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Research Article

Decoding the virome reveals diverse novel viruses in tree shrews (*Tupaia belangeri*) in Yunnan Province

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ABSTRACT

Viruses circulating in small mammals possess the potential to infect humans. Tree shrews are a group of small mammals inhabiting widely in forests and plantations, but studies on viruses in tree shrews are quite limited. Herein, viral metagenomic sequencing was employed to detect the virome in the tissue and swab samples from seventy-six tree shrews that we collected in Yunnan Province. As the results, genomic fragments belonging to eighteen viral families were identified, thirteen of which contain mammalian viruses. Through polymerase chain reaction (PCR) and Sanger sequencing, twelve complete genomes were determined, including five parvoviruses, three torque teno viruses (TTVs), two adenoviruses, one pneumovirus, and one hepacivirus, together with three partial genomes, including two hepatitis E viruses and one paramyxovirus. Notably, the three TTVs, named TSTTV-HNU1, TSTTV-HNU2, and TSTTV-HNU3, may compose a new genus within the family *Anelloviridae*. Notably, TSParvoV-HNU5, one of the tree shrew parvoviruses detected, was likely to be a recombination of two murine viruses. Divergence time estimation further revealed the potential cross-species-transmission history of the tree shrew pneumovirus TSPneV-HNU1. Our study provides a comprehensive exploration of viral diversity in wild tree shrews, significantly enhancing our understanding of their roles as natural virus reservoirs.

INTRODUCTION

Over two thirds of the viruses responsible for emerging infectious diseases (EIDs) in humans originate from wildlife and transmit to humans through zoonotic spillover events (Jones et al., 2008; Watsa, 2020). Although predicting the pathogenic potential of animal viruses is challenging, numerous viruses have been detected in animals prior to the occurrence of the human diseases caused by them (Blasdell et al., 2016; Geoghegan and Holmes, 2017). Consequently, timely monitoring of zoonotic viruses and their hosts is essential to mitigate the threats of EIDs (Chen, Y.M. et al., 2023). Among wildlife, small mammals have drawn increasing attention due to their widespread distribution and proximity to human habitats. Over the past decades, a myriad of viruses have been detected in small mammals like rodents, bats, and shrews (Luis et al., 2013; Nie et al., 2019; Feng et al., 2024). Historically, rodents have represented the most consequential reservoirs of zoonotic pathogens affecting human populations. More recently, bats have been identified as natural reservoirs for a lot of emerging viruses with pandemic potential (Meerburg et al., 2009; Letko et al., 2020).

Tree shrews are small mammals resembling squirrels classified within genus *Tupaia*, family *Tupaiidae*, order *Scandentia* (Campbell, 1966). They are widely distributed across South Asia, Southeast Asia, and Southwest China (Fan et al., 2013). Chinese tree shrews (*Tupaia belangeri chinensis*) and Northern tree shrews (*Tupaia belangeri*) were both found in China, primarily inhabiting humid tropical and subtropical forests (Zhou et al., 2024). Given their closer genetic relationship to humans compared to rodents, researchers have been using tree shrews as models for studying human diseases, particularly viral infections (Zhao et al., 2014). Actually,

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tree shrews have been successfully infected with hepatitis virus B and C, influenza virus, cytomegalovirus, herpes simplex virus, dengue virus, and SARS-CoV-2 (Darai et al., 1983; Yang et al., 2013; Feng, Y. et al., 2017; Xu et al., 2020; Jiang et al., 2021). Therefore, it is of practical significance to investigate the virome background of tree shrews before further laboratory investigation.

In the present study, we investigated the virome of seventy-six tree shrews (*Tupaia belangeri*) living near human settlements in Yunnan Province using viral metagenomic sequencing, followed by genome amplification and Sanger sequencing. As a results, twelve complete viral genomes and three partial viral genomes were identified, encomprising fifteen viruses, including five parvoviruses, three torque teno viruses (TTVs), two adenoviruses, two hepatitis E viruses (HEVs), one paramyxovirus, one pneumovirus, and one hepacivirus. These viruses were subjected to further genetic analysis, revealing novel genetic features and evolutionary insights.

RESULTS

Virome profile in the tree shrews

Viral metagenomic analysis revealed a diverse range of viruses in tree shrews, with the highest abundance of reads belonging to families *Parvoviridae*, *Siphoviridae*, and *Anelloviridae*. Reads of *Parvoviridae* were predominantly found in the oral swab samples, spleen samples, liver samples, and heart-kidney samples, while reads of *Anelloviridae* were mainly detected in the lung samples (Supplementary Table S1).

High-throughput sequencing generated a total of 10,378,681 reads, comprising 5,442,092 reads from swab samples and 4,936,589 reads from tissue samples. Specifically, 1,520,091 reads were obtained from oral swab samples, of which 6,6354 reads (4.4%) were assigned as virus-related sequences. Among these virus-related sequence reads, 94.1% were from DNA viruses and 5.2% belonged to RNA viruses. Notably, more than 80% of the reads were aligned to animal viruses, and few reads (0.4%) hit plant or insect viruses (Supplementary Table S1). Likewise, 3,922,001 reads were generated from anal swab samples, of which 281,135 reads (7.2%) were allocated as virus-related sequence reads among which the most (99.0%) were aligned to DNA viruses. Remarkably, the majority of the reads (98.7%) were related to bacteriophage, while few reads (<0.1%) were relevant to plant and insect virus (Supplementary Table S1).

As for tissue samples, 1,115,181 reads were generated from spleen samples, of which 158,199 (14.2%) were assigned as virus-related sequences. Among them, 98.6% were grouped into DNA viruses and more than 80% of the reads were allocated to the animal virus (Supplementary Table S1). 1,871,358 reads were generated from lung samples, of which 48,533 reads (2.6%) were related to viral sequences. Among them, 96.4% were grouped into DNA viruses. Additionally, the majority of the reads (97.4%) belonged to animal virus (Supplementary Table S1). 854,373 reads were generated from liver samples, of which 605,440 reads were allocated as virus-related sequence reads. Among them, 99.9% were grouped into DNA viruses and 99.99% belonged to animal virus (Supplementary Table S1). 1,095,677 reads were generated from heart-kidney samples, of which 37,2991 reads (34.0%) were related to viral sequences. Among them, 99.9% were grouped into DNA viruses and 99.9% belonged to animal virus (Supplementary Table S1).

These sequence reads were assembled into contigs by *de novo* assembly, but most contigs did not cover the complete genome. Therefore, we further validated the sequence by conventional PCR to determine the whole or near-whole genome sequences of the viruses, and further characterized them.

