Fig. 1 Schematic representation of the five partial viral genomes identified in the present study. The serrated margins at the 5' end indicate missing sequence. The main detectable viral domains (i.e. the capsid proteins, the RNA-directed RNA polymerase, the 3C peptidase and the 2C ATPase/helicase) are indicated. White regions indicate the spacer regions located between ORF1 and ORF2 and the 3'UTR

in this virus are encoded by the N-terminal part of the same polyprotein or by a second ORF located in the 5' missing end of the viral genome.

The genome of *Congeria* picorna-like virus 5 (Cplv5) was 5,230 bp long and it was predicted to lack ~3 Kb of sequence at its 5' end. The first, incomplete ORF displayed the usual picornain/RdRp domain pair at the C-terminal end, whereas the second complete ORF encoded the structural polyprotein (Fig. 1).

Phylogenetic placement of the Congeria *picorna-like viruses*

Although all the five *Congeria* picorna-like viruses were novel, Cplv3 was placed in a long branch of the phylogenetic tree, distantly related with most known Picornaviridae, but displayed a very high sequence identity (75-90% at the amino acid level) with a small group of viruses previously described in freshwater environments and occasionally found associated to Mollusca (Fig. 2). In detail, Cplv3 was phylogenetically related with a few nearly-identical viruses, placed in a single cluster by CD-HIT and indicated by "freshwater picorna-like virus cluster 1" in Figure 2. This included the *Trichosanthes kirilowii* picorna-like virus, the *Forsythia suspensa* picorna-like virus (Yang *et al*., 2021), the snail Beihai picorna-like virus 105/niflavirus (Ng *et al*., 2012; Shi *et al*., 2016) and with the freshwater mussel Clinch dicistro-like virus 1 (Richard *et al*., 2020).

On the other hand, Cplv1 belonged to a large monophyletic and heterogeneous group of Picornaviridae (Fig. 2). The closest know relative to Cplv1 included the Beihai picorna-like virus 70, 71 and 72, as well as the Wenzhou picorna-like virus 26 and Atrpec virus 1, which displayed sequence homology at the amino acid in the range of ~40% for the structural polyprotein and ~50% for the replicative polyprotein. These viruses have been reported in different invertebrates, including Cnidaria and Crustacea, but also in Mollusca (i.e. the Beihai picorna-like virus 70 and Wenzhou picorna-like virus 26) (Shi *et al*., 2016). Intriguingly, albeit quite distantly related with Cplv1, we found that another virus detected in *D. rostriformis bugensis* (GenBank accession ID: GHIX01007600.1), one of the closest extant relatives of *Congeria* spp. (Stepien *et al*., 2001; Bilandžija *et al*., 2013;), was placed in the same large clade of Picornaviridae (Fig. 2), which may therefore show some degree of host specificity to Mollusca.

Cplv4 was placed in a different, well distinct group of Picornaviridae (Fig. 2) and only showed weak sequence homology with known picorna-like viruses isolated from several different invertebrates, which most notably included Mollusca, such as in the case of the Beihai mollusks virus 1 and 2 (Shi et al., 2016). Interestingly, albeit distantly related, two viruses detected in *D. rostriformis bugensis* (GenBank accession IDs: GHIX01044252.1 and GHIX01014380.1) also belonged to this clade, which

