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Virome diversity and potential sharing of wild mammals in a biodiversity hotspot, Yunnan, China

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Abstract

Background Small mammals, including rodents, shrews and moonrats are widespread and serve as natural reservoirs for many viral pathogens. However, the composition and distribution of wild animal viromes remain poorly understood. At least 10,000 virus species have the ability to infect humans, but the vast majority are circulating silently in wild mammals. Understanding the virome profiles of these wild animals is crucial for outbreak preparedness, particularly in regions with high mammalian diversity.

Methods In this study, we enriched and extracted viral RNA from fecal samples of 459 wild mammals, representing 16 species, in the Xishuangbanna Dai Autonomous Prefecture of China, a recognized biodiversity hotspot in China. We then performed next-generation sequencing and comprehensive virome analyses across these different animal species.

Results We identified 5,346 nearly complete contigs annotated to 64 viral families, with 45 viral families identified in rodents and 46 viral families in shrews and moonrats, showing significant variation in viral diversity across different host species. Among these, 28 viral families were shared across species, including 11 identified viruses that were potential zoonotic pathogens. Additionally, numerous unidentified viral contigs containing the RdRp-gene showing close evolutionary relationships with viral families known to cause infections in animals. Importantly, several viruses detected in these animals, belonging to the family *Hepeviridae*, *Flaviviridae*, *Astroviridae*, *Picornaviridae*, and *Picobirnaviridae*, exhibited > 70% nucleotide sequence identity to viruses known to cause diseases in other wildlife species, domestic animals or even humans.

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Conclusions These findings significantly increase our knowledge of viral diversity and potential viral transmission within rodents and other sympatric small mammals in an emerging disease hotspot, shedding light on the need for continued surveillance of these small mammal populations.

Keywords Rodents, Shrews, Moonrats, Fecal samples, Virus diversity

Background

In recent years, the prevalence of emerging and re-emerging infectious diseases has risen significantly, leading to billions of illnesses and millions of deaths worldwide [1]. This escalating trend presents a serious threat to global public health and socio-economic development. Approximately 60.3% of human emerging infectious diseases are caused by zoonotic pathogens, originating from rodents [2], bats [3], birds [4], and other wildlife [5], with viral or prion pathogens accounting for 25.4% of these cases [6]. Viruses with RNA genomes are overrepresented as zoonoses [7], in particular, are of major concern due to their high mutation rates and potential for rapid evolution, which enhances their ability to cross species barriers [8]. Recent emergence events (such as coronavirus disease 2019, Lassa fever and hemorrhagic fever with renal syndrome) have highlighted the role of well-known viral families, which include both established viruses and newly identified strains with zoonotic potential. It is estimated that at least 10,000 virus species have the potential to infect humans, yet the majority of these viruses circulate silently within wild mammal populations, undetected and uncharacterized [9].

Climate change, urbanization, and agricultural expansion have profoundly transformed natural ecosystems, amplifying human exposure to wildlife, intensifying human-animal interactions and increasing the risk of viral spillover [10]. Although accurately predicting the next emerging virus is impossible, detecting potential zoonotic agents is crucial for preventing their spread and mitigating the risks associated with viral outbreaks. Alarmingly, viral metagenomic has explored less than 1% of the extent viral diversity to date [11]. This underscores the global vulnerability to emerging diseases and highlights the urgent need for comprehensive wildlife viral surveillance to better understand and mitigate the risks of viral transmission from animals to humans.

Wild mammals, particularly small species such as rodents, shrews, and moonrats, play a critical role in the ecology of zoonotic diseases [5]. These animals are widely distributed and recognized as natural reservoirs for a majority of the world's harmful pathogens, many of which have the potential to spill over into human populations [2]. Despite their ecological importance, our understanding of the viral diversity present in these hosts, as well as the distribution and composition of these viruses, remains limited. Collectively, these mammals exhibit diverse ecologies and lifestyles, occupying nearly every terrestrial habitat, including human-dominated environments that offer numerous opportunities for cross-species viral transmission through their urine, feces, or their arthropod ectoparasites such as ticks, mites, and fleas [12, 13]. In particular, certain viruses appear to be well adapted to their hosts, with little or less observable evidence of asymptomatic infection in wild reservoir host species [14, 15]. However, when these viruses spill over into domestic animals and humans, the consequences can sometimes be devastating.

Yunnan province, located on the southwestern border of China, shares cultural and geographical connections to Myanmar, Laos, and Vietnam in mainland Southeast Asia. It is recognized as a hotspot for both wild animal biodiversity and infectious diseases [16, 17]. This region has already witnessed the emergence of several zoonotic diseases, including hemorrhagic fever with renal syndrome (HERS) caused by the rodent-transmitted *Orthohantaviruses*, severe acute respiratory syndrome caused by the SARS-CoV coronavirus, and Nipah Virus associated with encephalitis [18, 19]. Xishuangbanna Dai Autonomous Prefecture within Yunnan is a popular tourist destination, renowned for its numerous natural reserves. However, extensive deforestation driven by increased tourism, human activities and climate change, is affecting wild animal populations and intensifying human-animal interactions, which heightens the risks of viral pathogens sharing and spillover [17]. Understanding the virome profiles of these animals in such a critical hotspot is essential for identifying potential risks and preventing future outbreaks.

Wildlife virome research aims to explore the diversity, ecology, and potential transmission pathways of viruses in wildlife [20]. Moreover, large-scale viromes can generally offer a more focused view of viruses in samples at specific times and locations [21]. Despite a growing body of research on viromes, our understanding of the spectrum of viruses across various wild animal species remains limited, particularly regarding viral sharing and interactions among different species. Large sample pooling is an efficient approach to examine virus diversity and evolution within different animal populations, as well as their viral sharing and evolutionary relationships [22]. In this study, we aim to characterize the total viromes from these wild mammals and to reveal viral sharing and interactions between viruses and their mammalian hosts. Additionally, we seek to assess the co-infection risks of

infectious viruses circulating among wild animals and evaluate their potential threats to public health.

Methods

Study site

Wildlife was captured in Xishuangbanna Dai Autonomous Prefecture in the southernmost part of Yunnan Province, China (Fig. 1). This region features tropical/subtropical monsoon climate, characterized by a dry season from November to April and a rainy season between May and October [23]. The average annual temperature is around 18 to 22 °C, with annual rainfall ranges between 1,193 and 2,491 mm [24]. Elevation ranges from 475 to 2,429 m [25]. The dominant natural forests include tropical rainforest, tropical monsoonal, tropical seasonal moist forest, and tropical montane evergreen broad-leaved [24]. Xishuangbanna is recognized as a global biodiversity hotspot, hosting approximately 88 rodents species [26], along with about 10 species of shrews and moonrats that live symbiotically with these rodents [27]. The region has well-developed tourism and agriculture sectors, leading to increased human-animal interactions. Additionally, Xishuangbanna is home to several ethnic minorities whose traditions and cultural practices, including the use of wild mammals in traditional medicine and trade in wildlife products, may facilitate the transmission of wildlife viruses and their spillover to humans [28, 29].

Sample collection

Animal samples was collected as part of the National Plague Surveillance Network. Wild mammals were captured using fold metal case traps provided by the Yunnan Institute of Endemic Diseases Control and Prevention (YIEDC). Traps, baited with peanuts, were set in forests, farmland, and residential areas during the afternoon and evening. A minimum of 300 traps were deployed each time, both inside and outside the designated areas. Captured wild animals were collected the following morning in separate biohazard specimen bags [30]. All animals were sent to local CDC laboratory for initial identification based on morphological criteria [3], and subsequently dissected in the Biosafety Level III laboratory for fecal sample collection. Fresh fecal samples were collected in separate sterilized 5 ml centrifuge tubes, transported on dry ice, and stored at -80 °C at YIEDC for further processing. To prevent cross-contamination, strict aseptic conditions were maintained, including the disinfection of equipment and the use of sterile instruments for dissection. Trained specialists at YIEDC conducted morphological identification of each captured animals and recorded detailed information, referencing the 'Field Guide to the Mammals of South-east Asia' and 'China's Mammal Diversity and Geographic Distribution' manual [31, 32].