Identification of viruses in samples

To confirm the prevalence of tree shrew parvovirus (TSParvoV), tree shrew adenovirus (TSAdV), tree shrew torque teno virus (TSTTV), tree shrew hepacivirus (TSHV), tree shrew hepatitis E virus (TSHEV), tree shrew paramyxovirus (TSParaV), and tree shrew pneumovirus (TSPneV), specific primer sets were designed and used to detect viral genomes in the samples. The result showed the positive rates as 9.9% for TSTTV, 4.2% for TSAdV-HNU2, 7.0% for TSAdV-HNU3, 1.0% for TSHEV, 4.2% for TSParaV, 13.9% for TSHV, 6.3% for TSPneV, 47.9% for TSParvoV-HNU1, 11.3% for TSParvoV-HNU2, 11.3% for TSParvoV-HNU3, 39.4% for TSParvoV-HNU4, and 7.0% for TSParvoV-HNU5. The TSParvoV-HNU1 exhibited the highest positive rate of 47.9%. The oral swab samples showed positive rates of 29.3% for TSHV and 15.5% for TSPneV, while the anal swab samples exhibited positive rates of 1.7% for TSHEV and 13.8% for TSPneV. Moreover, all viruses except TSParaV were detected in spleen samples, while TSAdV and TSParaV were identified only in spleen and kidney samples (Supplementary Table S2).

Genetic characteristics of the parvoviruses

Parvoviruses (family *Parvoviridae*) are small, non-enveloped viruses with a linear, single-stranded and positive DNA genome of about 5 kb in length. There are two large open reading frames (ORFs) in parvovirus genome, encoding the nonstructural protein (NS) and the shell protein (VP), respectively. Parvoviruses infecting vertebrates were further classified in the *Parvovirinae* subfamily in which at least five species infect humans.

We determined five tree shrew parvoviruses, TSParvoV-HNU1, TSParvoV-HNU2, TSParvoV-HNU3, TSParvoV-HNU4, and TSParvoV-HNU5, in this study. The complete genomic sizes of TSParvoV-HNU1, TSParvoV-HNU2, TSParvoV-HNU3, TSParvoV-HNU4, and TSParvoV-HNU5 were 5,232, 3,856, 5,071, 4,608, and 4,534 nucleotides with the GC contents of 49.9%, 41.6%, 49.7%, 43.6%, and 44.7%, respectively (Supplementary Table S3). Using BLASTX and BLASTP, we identified NS1 and VP in TSParvoV-HNU1, TSParvoV-HNU3, and TSParvoV-HNU4, as well as NS1, NS2, and VP in TSParvoV-HNU2 and TSParvoV-HNU5. (Fig. 1A). Moreover, calcium-binding motif (YXGXG) and phospholipase A2 enzymes (PLA2) secretory motif (HDXXY) were identified in TSParvoV-HNU1, TSParvoV-HNU3, TSParvoV-HNU4, and TSParvoV-HNU5, but these motifs did not be identified in TSParvoV-HNU2 (Supplementary Fig. S1).

Regarding NS1, TSParvoV-HNU1 (50.5%) and TSParvoV-HNU3 (50.0%) exhibited the highest amino acid sequence identity to a chipmunk parvovirus (GenBank accession No. GQ200736). TSParvoV-HNU2 showed 70.3% identity to a murine chapparvovirus (GenBank accession No. MF175078), while TSParvoV-HNU4 shared 50.9% identity with a rat parvovirus (GenBank accession No. AF036710). In contrast, TSParvoV-HNU5 displayed the highest identity (83.6%) to a minute virus (GenBank accession No. J02275) (Supplementary Table S4).

To determine the evolutionary histories of these tree shrew parvoviruses, we performed phylogenetic analysis of the amino acid sequences of their NS1 together with those of representative parvovirus species. Phylogenetic analysis revealed that TSParvoV-HNU1 and TSParvoV-HNU3 were clustered together, forming a sister clade to a group containing chipmunk parvovirus. TSParvoV-HNU5 was grouped with minute virus of mice, forming a sister clade to a cluster of canine and rat parvoviruses. TSParvoV-HNU4 was positioned within the genus *Protoparvovirus*, forming a subclade with parvoviruses from multiple host species. Finally, TSParvoV-HNU2 was clustered within the genus *Chaphamaparvovirus*, forming a subclade with several animal parvoviruses (Fig. 1B). Notably, SimPlot sliding window analysis revealed that TSParvoV-HNU5 harbored several recombination sites, indicating TSParvoV-HNU5 was likely derived from recombination between minute virus of mice (GenBank accession No. J02275) and rat parvovirus 1 (GenBank accession No. AF036710) (Fig. 1C).

Genetic characteristics of the torque teno viruses

TTV (family *Anelloviridae*), also known as transfusion transmitted virus, is small, non-enveloped virus with a single-stranded, circular DNA