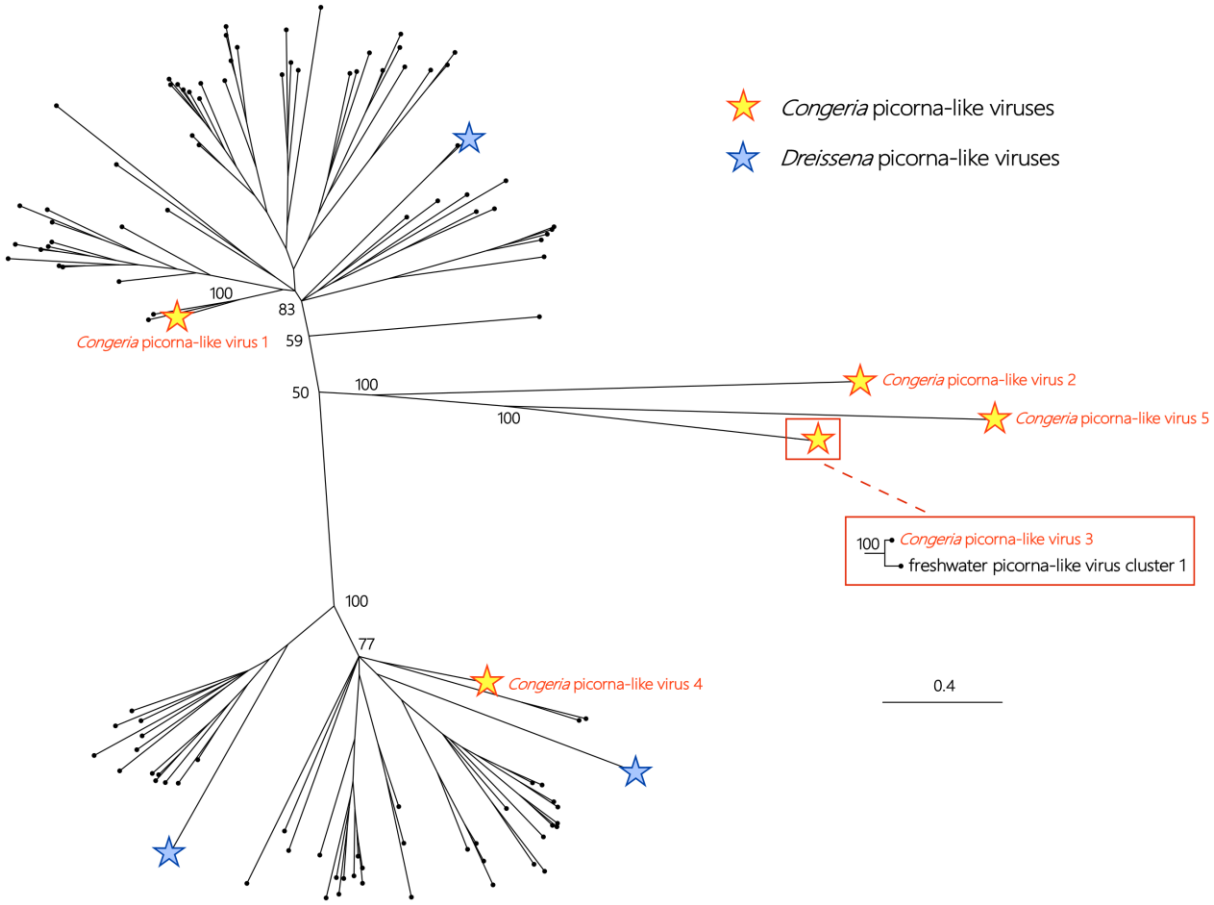

Fig. 2 Unrooted phylogenetic tree displaying the evolutionary relationship between the five *Congeria* picorna-like viruses (indicated by red dots), other picorna-like viruses identified in the *D. rostriformis bugensis* transcriptome (indicated by blue dots) and other known Picornaviridae. The tree is represented as a 50% majority consensus tree and posterior probability support values are only shown for major nodes. The scale bar indicates the number of substitutions per site

suggests that these viruses may be part of the regular virome of Dreissenidae and possibly of other bivalves (Fig. 2).

Cplv2 and Cplv5 did not show any significant match with known Picornaviridae, only showing sequence homologies with less than 30% identity at the amino acid level. They were therefore both placed as orphan taxa in long branches of the phylogenetic tree, which nevertheless created a highly supported monophyletic clade with the Cplv3 and the freshwater picorna-like virus cluster 1 (Fig. 2).