Between 2021 and 2023, fecal samples were collected from 459 individuals, comprising 9 species of rodents from the order Rodentia and 7 species of shrews and moonrats under from the order Eulipotyphla. The rodent species included *Rattus tanezumi*, *R. brunneusculus*, *R. nitidus*, *R. andamanensis*, *Niviventer confucianus*, *N. fulvescens*, *Mus Pahari*, *M. caroli*, and *Eothenomys eleusis*. The shrews and moonrats comprised *Hylomys suillus*, *Anourosorex squamipes*, *Tupaia belangeri*, *Crocidura attenuata*, *C. fuliginosa*, *C. rapax*, and *Suncus murinus*. All samples were obtained from the border area

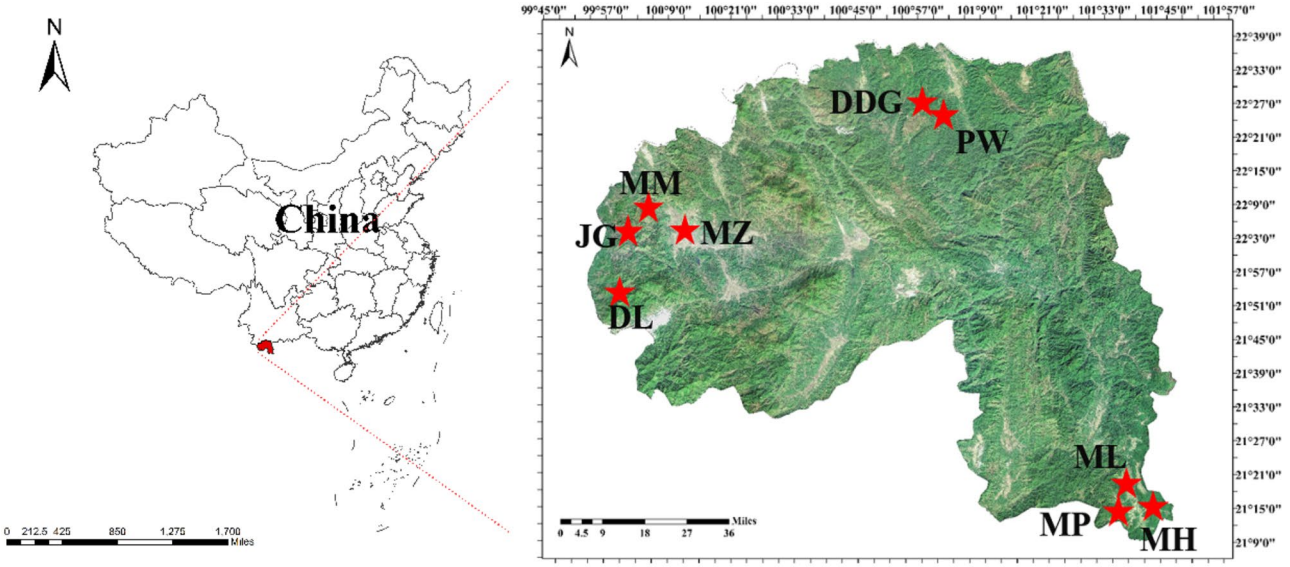

Fig. 1 Sampling sites located in Xishuangbanna Dai Autonomous Prefecture, Yunnan province, China. Each star represents the location of the sampling site

in Xishuangbanna Dai Autonomous Prefecture, Yunnan province (Fig. 1), which were categorized into two groups: rodents and small mammals, with the latter including shrews and moonrats.

Virus-like particle (VLP) enrichment and viral RNA extraction, library preparation and sequencing

To enrich VLP, each fecal sample was combined with grinding beads and phosphate-buffered saline (1×PBS) in a centrifuge tube and vortexed for 90 s at 4 °C. Aliquots (1 ml) from resulting mixtures of the same species were pooled into a new centrifuge tube. After centrifuging at 8,000 rpm for 10 min, the supernatant was filtered through 0.45 μm and 0.22 μm polyvinylidene difluoride filter (Millipore) to eliminate eukaryotic cell- and bacterium-sized particles. The filtered samples were then centrifuged at 60,000×g for 2 h at 4 °C, and the resulting pellets were re-suspended in 200 μl PBS to enrich the viral content. To remove the naked DNA and RNA, 200 μl of the resuspended pellet from each pooled sample was digested in a cocktail of DNase and RNase enzymes consisting of 14 μl of DNase I (NEB), and 7 μl of RNase One (Promega) at 37 °C for 2 h [33].

Viral RNA was extracted from each individual's fecal sample, using TRIzol Reagent (Invitrogen) following manufacturer instructions and eluted into 20 μl DEPC water. In this process, viral RNA and residual DNA in highly stable viral particles were extracted. To increase the concentration of viral RNA, we pooled RNA samples from 6 to 10 individuals of the same species collected at the same sample site, creating 44 pools for analysis. RNA concentration was then quantified using the NanoDrop 2000 (Thermo Fisher) and Qubit 4.0 Fluorometer (Thermo Fisher Scientific) before library construction and sequencing. When RNA concentration measured by NanoDrop 2000 was less than 100ng/μl and the concentration measured by Qubit was less than 1000ng/ml, the viral RNA was extracted again.

Library construction was performed using the NEB Next Ultra RNA library preparation kit for Illumina (NEB), and each pool was labeled with a specific index using Next Multiplex Oligos for Illumina (NEB) and then processed for Next Generation Sequencing (NGS) and paired-end (150 bp reads) sequencing for each RNA library performed on the Illumina HiSeq 2500 sequencer (Illumina). For each library, the sequencing reads underwent adaptor and quality trimming using the Trimmomatic (v0.39) [34] with application parameters are LEADING 3, TRAILING 3, SLIDINGWINDOW 4:15, and MINLEN 36. Subsequently, the trimmed reads were assembled *de novo* using MEGAHIT (v1.1.3) deploying default settings [35]. No filtering of host or bacterial reads was conducted prior to assembly.

Virus identification, quantification and vOTU

For viral contig identification, all assembled contigs were compared to the non-redundant protein (NR) database using DIAMOND BLASTX (v2.0.15.153) with an e-value threshold of 1×10^{-5} [36]. Sequences annotated as belonging to the kingdom 'Viruses' based on the top blast hit were initially marked as potential viral sequences. Additionally, the contigs were compared to the non-redundant nucleotide (NT) database using BLASTN (v2.12.0) to identify further potential viral sequences [37]. For viral sequences longer than 1,500 bp, CheckV was employed to estimate completeness by comparing the sequences with large databases of complete viral genomes, including publicly available metagenomes, meta-transcriptomes, and meta-viromes [38]. Furthermore, we used VirBot, a simple yet effective tool for RNA virus detection, which applies a custom protein domain family and tailored scoring thresholds to identify RNA viruses [39]. VirBot shows its high specificity in metagenomic datasets and superior sensitivity in detecting novel RNA viruses [39].

To quantify viral abundance, quality-trimmed sequencing reads were aligned to the identified potential viral contigs using Bowtie 2 (v2.2.3) with end-to-end alignment [40]. The resulting alignments were sorted and indexed using SAMtools (v1.13) to extract read counts for each contig [41]. Viral abundance was calculated and normalized as viral Reads Per Kilobase per Million mapped reads (RPKM), with the abundance of each viral family was determined by summing the abundance values of contigs annotated within that family.