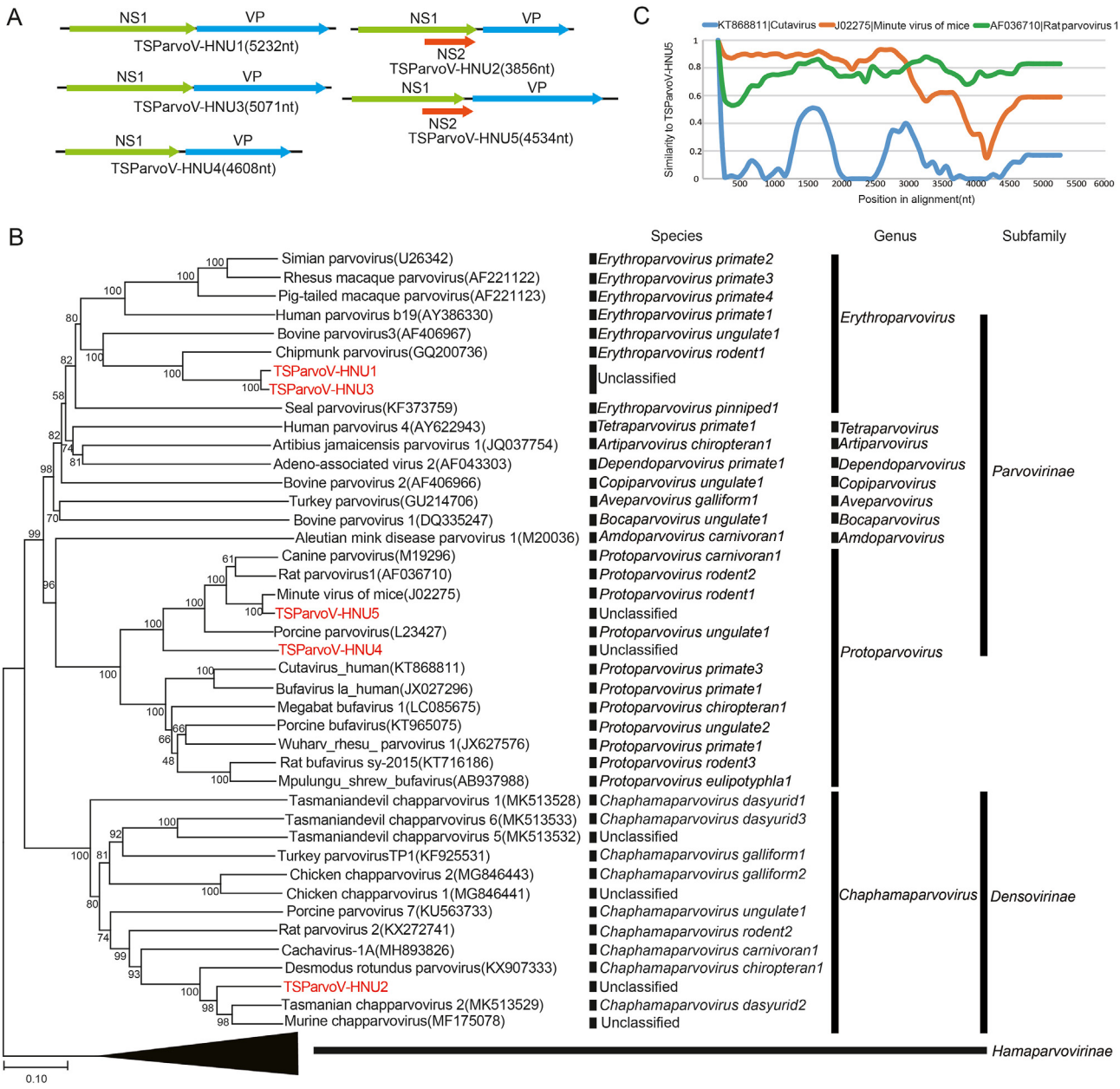

Fig. 1. Genomic characterization and evolutionary relationships of five tree shrew parvoviruses (TSParvoVs). **A** Genomic characterization of TSParvoV-HNU1, TSParvoV-HNU2, TSParvoV-HNU3, TSParvoV-HNU4, and TSParvoV-HNU5. **B** Phylogenetic analysis of TSParvoV-HNU1 to TSParvoV-HNU5 (highlighted with red). Neighbor-joining (NJ) tree was established based on the amino acid sequences of NS1 using MEGA7 with the bootstrap value set at 1000. **C** Results from SimPlot analysis of TSParvoV-HNU5. The y-axis gave the percentage of identity within a sliding window of 500 bp wide centered on the position plotted, with a step size between plots of 20 bp. Comparison of TSParvoV-HNU5 with cutavirus (GenBank accession No. KT868811), minute virus of mice (GenBank accession No. J02275) and rat parvovirus1 (GenBank accession No. AF036710) were shown.

genome. The genomic size varies among different TTV species. The genome of TTV contains a translational region and an untranslated region (UTR). TTV has two main ORFs, ORF1 and ORF2, encoding the viral replication-associated protein (Rep) and the viral capsid protein (Cap), respectively.

Three tree shrew TTVs were identified in this study, TSTTV-HNU1, TSTTV-HNU2, and TSTTV-HNU3. The complete genomes of TSTTV-HNU1, TSTTV-HNU2, and TSTTV-HNU3 were 2,224 nt, 2,259 nt, and 2,292 nt in length with GC content of 49.7%, 50.5%, and 50.8%, respectively (Supplementary Table S3), and they exhibited typical TTV genome structures (Fig. 2A). Interestingly, the sequence identities among TSTTV-HNU1, TSTTV-HNU2, and TSTTV-HNU3 ranged from 96.4% to 99.6% at ORF1. Moreover, TSTTV-HNU1, TSTTV-HNU2, and TSTTV-HNU3 exhibited the highest nucleotide identities to Mosquito VEM Anellovirus SDRB A (GenBank accession No. HQ335084) at ORF1 (Supplementary Table S4). Phylogenetic analyses revealed that the TSTTV-HNU1, TSTTV-HNU2, and TSTTV-HNU3 were clustered together, forming a new genus in family *Anelloviridae*, according to the genus demarcation criteria proposed by ICTV (Proposal, 2020.015D) (Fig. 2B).

Genetic characteristics of the adenoviruses

Adenoviruses (family *Adenoviridae*) were first detected in human adenoids. These icosahedral non-enveloped, double-stranded DNA viruses with 26–45 kbp genome are the common pathogens of vertebrates. Adenoviruses can be classified into five genera: *Mastadenovirus*,