Tissue and temporal distribution of the five C. kusceri *picorna-like viruses*

Overall, the five viruses displayed a very similar pattern of relative abundance, both across tissues and between the two sampling time points, even though it was not possible, due to the pooling strategy used prior to RNA-sequencing, to evaluate whether such abundances were influenced by significant inter-individual differences. All viruses could be detected in *C. kusceri* in July 2018, albeit at relatively low levels. Higher viral loads were identified in the mantle tissue, followed by gonads and digestive gland, with little or no detectable signal in the gills and adductor muscle (Fig. 3), suggesting that the viruses were poorly replicating in bivalve tissues. In September 2018, higher viral loads were recorded in all tissues, with an increase by several orders of magnitude in the digestive gland (by ~50X for Cplv5 and by > 100X for the other 4 viruses). Cplv3 was the most abundant in this tissue, reaching an average read coverage close to 800X. The relative abundance of Cplv3 was ~3.7 higher than Cplv1, ~5.5X higher than Cplv2, ~13.1X higher than Cplv4 and ~17.4X higher than Cplv5. Four out of the five viral sequences were identified as differentially expressed transcripts in the statistical analysis for differential gene expression (a Kal's Z-test on proportions (Kal *et al*., 1999) carried out between the September 2018 and July 2018 digestive gland samples (Scapolatiello *et al*., 2021). In detail, differential expression was

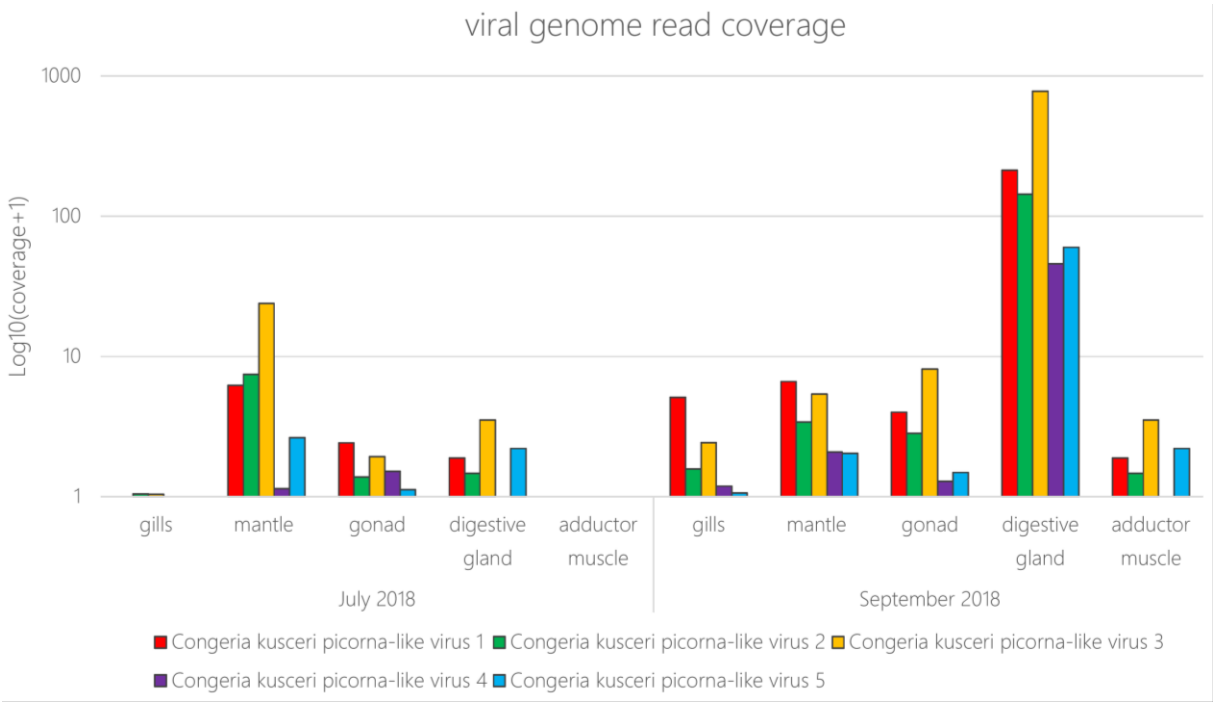

Fig. 3 Relative abundance of the 5 *Congeria kusceri* picorna-like viruses in five different tissues in June and September 2018, calculated based on the number of reads mapped on the reference genomes

supported by false Discovery Rate-corrected p-values equal to 0 (Cplv3), 1.82 x 10^{-12} (Cplv1), 4.26 x 10^{-9} (Cplv2) and 8.31 x 10^{-3} (Cplv5).