Following the methods outlined by Shah et al., we constructed virus Operational Taxonomic Units (vOTUs) after assembly and species-level de-duplication [42]. To cluster similar viruses into species-level groups, assembled contigs from all samples were pooled into a single file. An all-against-all alignment was performed using BLAST, and the resulting alignments were used to create clusters with >95% sequence similarity. The longest non-chimeric assembly sequence within each cluster was selected as the vOTU representative. The abundance of each vOTU was determined based on the contig abundance.

Construction of RNA-dependent RNA polymerases (RdRp) phylogenetic tree and taxonomic placement of new viral candidates

As the most conserved proteins in RNA viruses [43], viral RNA-dependent RNA polymerases (RdRp) are commonly used to infer RNA virus phylogenies [44]. By combining RdRp-specific Hidden Markov Models (HMMs) and structural comparisons, we found that RdRp-scan can efficiently detect RdRp sequences with identity levels as low as 10% to known viruses and unidentifiable

sequences using standard sequence-to-sequence comparisons [44]. Briefly, we first translated viral open reading frames (ORFs) from the contigs using getorf, applying the appropriate translation Tables [45]. The resulting ORFs were subjected to redundancy removal using CD-HIT with a similarity threshold of 0.98 [46]. Subsequently, we scanned the RdRp HMM profile database from the RdRp-scan workflow using HMMscan [44]. Additionally, we directly aligned the viral ORFs to the RdRp sequence database from RdRp-scan using DIAMOND with the parameters (min-orf 600, E value $< 10^{-5}$) to obtain ORFs containing RdRp sequences. The combined results from these two methods yielded RdRp-like candidates.

For the RdRp-like candidate sequences, we conducted a multiple sequence alignment using Clustal Omega, employing reference RdRp sequences from the RdRp-scan database. The alignment results were then used to construct a phylogenetic tree using FastTree (wag -gamma) [47], and the tree was visualized with iTOL (v6) [48]. Based on the homology of RdRp sequences, we speculated the taxonomic of unclassified sequences as new viral candidates.

Viral sharing and zoonotic potential prediction

To elucidate potential viral cross-species transmission events, we visualized the virus sharing patterns among different species using a bipartite network. In this network, nodes represent either host or vOTU, with edges connecting host nodes to virus nodes indicating the presence of the virus in that species. To ensure the accuracy of the species-virus connections, we required that vOTUs have more than 1000 reads to establish a network connection. The host-vOTU network consisted of 15 host species, 13 virus families, and 128 interactions between these sets of nodes. Viral sharing events among species were identified in the network using the Igraph package in R (v 4.1.1) and visualized in Cytoscape (v 3.9.1) [49].

Zoonotic viruses are defined as those that can be transmitted from animals to humans., causing co-infections in both animals and humans. High-risk viruses were classified as those that have caused at least one pandemic disease. Currently, determining which animal viruses may have the potential to infect humans is challenging. To address this, we employed a machine learning model designed to predict the probability of animal-infecting viruses causing human infection under biologically relevant exposures, specifically their zoonotic potential, based on features derived from viral and human genomic sequences [50]. Additionally, phylogenetic relationships of viruses can predict the potential for cross-species infectivity, as closely related viruses are generally thought to share a common phenotype and host range [50], and a potential risk virus was defined as a virus exhibiting greater than 70% amino acid similarity to known infectious viruses.

For phylogenetic analysis, pathogenicity reference sequences were obtained from GenBank in National Center for Biotechnology Information (NCBI). Nucleotide sequences and deduced amino acid sequences were aligned using the MEGA software (version 11.0). The best substitution model was then evaluated with the Model Selection package. Subsequently, a Maximum Likelihood Method was employed with the appropriate model for conducting phylogenetic analyses, and phylogenetic trees were generated with 1,000 bootstrap replicates.

Results

Distribution and abundance of virus in wild animals

Total RNA was extracted using targeted VLP enrichment and then 44 libraries were constructed based on species of rodents, shrew and moonrats. The raw data totaled 353.72 Gb, yielding 87.13 Gb of high-quality data for analysis. After removing adapter and ribosome sequences, 290,421,597 high-quality reads were obtained, resulting in 24,153 unique contigs for viral identification. Our study identified 5,338 contigs longer than 1,000 bp, representing 64 RNA and DNA viral families (Table S1). *R. tanezumi* had the highest number of high-quality contigs ($n = 1{,}718$ contigs), while *C. attenuate* had the fewest contigs ($n = 40$). In addition, we searched for assembled contigs that encode hallmark genes of viruses (RdRp-gene) for RNA viruses, from which 1,836 viral contigs were identified after filtering for contig length and hallmark gene completeness for the prediction of new viral candidates. (Table S2). The machine learning prediction results on the zoonotic potential of contigs revealed that among 3,944 contigs with zoonotic potential, 28 sequences exhibited very high zoonotic potential, belonging to 9 virus families: *Endornaviridae*, *Flaviviridae*, *Herpesviridae*, *Nodaviridae*, *Partitiviridae*, *Picobirnaviridae*, *Picornaviridae*, *Tombusviridae*, and *Totiviridae* (Table S3).

A total of 45 viral families were identified in rodent hosts, while 46 viral families were found in shrew and moonrat hosts. An overview of the viral composition of different animal species is presented in Fig. 2. The number of virus family per host was uneven across the examined rodent and small mammal hosts. *Picobirnaviridae* was the most dominant viral family, detected in 56.58% of rodent hosts and 18.79% of other small mammal hosts (Fig. 2A and B). Among rodents, *Marmot picobirnavirus* and *Picobirnavirus* represented the largest proportions of viruses, accounting for 21.46% and 11.35%, respectively. Similarly, in small mammals, *Picobirnavirus* and *Marmot picobirnavirus* were also the most prevalent within *Picornaviridae*, representing 5.51% and 3.67%, respectively. Following *Picobirnaviridae*, the viral families *Uroviricota*