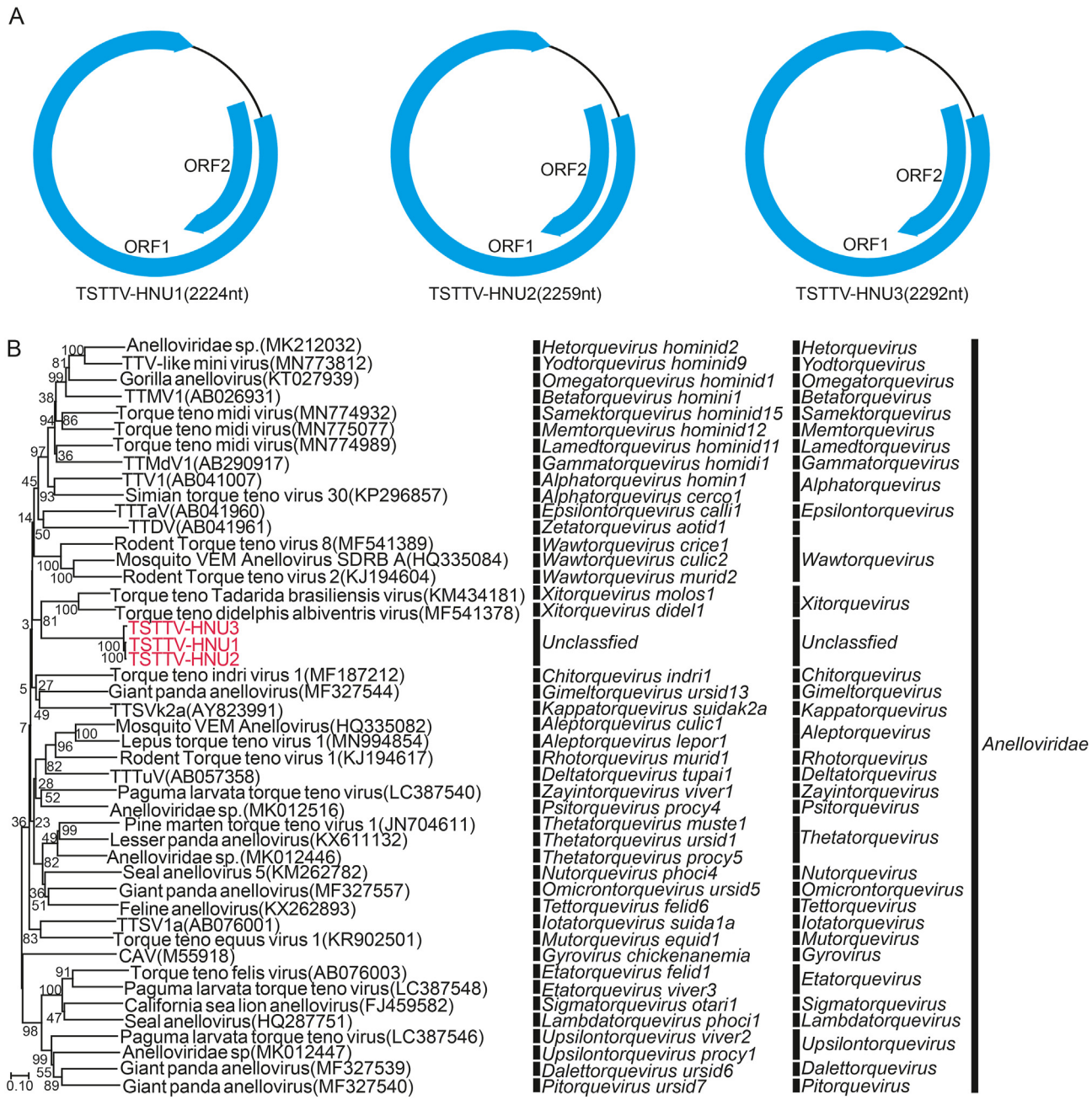

Fig. 2. Genomic characterization and evolutionary relationships of three tree shrew torque teno viruses (TSTTVs). **A** Genomic characterization of TSTTV-HNU1, TSTTV-HNU2, and TSTTV-HNU3. **B** Phylogenetic analysis of TSTTV-HNU1, TSTTV-HNU2, and TSTTV-HNU3 (highlighted with red). Neighbor-joining (NJ) tree was established based on the nucleotide sequences of ORF1 using MEGA7 with the bootstrap value set at 1000.

Aviadenovirus, *Atadenovirus*, *Siadenovirus*, and *Ichtadenovirus*. In infants, young children, and immunocompromised individuals, adenovirus infection can lead to severe pneumonia and even death.

We determined two adenoviruses, TSAdV-HNU2 and TSAdV-HNU3 in this study. The complete genome of TSAdV-HNU2 was 31,494 bp in length, the complete genome of TSAdV-HNU3 was 29,559 bp in length, and their GC content were 48.9% and 48.2%, respectively (Supplementary Table S3). The two tree shrew adenoviruses both exhibited typical adenovirus genome structure (Fig. 3A). As for their DNA polymerases, TSAdV-HNU2 shared 97.5% amino acid identity with the TSAdV-1 (GenBank accession No. AF258784), and TSAdV-HNU3 shared 58.7% amino acid identity with the PAdV-5 (GenBank accession No. AF289262) (Supplementary Table S4). According to the phylogenetic tree based on DNA polymerase of representative adenoviruses, TSAdV-HNU2 was grouped with TSAdV-1, forming a sister clade parallel with to adenoviruses from porcine, bovine, and ovine. TSAdV-HNU3 fell within the genus *Mastadenovirus* and formed a subclade containing human and other animal adenoviruses (Fig. 3B).

Genetic characteristics of the pneumovirus

Pneumoviruses (family *Pneumoviridae*) are enveloped, single-stranded negative RNA viruses. They are classified into two genera, *Orthopneumovirus* and *Metapneumovirus*. The size of pneumovirus genome is 13.2–15.3 kb, encoding NS1, NS2, N, P, M, SH, G, F, M2, and other proteins, among which NS1 and NS2 proteins are unique to the genus *Orthopneumovirus*.

One pneumovirus, TSPneV-HNU1, was determined in our study. The complete genome of TSPneV-HNU1 was 14,812 nt in length, possessing a