The significant viral loads recorded in the digestive gland may have different interpretations. In absence of ultrastructural observations, the precise location of the viral particles cannot be defined, leaving three alternative explanations open for the detection of viral RNA: (i) bioaccumulation of the virus from exogenous sources by filter-feeding; (ii) association of the virus with parasites or symbionts of *C. kusceri*; (iii) active viral replication in the digestive gland of *C. kusceri*.

The bioaccumulation of Picornaviridae by filter-feeding has been documented in bivalves on several occasions, in particular for what concerns wastewater-borne human enteric viruses, even though such viruses are likely unable to infect bivalves and replicate in their tissues (Hansman *et al*., 2008). It is therefore possible that the five picorna-like viruses described in this work are the result of influx of surface waters containing virus-associated food particles in the caves, with no direct impact on the health of *C. kusceri*. Nevertheless, this hypothesis is challenged by relatively poor influx of water from the external environment which occurs during the summer season at the Jama u Predolcu (Croatia). The possibility that the detection of these five viruses was linked with the presence of other parasitic infections, as previously reported in some marine species (Crespo-González *et al*., 2008), would certainly deserve further study. Although several freshwater bivalve species have been previously identified as viable trematode hosts (Brian and Aldridge, 2019; Taskinen, 1998; Marszewska and Cichy, 2015; Müller et al., 2015), no scientific literature is available on this subject for *C. kusceri*. As of note, no 18S/28S rRNA or COI markers suggestive of the presence of eukaryotic parasites was detected in the assembled transcriptome. Future studies should also aim at investigating possible host-virus links, such as the presence of Endogenous Viral Elements (EVEs) (Blair et al., 2020), or at mapping CRISPR spaceromes (Shmakov *et al*., 2020) in the genome of *C. kusceri*, once this resource will become available.

Concerning the possibility of active viral replication in the digestive gland, it needs to be remarked that histological lesions linked with picorna-like VLPs have been previously observed in this tissue in other bivalve species, supporting the idea that the digestive gland may serve as a preferential viral replication site also in *C. kusceri* (Hine and Wesney, 1997).

Conclusions

The five different picorna-like viruses identified in *C. kusceri* represent novel additions to the poorly known group of bivalve-associated RNA viruses and provides some insights in the virtually unexplored viral landscape associated with subterranean waters. Moreover, our bioinformatics-based detection approach confirms the usefulness of analyzing RNA-sequencing datasets for the identification of uncharacterized RNA viruses in bivalve hosts (Rosani and Gerdol, 2017; Rosani *et al*., 2019).

It is worth noting that Cplv2, Cplv3 and Cplv5 belonged to a well-supported monophyletic clade that only comprised a few known viruses previously isolated from freshwater sources, which may therefore represent an understudied group of picorna-like viruses associated with freshwater bivalves, and possibly with other freshwater invertebrates. All viruses displayed a marked increase in abundance throughout the summer season, which clearly indicates that this threatened bivalve species can be exposed to high viral loads in its natural environment. While it is presently unknown whether these viruses may have a detrimental effect on the health of this species, their phylogenetic relatedness with other Picornaviridae found in different molluscan species suggest they may be not the product of bioaccumulation by filter-feeding of food particles. Further ultrastructural investigations could clarify whether the presence of VLPs in the tissues of *Congeria* are linked with tissue lesions, helping to define whether alterations of the virome associated with this species should be considered as an additional risk factor to be monitored for the conservation of this endangered cave-dwelling bivalve. In light of the previous experience built in the conservation of freshwater bivalves living in surface waters (Brian *et al*., 2021), we believe that an improved characterization of the potentially pathogenic microorganisms associated with *Congeria* and its unique habitat, should be considered as a priority for a better planning of future translocation-based conservation efforts.

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