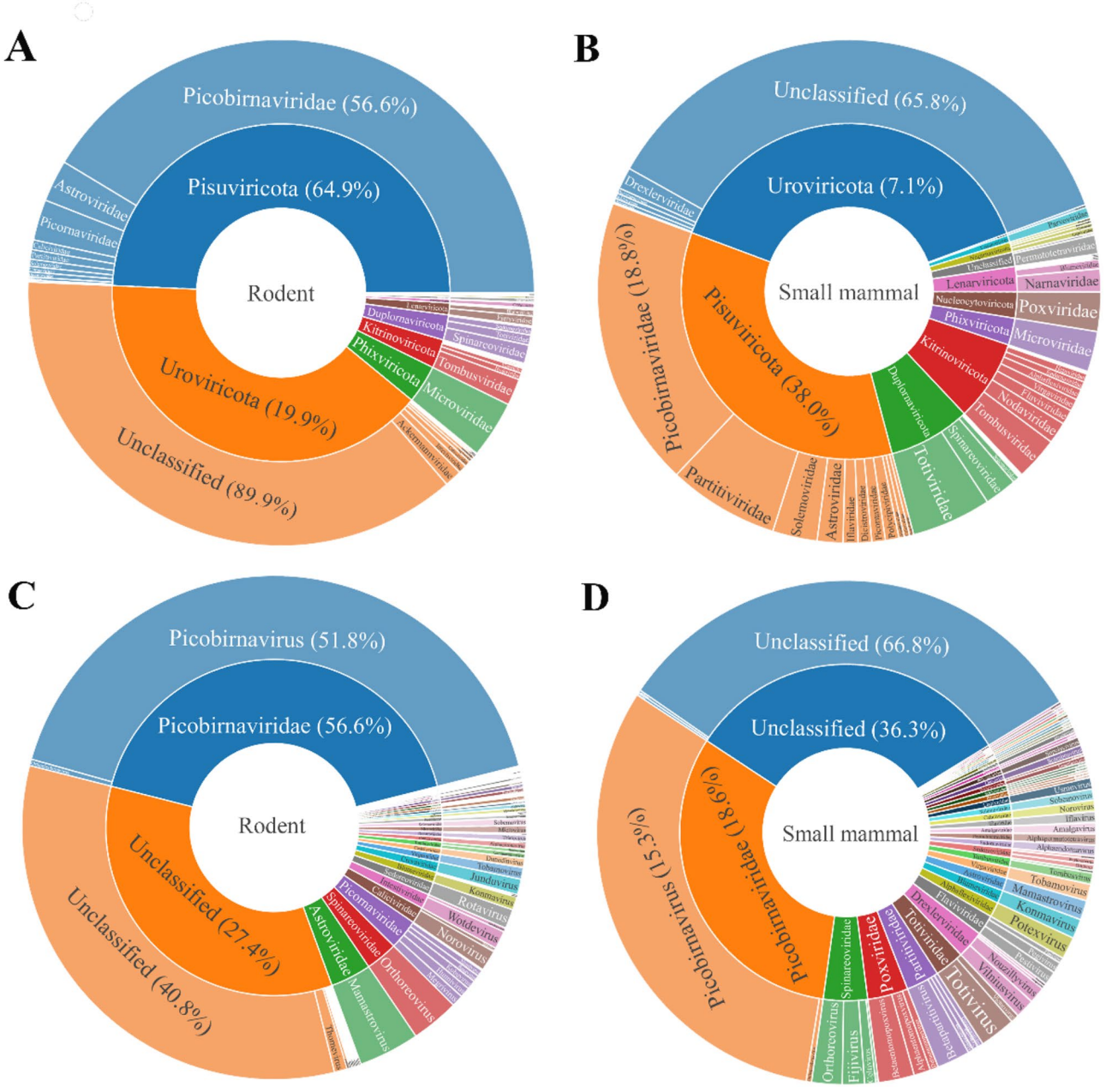

Fig. 2 Overview of virus classifications identified in rodents and small mammals. Phylum to family (**A** and **B**) and family to genus (**C** and **D**). Different families are labeled in distinct colors

and *Kitrinoviricota* were also prevalent in rodent and small mammal hosts. Moreover, a significant proportion of viral contigs remained unclassified, accounting for 27.42% in rodents and 36.27% in shrews and moonrats (Fig. 2C and D).

Our analysis revealed significant differences among hosts in terms of viral composition and abundance (Fig. 3). In terms of virus composition, *R. tanezumi*, *A. squamipes*, and *C. fuliginosa* exhibited the most diverse viral profiles, while *R. nitidus*, *C. attenuata*, and *T. belangeri* had the least abundant viral compositions. Viral families such as *Circoviridae*, *Tombusviridae*, *Picobirnaviridae*, *Picornaviridae*, and *Caliciviridae* were widely distributed, with varying abundances across species. Among the species, the highest virus abundance was found in *R. tanezumi* (32.18%), followed by *E. eleusis* (10.58%) and *R. brunneusculus* (14.31%). Additionally, 13 of the 38 viral families with zoonotic potential were detected in our 44 sample pools according to the Global Virome Project (Fig. 3) [51]. Among these, *Picobirnaviridae*, *Picornaviridae*, *Caliciviridae*, and *Astroviridae* were widely distributed across species, whereas *Flaviviridae*,

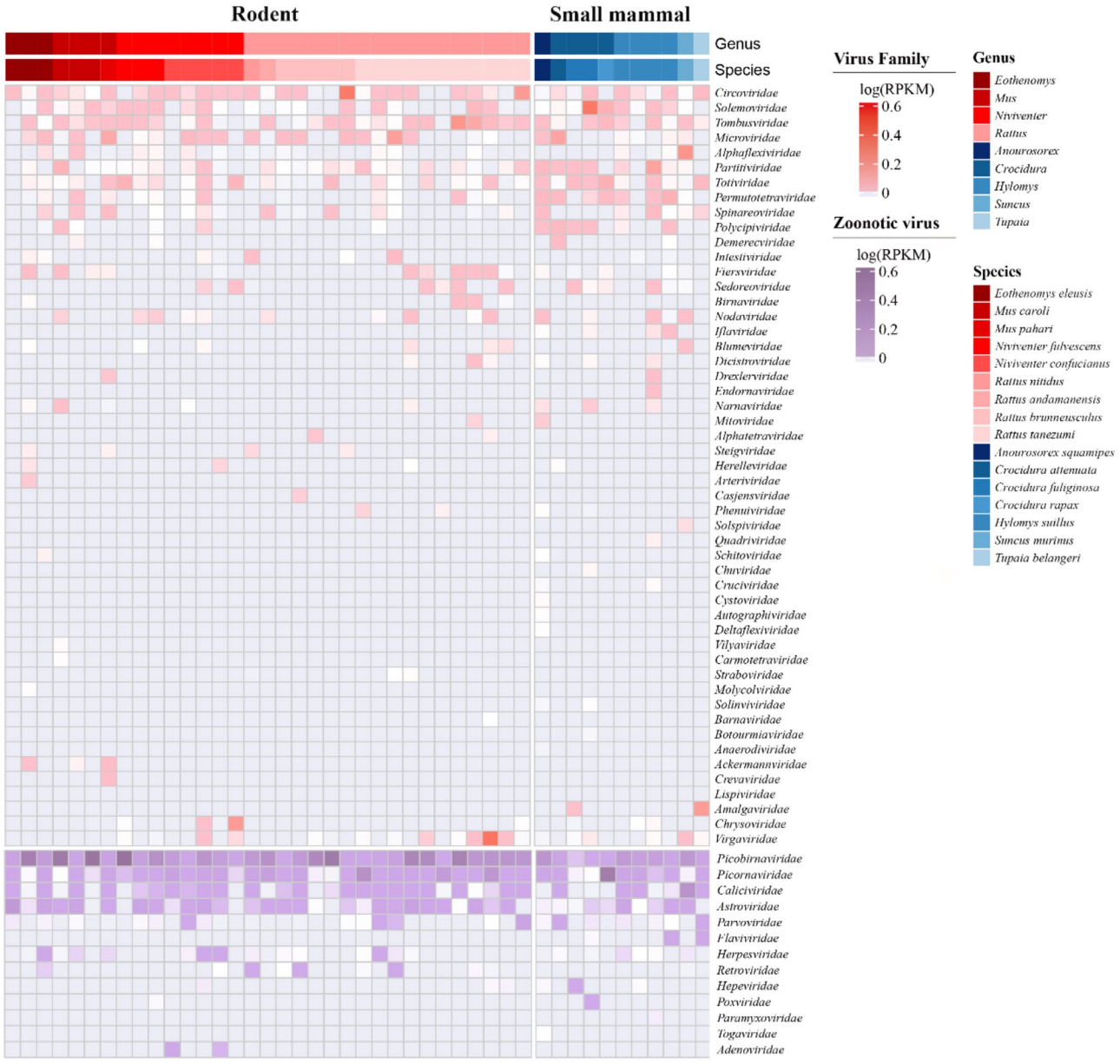

Fig. 3 Heatmap of normalized vOTU abundance in wild mammals. Genus and species are listed in the right column, while rodent and small mammal poolings are presented in the top row. Colored boxes, ranging from grey to red, represent viral abundance, with grey to purple indicating the abundance of zoonotic viral families. The relative abundance (RPKM) of each viral family across all samples is shown, with viral family names listed in the right column

Paramyxoviridae, and *Poxviridae* were found exclusively found in shrew and moonrat hosts.

Viruses sharing network and potential risk

According to the vOTU abundance, the comparison of viral communities using Non-metric Multidimensional Scaling (NMDS) analysis with Bray-Curtis indicated that there was no difference between rodents and small mammalian hosts ($R^2 = 0.030$, $P = 0.11$) (Fig. 4A, Table S4), suggesting that the viral communities in these two group populations were similar. Additionally, we observed 28 viral families sharing among rodents, shrews and moonrats at species level through a bipartite incidence matrix analysis (Table S5). These shared viruses comprised 13 viral clades (families or classes), including 11 identified zoonotic viruses and eight other non-zoonotic viruses (Fig. 4B). Among rodents, *R. tanezumi*, *R. brunneusculus* and *R. nitidus* had the most virus sharing events (with 26, 21, and 16 virus sharing nodes, respectively), and shared the most viruses with zoonotic potential (Table S6). These differences in viral composition among species suggest that viral sharing is more common and frequent within species of the same genera than across genera.