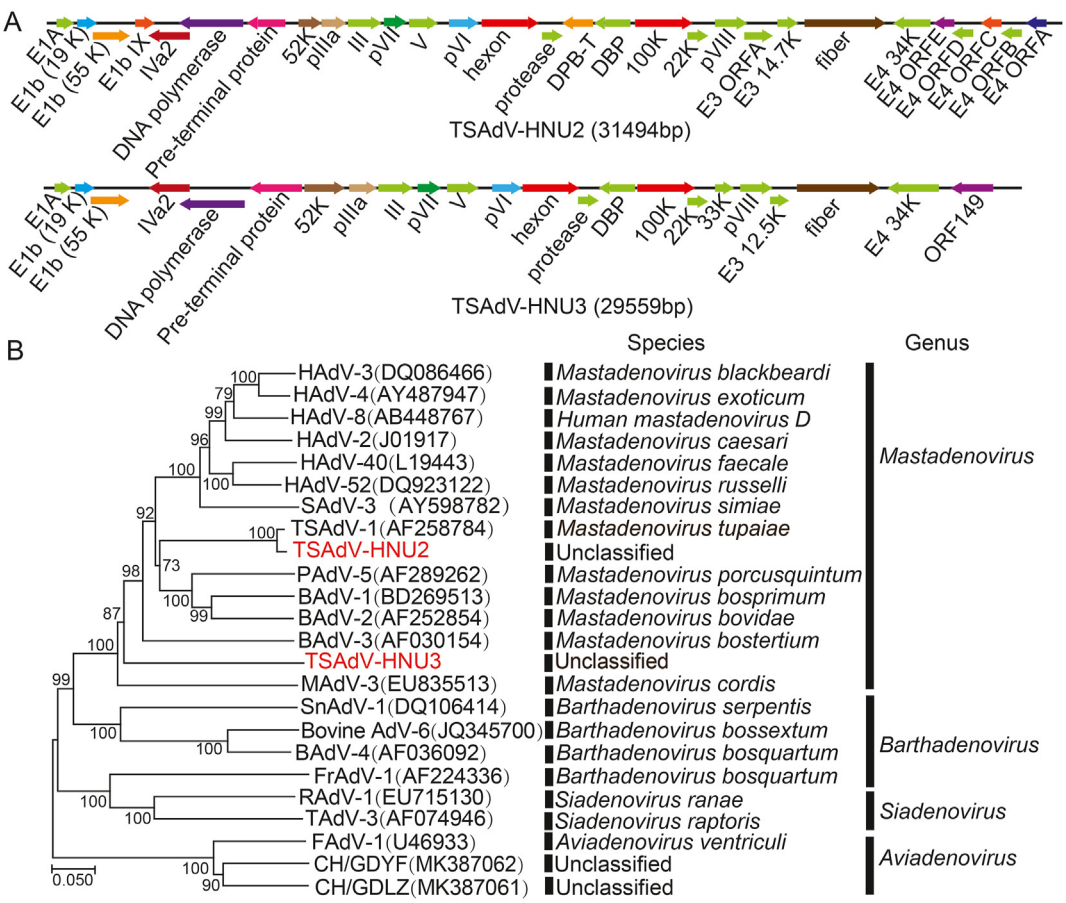

Fig. 3. Genomic characterization and evolutionary relationships of two tree shrew adenoviruses (TSAdVs). **A** Genomic characterization of TSAdV-HNU2 and TSAdV-HNU3. **B** Phylogenetic analysis of TSAdV-HNU2 and TSAdV-HNU3 (highlighted with red). Neighbor-joining (NJ) tree was established based on the amino acid sequences of DNA polymerase using MEGA7 with the bootstrap value set at 1000.

typical genome organization similar to that of other members of the family *Pneumoviridae* (Fig. 4A). TSPneV-HNU1 shared an amino acid identity of 99.2% with SPV_57 (GenBank accession No. KX364383) at L protein (Supplementary Table S4). Phylogenetic analysis of the amino acid sequences of L protein revealed that TSPneV-HNU1 was clustered with the SPV_57, forming a sister clade parallel to two other clades of canine orthopneumovirus and murine orthopneumovirus within the genus *Orthopneumovirus* (Fig. 4B). The estimated divergence time showed that all pneumoviruses originated from a common ancestor in about 3901 BC to 98 BC. Moreover, TSPneV-HNU1 had the highest homology with

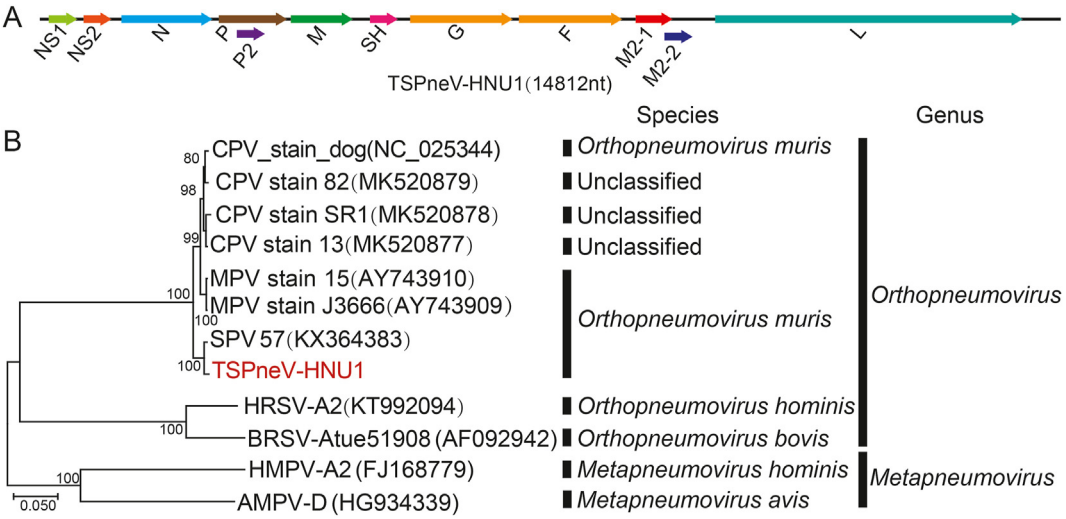

Fig. 4. Genomic characterization and evolutionary relationships of a tree shrew pneumovirus (TSPneV) in this study. **A** Genomic characterization of TSPneV-HNU1. **B** Phylogenetic analysis of TSPneV-HNU1 (highlighted with red). Neighbor-joining (NJ) tree was established based on the amino acid sequences of L protein using MEGA7 with the bootstrap value set at 1000.

SPV_57, and their estimated divergence time was between 1993 and 2007 (Supplementary Fig. S2).

Genetic characteristics of the hepacivirus

Hepaciviruses (family *Flaviviridae*) are enveloped viruses with a single-stranded positive RNA genome approximately 8.9–10.5 kb in length. The genus *Hepacivirus* comprises 14 designated species (A-N), among which the hepatitis C virus (HCV) is the best characterized. Humans and chimpanzees serve as the natural hosts for HCV.

Here, we determined the complete genome of a hepacivirus, TSHV-HNU1. The complete genome sequence of TSHV-HNU1 comprised 8,311 nt with a GC content of 54.4%. According to the predicted cleavage site of signal peptidase, the polyprotein encoded by TSHV-HNU1 genome could be cleaved to C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig. 5A). TSHV-HNU1 shared 63.4% and 67.2% amino acid identities with hepacivirus P isolate RHV-GS2015 (GenBank accession No. NC_040815) at NS3 and NS5B, respectively (Supplementary Table S4). Phylogenetic analysis of the amino acid sequences of NS3 revealed that TSHV-HNU1 was clustered with hepacivirus P isolate RHV-GS2015, forming a sister clade parallel to Hepacivirus norvegici (GenBank accession No. KJ950939) and Norway rat hepacivirus 2 (GenBank accession No. NC_025673) within the genus *Hepacivirus* (Fig. 5B).

Genetic characteristics of the hepatitis E viruses

HEVs (family *Flaviviridae*) are single-stranded, positive RNA viruses. Members of this family are classified into three genera, *Hepacivirus*, *Orthoflavivirus*, and *Pestivirus*. HEV is one of the most common causes of hepatitis worldwide, and studies have suggested that rodents may be a potential source of HEV infection in humans and livestock.

Partial genomes of two HEVs, TSHEV-HNU1 and TSHEV-HNU2, were determined in this study. Through genome comparison and ORF prediction, we determined the two fragments to be parts of ORF2. TSHEV-HNU1 and TSHEV-HNU2 showed 97.8% and 89.0% of nucleotide identities with TSHEV (GenBank accession No. KR905549) at ORF2 (Supplementary Table S4). In the ORF2 phylogenetic tree, TSHEV-HNU1, TSHEV-HNU2 formed a clade together with TSHEV (Fig. 6).