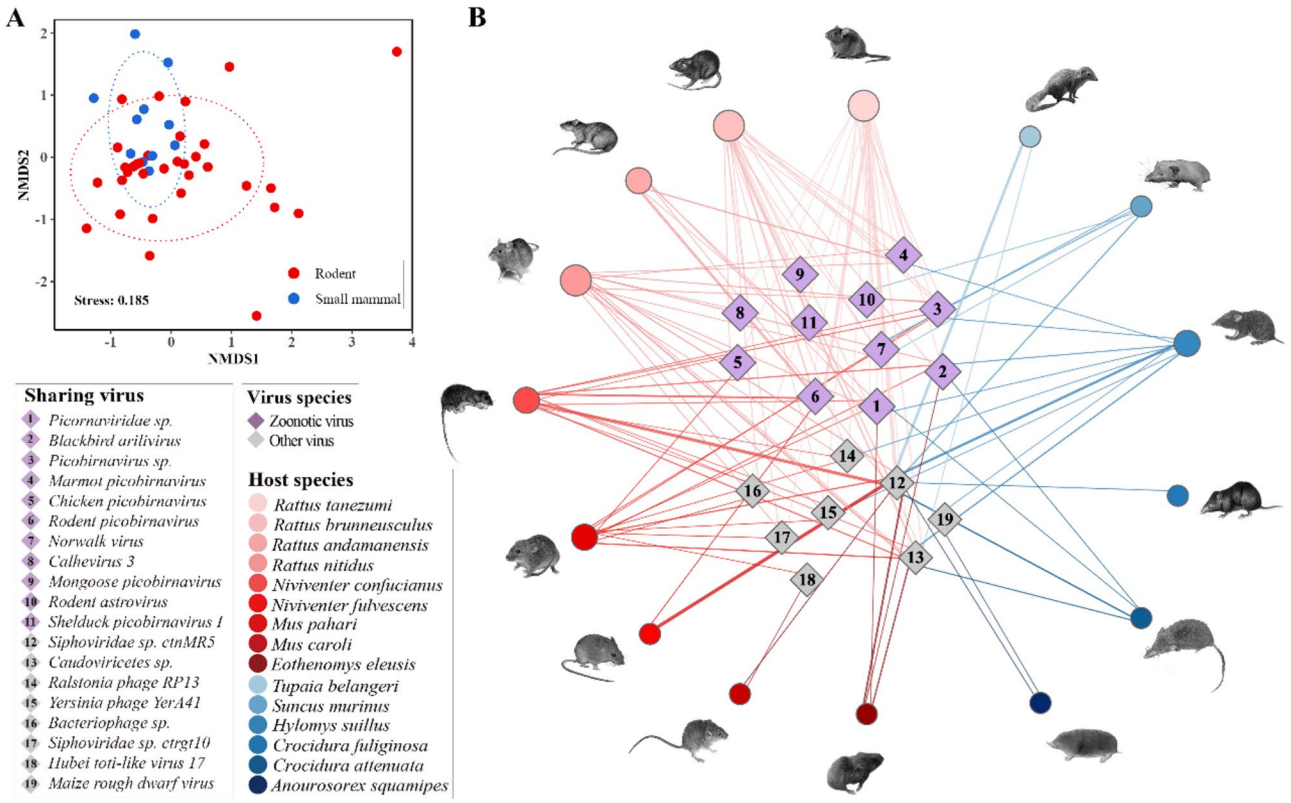

Fig. 4 Viral community analyses at the vOUT level for wild mammals (**A**). Virus-host sharing network for known viruses (**B**). The NMDS plot shows the similarity of viral communities based on the Bray-Curtis ecological distance matrix, with *P* value was calculated using PERMANOVA. Red circles represent rodent hosts and blue circles represent small mammal hosts. Purple diamonds indicate zoonotic shared viruses, while gray diamonds represent other shared viruses. The line thickness corresponds to the weight of the host-virus association, with thicker lines representing stronger associations

Our analysis revealed the diversity of viral families known to cause infections in both humans and animals (Fig. 3), and these zoonotic viruses are widely distributed across different species. We described viruses that pose an ongoing threat to global health and those that have emerged in the population and caused significant diseases as high-risk pathogenic viruses. We identified 1,998 open reading frames (ORF) in our samples (Table S2). Apart from the contigs assigned to known viral families, a significant number of viral contigs belonged to unclassified sequences. For these unidentified viral contigs containing the Rdrp-gene, phylogenetic analyses showing close evolutionary relationships with those identified viral families, indicating that they belong to potentially identified viral families. As well as viruses of concern, 321 ORFs were classed as newly discovered viral candidates and clustered with 1,515 known pathogenic ORFs. These ORFs representing 11 pathogenic virus families, with *Flaviviridae* and *Hepeviridae* posing high risk of pathogenicity (Fig. 5). The *Picornaviridae* family contained the highest number of unclassified viral candidates, followed by *Astroviridae*. Interestingly, most of these newly identified viral candidates may represent potentially new taxa or mutated strains of viruses found in these wild mammal-derived samples.

Phylogenetic analyses of the identified viruses predicted the potential risk of cross-species transmission. The results showed that many viral contigs identified in this study were closely related to (with >70% nucleotide sequence identity) viruses known to cause disease in other wildlife species, domestic animals or humans (Figs. 6 and 7). The GenBank Number and virus name for all reference sequences can be found in Figs. 6 and 7.

Hepeviridae

Members of the family *Hepeviridae* are positive-strand RNA viruses, with its family name derived from the name of the clinical disease 'hepatitis E' [52]. Importantly, hepatitis E-like viruses from mammals can cause infection of hepatitis E virus (HEV) by zoonotic transmission [53]. A total of eight high-quality contigs detected in the *C. fuliginosa*, *A. squamipes*, and *R. tanezumi* poolings fell as basal lineages to the genus *Orthohepeviruses*. Sequences similarity and phylogenetic analysis of RdRp-gene indicated two of these contigs were closely related to known Swiper virus (GenBank: MT833875.1) and Bat Hepatitis E

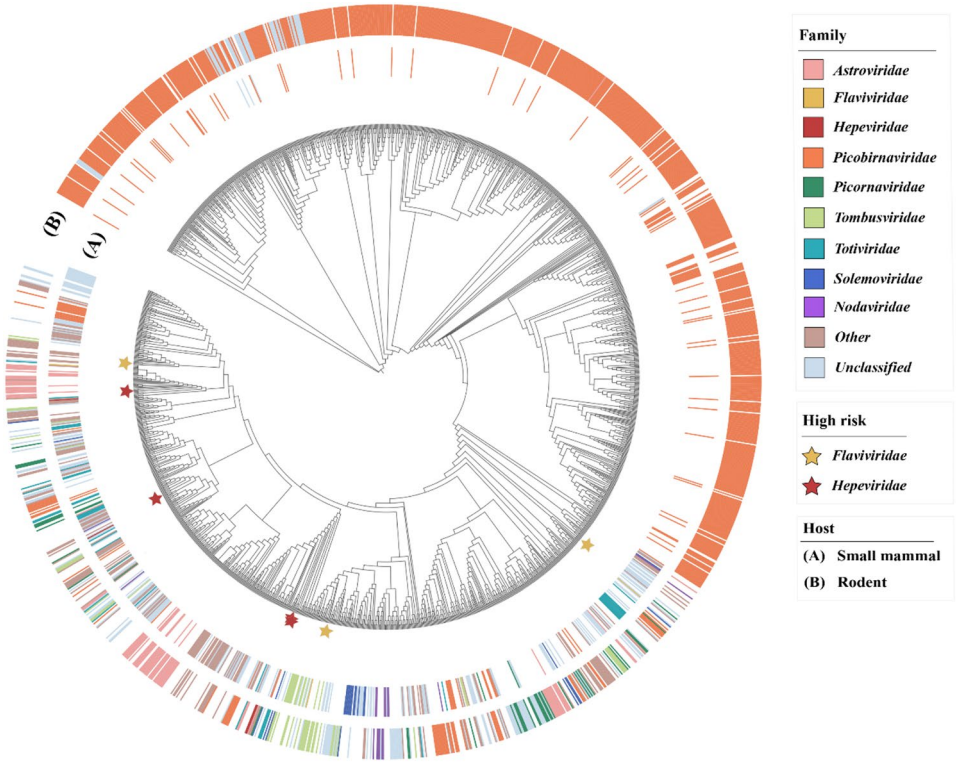

Fig. 5 Phylogenetic tree of RdRp protein sequence from wild mammals. The inner ring represents the phylogenetic tree of small mammals and the outer ring represents the phylogenetic tree of rodents. An asterisk denotes high-risk viruses

(accession: NC_076569.1) virus with nucleotide (nt) sequence identities between 98 and 100% (Fig. 6), which indicated these contigs detected in these samples could be pathogenic potential to other wildlife. Moreover, the contigs obtained from *R. tanezumi* (MLML F Rt.k141 3467) was closely related to the contigs from *A. squamipes* (MHC27-37.k141 13648) with 100% nt sequence identity, which means there would have possibility of cross-species transmission between our rodent samples and small mammal samples (Fig. 6).

Flaviviridae

Flaviviridae is a family composed of a large number of enveloped positive-strand RNA viruses, many of which pose serious risks to human health on a global scale [54]. The most recent classification of the *Flaviviridae* by the ICTV names 89 species divided between four genera within the family: *Flavivirus*, *Hepacivirus*, *Pegivirus* and *Pestivirus* [54]. Only *C. fuliginosa*, *H. suillus* and *T. belangeri* poolings showed positive, with 11 high-quality contigs were identified as *Flavivirus*, *Pegivirus* and *Pestivirus*. Viral contigs identified in *H. suillus* and *T. belangeri* showed high (99–100%) nt sequence identities with rodent pestivirus (accession: NC_077000.1), bat pestivirus (GenBank: MH282910.1) and shrew pegivirus (GenBank: JQ922489.1) (Fig. 6).

Astroviridae

The family *Astroviridae* comprise non-enveloped, positive sense, single-stranded RNA viruses, which have been classified into two genera, namely *Mamastroviruses* (MAstVs) and *Avastroviruses* (AAstVs) known to infect mammalian and avian species, respectively [55, 56]. In total, 102 high-quality contigs were identified to *Astroviridae* in all poolings, and 29 nearly complete *Astroviruses* genomes. The phylogenetic trees based on RdRp and capsid amino acid sequences were generated to study the evolutionary position of *Astroviruses*. We identified 13 contigs from rodent, shrew and moonrat hosts closely related to Wufeng rodent astrovirus 1 (GenBank: OQ802756.1), Rodent astrovirus (GenBank: MT549859.1), Rat astrovirus Rt/X104/Xiamen (GenBank: MF033386.1), Murine astrovirus isolate TZ (GenBank: ON571550.1), Nelson astrovirus-like 1 (GenBank: MZ443626.1) and Bastrovirus/VietNam/Rat (GenBank: KX907130.1) (with sequence identities between 77 and 100%) (Fig. 6).

Picornaviridae

Members of the *Picornaviridae* family are small, non-enveloped, positive single-stranded RNA viruses and include 40 genera, all of which contain viruses that infect vertebrates [57]. Diverse *Picornaviruses* cause

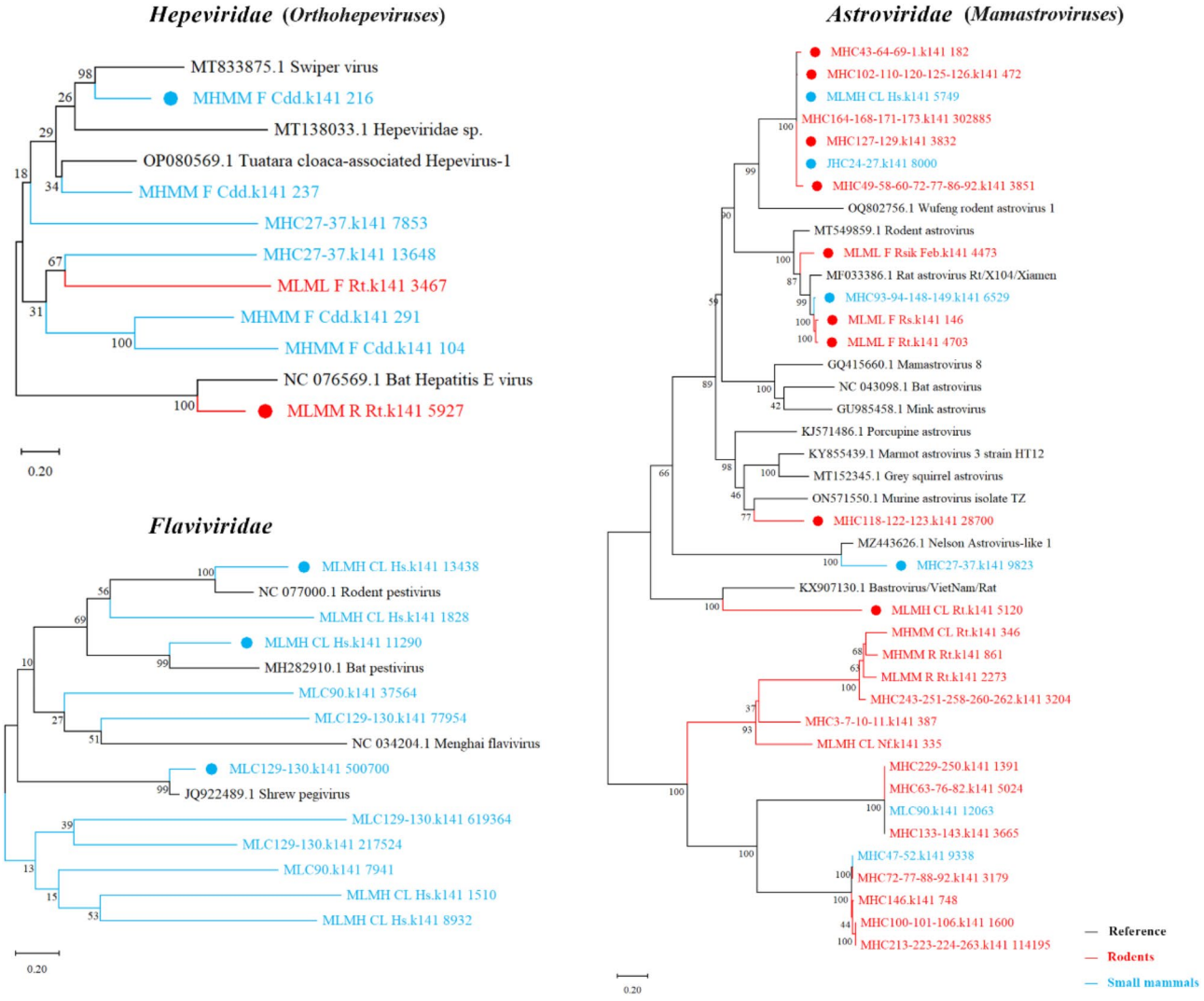

Fig. 6 Phylogenetic of *Hepeviridae*, *Flaviviridae* and *Astroviridae* constructed by maximum likelihood method. Trees were generated with 1000 bootstrap replicates using the best-fit models (LG + G + I for RdRp protein). Red lines represent sequencing contigs from rodents, blue lines represent sequencing contigs from small mammals, and black lines represent reference sequences obtained from NCBI. Circles have been added before sequences displaying high similarity (with sequence identity ≥ 90%) to pathogenic reference sequences