Genetic characteristics of the paramyxovirus

Paramyxoviruses (family *Paramyxoviridae*) are enveloped, single-strand, negative-sense RNA viruses. The paramyxovirus genome ranges from 14.6 to 20.1 kb in size, primarily encoding the N, P/V/C, M, F, RBP, and L proteins, with some species additionally expressing SH, tM, and U proteins. Paramyxoviruses are diverse in shape and mainly spherical, with particle diameters of about 150 nm.

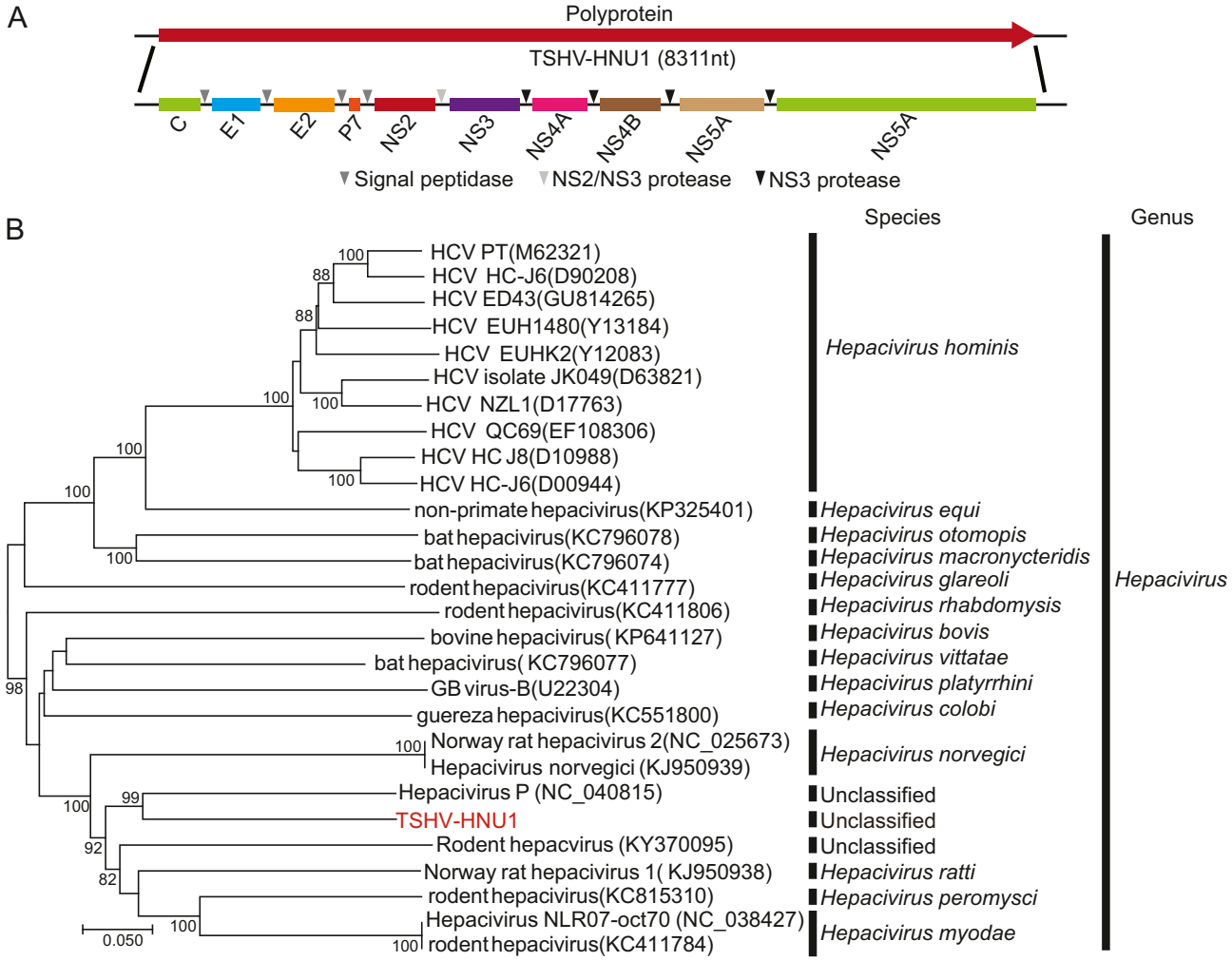

Fig. 5. Genomic characterization and evolutionary relationships of a tree shrew hepacivirus (TSHV). **A** Genomic characterization of TSHV-HNU1. **B** Phylogenetic analysis of TSHV-HNU1 (highlighted with red). Neighbor-joining (NJ) tree was established based on the amino acid sequences of NS3 using MEGA7 with the bootstrap value set at 1000.

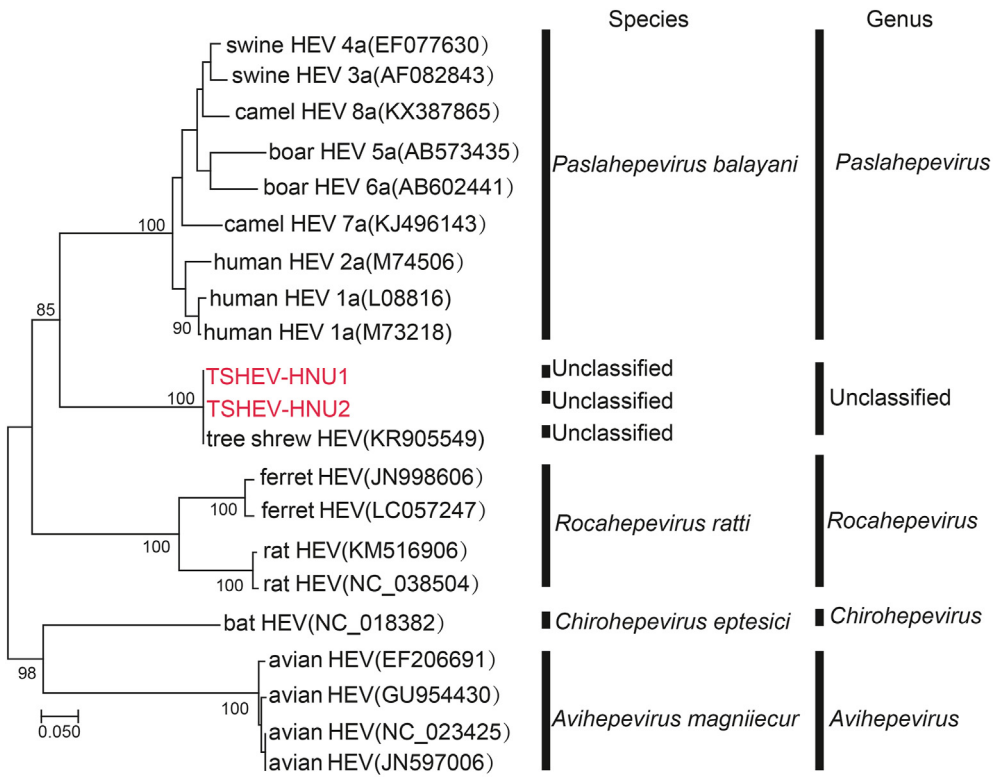

Fig. 6. Phylogenetic analysis of partial genomes of two tree shrew hepatitis E viruses (TSHEVs) TSHEV-HNU1 and TSHEV-HNU2 (highlighted with red). Neighbor-joining (NJ) tree was established based on the amino acid sequences of ORF2 using MEGA7 with the bootstrap value set at 1000.