mucocutaneous, encephalic, cardiac, hepatic, neurological, and respiratory diseases in a wide variety of vertebrate hosts [58]. In our samples, 91 complete contigs were identified as *Picornaviridae* family in rodents and small mammals. Phylogenetic trees based on ORFs were generated to study the evolutionary position of *Picornaviridae*, and six contigs from rodents, shrews and moonrats were closely related to known zoonotic Picornaviruses, such as Aichivirus A6 (GenBank: MF947444.1), Kobuvirus sp. (GenBank: MF947447.1), Rhinolophus picornavirus (GenBank: MF352416.1), R. tanezumi hunnivirus (GenBank: MF352430.1), Jingmen rodent picorna-like virus 2 (GenBank: OQ716096.1)with nt sequence identities between 90 and 99% (Fig. 7). Moreover, the contigs detected from *R. tanezumi* (MLML F Rt.k141 4760) was closely related to the contigs from *H. suillus* (MHC47-52. k141 9135) with 90% nt sequence identity, which suggests that there would have possibility of cross-species transmission between rodents and sympatric other small mammals, as well as a possible common ancestry or similar environmental selective pressures (Fig. 7).

Picobirnaviridae

Picobirnaviridae were initially discovered in humans and rodents, which are small, non-enveloped icosahedral viruses with a segmented double-stranded RNA genome [59], and have been detected in the feces of a variety of animals, such as humans, rabbits, dogs, pigs, rodents, and birds. The zoonotic nature of *Picobirnaviridae* infection is evident through the identification of genetically similar *Picobirnaviridae* in both humans and animals [60]. In our samples, a total of 2,588 contigs were identified

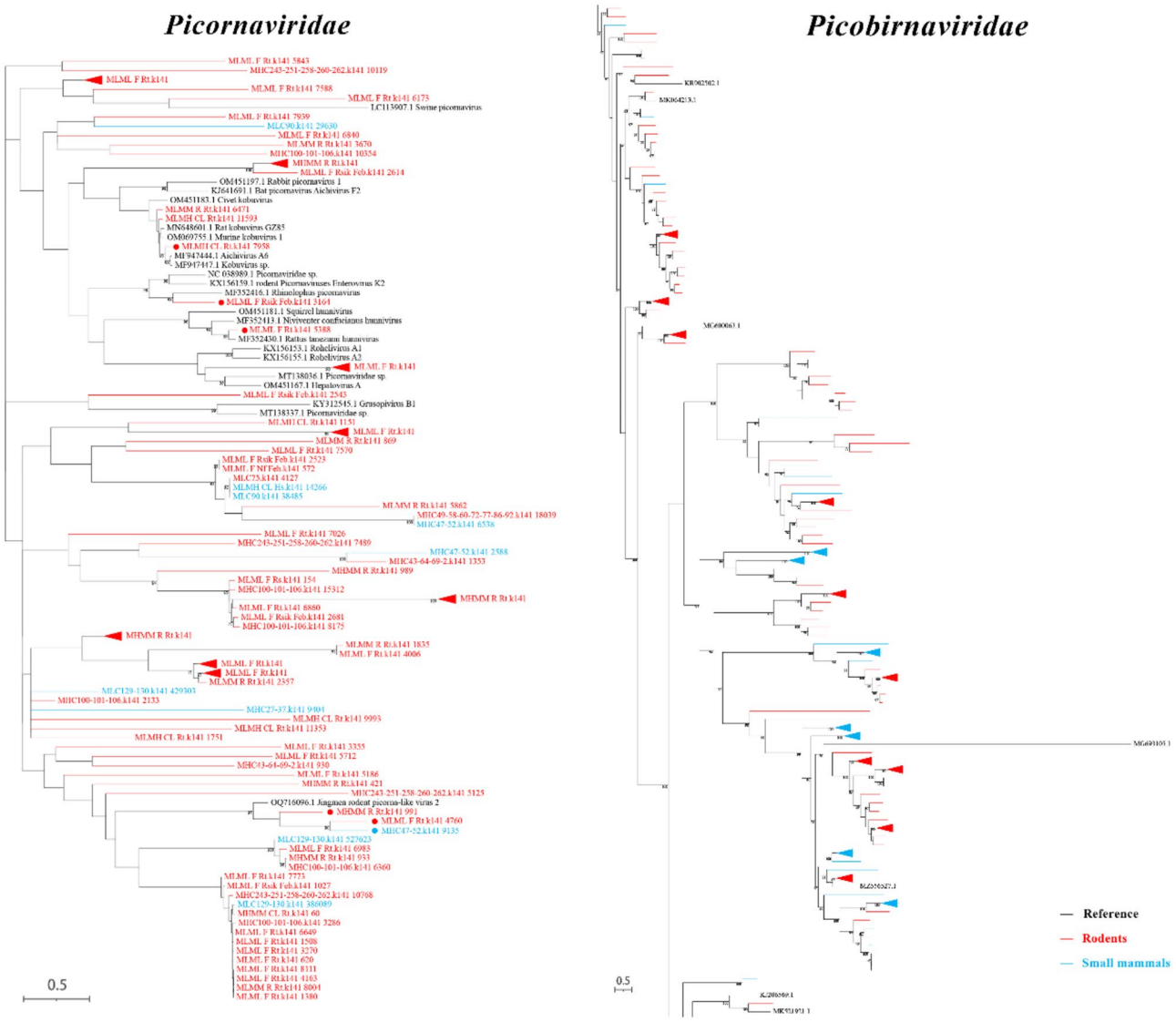

Fig. 7 Phylogenetic trees of *Picornaviridae* and *Picobirnaviridae*. The *Picobirnaviridae* tree shown here is a partial subtree representation due to its large size, for the complete tree, refer to Fig. S1

as *Picobirnaviridae*, of which 1038 contigs containing the RdRp-gene were used to construct the phylogenetic tree. Here, we identified various RdRp sequences create several new genogroups, and these sequences could not cluster with known *Picobirnaviridae*, suggesting that these viruses may be members of potential new taxa or genera in family *Picobirnaviridae*. Phylogenetic trees built from RdRp and capsid amino acid sequences is an important way to study the variation of these *Picobirnaviridae* (Fig. 7 and Fig. S1).

Discussion

Our study provides a comprehensive overview of the virome composition in wild small mammals from the Xishuangbanna Dai Autonomous Prefecture, revealing a remarkable diversity of DNA and RNA viruses across multiple host species, including rodents, shrews, and moonrats. The identification of 64 viral families, including 13 with zoonotic potential, underscores the rich viral reservoir harbored by these animals, particularly in a biodiversity hotspot. Importantly, the virome analysis revealed that the sharing of zoonotic viruses and the presence of newly unclassified viral candidates pose a potential risk for emerging infectious diseases.

Emerging zoonotic diseases have garnered considerable attention in recent years due to the advancements in molecular technologies, such as next-generation sequencing, metagenomics, and meta-transcriptomics. These cutting-edge tools have led to the development of the relatively new field of virome studies. Ongoing epidemics of emerging and re-emerging infectious diseases highlight the necessity for impartial research on

both known and unknown infectious viruses, especially related to mechanisms of cross-species transmission [61]. Understanding wildlife virome composition is crucial for identifying potential threats, as many emerging infectious diseases originate from wildlife reservoirs.

Historical knowledge of the ecological diversity of viruses harbored by wildlife has been limited [4]. Our analysis revealed that viral communities did not differ significantly between rodents and sympatric small mammals in this region. Notably, 28 viral families were found to be shared at the species level. Among these shared viral families, 11 viruses were identified with zoonotic potential, having an increased or high risk of cross-species transmission. These viruses likely possess the ability to jump species barriers, either between animals or between animals and humans, posing a risk of zoonotic spillover. The presence of these shared viruses suggests that interspecies viral transmission is not uncommon in these ecosystems, potentially facilitated by the overlapping habitats and ecological niches of these mammals [62]. The uniformity in viral communities among different species indicates that the barriers to interspecies viral transmission in this region may be lower than previously thought. This could mean that viruses capable of infecting one species may easily spill over into others, including humans, especially in areas where wildlife and human activities intersect [63]. The shared viral families with zoonotic potential identified in our study underscore the importance of monitoring these communities, as any changes in environmental or ecological factors could trigger an outbreak. As such, future investigation to predict and prevent zoonotic outbreaks in this region should consider the ecological dynamics that contribute to this viral homogeneity, as well as the potential for these viruses to spread across species boundaries.

The majority of recent pandemics have a viral etiology originating from mammals, and due to their inherent ability for interspecies transmission, viral zoonoses are likely to be responsible for the next pandemic [64]. Around 263 viruses from 38 viral families are known zoonotic viruses identified from wildlife [65], and our study identified 13 viral families with zoonotic potential in fecal samples obtained from rodents, shrew and moonrats. This high number of families with zoonotic potential underscores the importance of understanding and monitoring the viral diversity present in these wild mammals to assess the risk of transmission to humans. Specifically, viruses that primarily infect through the fecal-oral route, such as *Picobirnaviridae*, *Caliciviridae*, *Astroviridae*, *Parvoviridae* and *Reoviridae*, were widely distributed in our findings. Wild mammals can live in close proximity with humans and play an important role in the interaction between human and arthropod vectors and other wildlife. Therefore, these viruses have high potential for spillover in shared environments due to the characteristics of fecal-oral transmission [66]. Interestingly, potentially zoonotic viral families of *Retroviridae*, *Herpesviridae*, and *Adenoviridae* were exclusively found in rodent hosts, while *Flaviviridae*, *Poxviridae*, and *Paramyxoviridae* were limited to shrew and moonrat hosts. Although 29 vOTUs were annotated as *Entomopoxviruses*, which known to be insect-specific and incapable of infecting mammals, their presence warrants further investigation. This could help determine whether their detection is linked to the insectivorous feeding habits of the host animals or their resistance to enzymatic hydrolysis. The observed host-specific distribution of viral families underscores the potential role of unique life history traits and distinct co-evolutionary dynamics in shaping the virome composition of wild animal populations [22].

Given the exponential pace at which new viruses are being discovered, it is imperative to prioritize finite research and surveillance efforts on those viruses with the highest potential to endanger humanity [67]. However, determining zoonotic potential for newly discovered animal viruses, in the absence of documented human infection, is currently a major scientific challenge [12]. Based on RdRp-scan gene, we clustered the unclassified contigs with known pathogenic ORFs, representing 11 pathogenic viral families, with *Flaviviridae* and *Hepeviridae* posing high risk of pathogenicity. The identification of diverse, novel RdRp-like signals from metagenomic data, closely related to known pathogenic virus families, suggests the presence of new viral candidates with zoonotic potential and further investigation is required to confirm their virological characteristics and public health risks. Despite these limitations, these findings greatly increase our knowledge of the viral diversity in wildlife in a densely populated country in an emerging disease hotspot.

Further studies delving into the specific characteristics and transmission dynamics of high-risk viruses can provide valuable insights into preventing and controlling virus cross-transmission including members of the family *Hepeviridae*, *Flaviviridae*, *Astroviridae*, *Picornaviridae*, and *Picobinaviridaes* [53, 57, 68–70], and many of these viruses cause severe disease in humans (e.g., hepatitis E-like diseases, tick-borne encephalitis, and acute flaccid myelitis). In this study, we identified multiple viruses from wild mammals that shared high sequence identities and close genetic relationships with known mammal pathogens. Patterns observed across host-virus networks have been used to understand virus sharing among these vertebrate species, and there were indeed many pathogenic viruses shared among different species. Importantly, overlapping habitats, diets, and

environments provide the necessary conditions for the transmission and spillover of these shared viruses, but the origin of these viruses, whether host-derived, diet-derived, or from the fecal microbiome, requires careful consideration and can present challenges in distinguishing true infections from incidental viral presence. In addition, dozens of viral contigs from the *Astroviridae*, *Picornaviridae*, and *Picobirnaviridae* families were identified in rodents and small mammals. These contigs were distinct from all recognized viral references and formed separate clades. The discovery of these viral contigs provides new insight into the evolutionary origins of these viruses. The phylogenetic relationships we uncovered suggest that these rodent- and small-mammal-borne viruses have the potential for cross-species transmission, which may alongside intra- or inter-species contact, allowing for co-evolve with their hosts [5]. This lays a foundation for our subsequent research on the cross-species transmission mechanism of specific viruses between different species.

A fundamental requirement for virus spillover is the interaction between different species. Increased and frequent interactions between humans and other animal species raise the likelihood of introducing zoonotic or new pathogens into the human population [71]. In Yunnan Province, Xishuangbanna Prefecture is a border region with frequent population movements and wild-animal trade, increasing opportunities for human-wildlife contact and pathogens spillover risk. Climate change and habitat degradation have led to changes in wildlife populations, resulting in heightened human-animal interactions and an elevated risk of disease spillover [72]. As a likely hotspot of emerging zoonotic diseases in the future, Xishuangbanna Prefecture requires comprehensive surveillance, research, and public health measures to mitigate the potential zoonotic threats posed by rodents and other sympatric small mammals.

Given the role of these animals as reservoirs for potentially pathogenic viruses, future research should aim to expand the geographic and species scope of total virome studies, as well as investigating the environmental and ecological factors that drive viral diversity and transmission. While pooling samples for NGS sequencing is practical for large-scale surveys, it may obscure intra-species viral diversity, hinder the detection of low-abundance viruses, and limit the ability to examine host-virus interactions at the individual level. Including a known positive control of an infectious virus in NGS experiments could enhance the method's sensitivity and reliability, ensuring the robustness of the findings. Additionally, the identification of novel viral sequences highlights the need for continued efforts to characterize these viruses and evaluate their potential to cause disease in both humans and other animals.

Conclusions

In conclusion, this study enhanced our understanding of the virome in wild small mammals and highlights the critical role these animals play in the maintenance and sharing of viruses with zoonotic potential. These findings provide a foundation for future research aimed at mitigating the risk of emerging infectious diseases originating from wildlife. More surveillance of wildlife-borne viruses, particularly at the wildlife-domestic animal-human interface, is needed to prevent outbreaks of emerging and reemerging viral diseases.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-025-02702-0.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Author contributions

Experimental conceptualization and design: Lei Xu and Yongman Guo; Experimental execution and data analysis: Yongman Guo, Xueqi Jiang, Hanwei Liangand Junbin Ye; Laboratory/samples collection: Chao Su and Zihou Gao; Paper writing: Yongman Guo; Supervision: Lei Xu; Reviewing: Ruifu Yang; Editing and reviewing: Thomas R. Gillespie.

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Data availability

The raw sequence data reported in this paper have been deposited in the NCBI under the BioProject PRJNA1210821, which has been released on 2025-03-13. Everyone can access the data at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1210821.

Declarations

Ethical approval

Wild mammals were captured and treated in accordance with the guidelines outlined in the Regulations for the Administration of Laboratory Animals (Decree No. 2 of the State Science and Technology Commission of the People's Republic of China, 1988). This study, including the procedures and protocols of specimen collection and processing, was approved by the Ethics Committee of Yunnan Institute of Endemic Disease Control and Prevention (Approval number: 202313).

Competing interests

The authors declare no competing interests.

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