In our work, five genome segments of a paramyxovirus TSParaV-HNU1 were discovered. TSParaV-HNU1 exhibited 89.5%, 76.7%, and 66.2% with tupaia paramyxovirus (GenBank accession No. NC_002199) at amino acid sequences of N, P, and L protein, respectively (Supplementary Table S4). From the phylogenetic tree based on the amino acid sequences of L protein of representative paramyxoviruses, TSParaV-HNU1 was clustered with tupaia paramyxovirus (GenBank accession No. NC_002199), forming a sister clade parallel to a group containing mossman narmovirus, myodes narmovirus, and nariva narmovirus (Fig. 7).

Fig. 7. Phylogenetic analysis of partial genome of a tree shrew paramyxovirus (TSParaV) TSParaV-HNU1 (highlighted with red). Neighbor-joining (NJ) tree was established based on the amino acid sequences of L protein using MEGA7 with the bootstrap value set at 1000.

DISCUSSION

Over the past two decades, viral metagenomic analysis facilitated by next-generation sequencing have become a powerful tool in monitoring zoonotic viruses prior to their transmission to humans, leading to extensive research on the virome of wild and domestic animals (Edwards and Rohwer, 2005; Mokili et al., 2012; Temmam et al., 2014; Coutinho et al., 2017). Due to their close phylogenetic relationship to humans and wide geographical distribution, tree shrews have been identified as reservoirs for different viruses with potential of human infection. The concern about virus transmission from tree shrews to human is particularly raised in Yunnan Province where a high risk of disease emergence has been found at human-animal interfaces (Feng et al., 2024). Consequently, surveillance of viruses carried by tree shrew populations in Yunnan Province is expected of great significance for the prevention of future zoonotic outbreaks. Furthermore, tree shrews may serve as crucial intermediate hosts in cross-species virus transmission. In this study, we decoded the virome of wild tree shrews and uncovered that they were reservoirs for multiple viruses belonging to at least 13 families. The viral diversity observed here can largely be attributed to geographical landscapes and rich biodiversity of Yunnan Province (Feng et al., 2024). Notably, only 14% of reads showed significant similarity to the sequences of known viruses archived in the database. This phenomenon may result from the limitations of alignment-based classification and the presence of "viral dark matter" which refers to the substantial portion of nucleic acids with unknown viral origins (Sullivan, 2015; Krishnamurthy and Wang, 2017). Consistent with the wide range of host animals, we found a high abundance of parvoviruses-related reads in the tree shrews. Interestingly, we also detected several reads associated with plant and insect viruses, which may reflect the feeding habits and life cycles of the tree shrews (Chen, L. et al., 2019a; Fuchs, 2024). These findings underscore the importance of considering background virome when utilizing tree shrews as disease models in future research.

Previous studies have identified several zoonotic viruses in tree shrews, including adenovirus, paramyxovirus, papillomavirus, polyomavirus, mammalian orthoreoviruses, picornavirus, and rotavirus A (Tidona et al., 1999; Schöndorf et al., 2003; Liu et al., 2019; Zhou et al., 2024). In our study, through PCR and Sanger sequencing, we determined fifteen viruses belonging to seven families. The tree shrew parvoviruses, TSParvoV-HNU1 and TSParvoV-HNU3, showed high amino acid identities with a chipmunk parvovirus, while TSParvoV-HNU2, TSParvoV-HNU4 and TSParvoV-HNU5 shared high amino acid identities with murine viruses. More importantly, TSParvoV-HNU5 probably originated through viral recombination. In the previous study, an extensive number of parvovirus contigs and operational taxonomic units (OTUs) belonging to the subfamily *Parvovirinae* have been discovered in *Rattus rattus* in Yunnan Province (Kane et al., 2024). Additionally, another study has revealed that NS1 of Chipmunk Parvovirus (ChpPV) induces apoptosis in parvovirus-B19-semipermissive UT7/Epo-S1 cells (Chen, Z. et al., 2010). Therefore, attention should be paid on the cross-species potential of tree shrew parvoviruses.

Pneumoviruses, known to infect humans, domestic mammals, rodents, and birds (Easton et al., 2004), were also identified in the tree shrews. TSPneV-HNU1 exhibited a high amino acid identity with SPV_57 at L protein and they probably originated from a common ancestor, suggesting that the risk of cross-species transmission of TSPneV-HNU1. This finding also supports the potential use of tree shrews as laboratory models for human pneumovirus research. Furthermore, a mouse-associated orthopneumovirus was identified in game animal species, such as pangolins and Asian badgers (He et al., 2022). Canine pneumovirus causing canine infectious respiratory disease was reported in Italy, and an emerging orthopneumovirus was also detected from dogs with canine infectious respiratory disease in China (Decaro et al., 2016; Song et al., 2021). Therefore, the further study of pneumovirus in animal populations is of great significance. As for TTV, high prevalence rates of RoTTV3 have been documented in common murine rodents in China (Xiong et al., 2018). Our findings showed that TTV may have a high prevalence in tree shrews, too. The tree shrew TTVs we detected showed high sequence identities in ORF1, suggesting that they may have been circulating in the population of wild tree shrews.

The discovery of hepaciviruses with broad host ranges adds another dimension to our findings. We identified one hepacivirus (TSHV-HNU1) and two hepatitis E viruses (TSHEV-HNU1 and TSHEV-HNU2) in tree shrews. HEV is a significant human pathogen in China, with zoonotic transmission primarily occurring through exposure to wild boars, domestic pigs, and rabbits. Current evidence suggests domestic pigs serve as the principal natural reservoir for genotypes causing human hepatitis E infections (Hoan et al., 2019). TSHV-HNU1 and bovine hepacivirus form a subclade of genus *hepacivirus*, emphasizing the need for continued surveillance of hepaciviruses in tree shrews. Actually, hepaciviruses have been detected in tree shrews previously, which further support their potential as laboratory models for human hepatitis-associated viruses (Zhou et al., 2024). Additionally, we identified two adenoviruses, TSAdV-HNU2 and TSAdV-HNU3, in the tree shrews. Adenoviruses have been reported to infect almost all known vertebrates and fowl adenoviruses (FAdVs) caused huge economic losses to the poultry industry (Chen, L. et al., 2019b; Harrach et al., 2019), so the potential transmission of tree shrew adenoviruses to poultry deserves extensive studies. Lastly, the partial genome of a paramyxovirus TSParaV-HNU1 was detected in our study and which shared the closet genetic distance with some paramyxoviruses from several animal hosts. Paramyxoviruses were reported to have a cross-species transmission and potential outbreak risks in the northwest region of China, so further investigation of paramyxoviruses in tree shrews was warranted (Ren et al., 2017).

CONCLUSION

In conclusion, our virome analysis revealed a high abundance and diversity of viruses in wild tree shrews, encompassing at least thirteen families infecting mammals. The most viruses detected were DNA viruses and animal viruses, along with some plant and insect viruses. Notably, we determined the complete genomes of twelve viruses and the partial genomes of three ones. Several viruses we determined may have originated through recombination with potential spillover risks. Our findings suggest that tree shrews may serve as important reservoirs for viruses capable of infecting diverse animal hosts including humans, and tree shrews can be animal models for studying a variety of human viruses.

MATERIALS AND METHODS

Sampling

During routine surveillance of pathogen control from 2016 to 2018, a total of 76 tree shrews (58 live and 18 dead) were captured near human settlement such as fields, shrubs, and grasslands in several border areas of Yunnan Province, China. Animal species were confirmed by morphology and sequencing the mitochondrial cytochrome b (CytB) gene. Swab samples were collected from living individuals and then animals were released into nature. Tissue samples were collected from dead ones. The swabs were stored in cryopreserved tubes containing virus transport medium (VTM) while tissue samples were placed in same tubes without VTM. All samples were immediately placed in liquid nitrogen for short-term storage, and then transported to the laboratory with dry ice and stored at -80 °C until nucleic acid isolation.

Viral metagenomic sequencing and data analysis

The samples were divided into six pools: oral swab samples, anal swab samples, heart-kidney samples, spleen samples, lung samples, and liver samples. The samples were homogenized with steel beads before total nucleic extraction using phosphate buffer saline (PBS). Before the extraction of nucleic acids, the suspension was filtered and treated with

nucleases to reduce incorporation of nucleic acids derived from the host and bacteria. Total viral nucleic acids were extracted using the TIANamp Virus DNA/RNA Kit (TIANGEN, China). After total nucleic extraction, quantity and quality were checked, followed by the library construction. The total viral nucleic acid was used for random PCR (rPCR) and the purified products were then used to build a sequencing library for HiSeq-PE150 instrument (Illumina platform) according to the manufacturer's instructions. Raw reads were trimmed and assembled using Geneious primer software (version R11.1) (Kearse et al., 2012). To identify potential viral sequences, the assembled contigs were compared against the NCBI protein (nr) databases using BLASTX with an e-value cutoff of 1×10^{-5} (Johnson et al., 2008).

PCR detection of viruses

According to the sequence data analysis, specific primers for PCR detection were designed to detect contig sequences of TSParvoV, TSAdV, TSTTV, TSHV, TSHEV, TSParaV, and TSPneV in all samples (Supplementary Table S5). Detection of TSParvoV, TSAdV, and TSTTV were performed using TSINGKE Master Mix (TSINGKE, China) according to the manufacturer's instructions and run on a PCR detection system (Bio-Rad, Germany) with the following protocol: 94 °C at 2 min, 94 °C at 30 s, 55 °C at 30 s, 72 °C at 30 s, 72 °C at 5 min. As for detection of TSHV, TSHEV, TSParaV, and TSPneV, the first-round were performed using One-Step RT-PCR Kit (TAKARA Bio, China) according to the manufacturer's instructions with the following thermal cycling protocol: 50 °C for 30 min, 94 °C for 2 min, followed by the 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. A second-round PCR was performed using TSINGKE Master Mix (TSINGKE, China) according to the manufacturer's instructions with the following thermal cycling protocol: 94 °C for 2 min, following by the 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and terminated by 72 °C for 5 min. The PCR products were purified using MiniBEST Agarose Gel DNA Extraction Kit Ver. 4.0 (TAKARA, China) and then sent to Tsingke Biotechnology Co., Ltd. for sequencing.

Amplification of viral genomes

After PCR screening and sequencing, samples with a single positive result were selected to amplify the complete viral genomes. Primers were designed to amplify the complete genomes of the viruses respectively (Supplementary Table S5). The PCR replicons of each fragment were cloned into T-vector using pClone007 Vector Kit (TSINGKE, China) and at least three positive clones were selected for sequencing using the Sanger method. The sequence fragments obtained were utilized to assemble the complete viral genomes.

Prediction of ORFs and genetic analysis

ORFs were predicted using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/, accessed on December 10, 2024) with genes annotated against the NCBI conserved domain database (CDD) (Sayers et al., 2022). Sequences were aligned using Geneious (version 9.0.2) and MEGA (version 7.0) (Kumar et al., 2016). Phylogenetic trees were estimated using the neighbor-joining (NJ) method in MEGA, employing the best-fit substitution model and 1000 bootstrap replicates. For recombination detection, the multiple alignments were analysed using a bootscan approach implemented in SimPlot (version 3.5.1), and a window size of 500 bp and a step size of 20 bp were set (Lole et al., 1999). For estimation of divergence time, strict clock models were run for 150 million Markov Chain Monte Carlo (MCMC) samples, with a 10% burn-in and sampling every 5000 steps. Logs were visualized in Tracer (version 1.7.1) and trees plotted in TreeAnnotator in BEAST (version 1.8) (Drummond and Rambaut, 2007).

DATA AVAILABILITY

All the sequences in this manuscript can be obtained from the Genbank Genedatabase with the following accession numbers: PQ855815 to PQ855819, PQ855823 to PQ855836 (https://www.ncbi.nlm.nih.gov). A fully annotated mitochondrial whole genome sequence from a tissue sequencing source is presented in the Supplementary Material.

ETHICS STATEMENT

The collection of small animals was performed by veterinarians with approval from the Animal Ethics Committee of Dali University (DLDXLL2013002).

AUTHOR CONTRIBUTIONS

Zhong-Hao Lian and Zhi You: data curation, formal analysis, investigation, validation, visualization, writing-original draft. Pei-Yu Han: samples collection, data curation, formal analysis. Yun-Zhi Zhang: samples collection, funding acquisition, project administration, methodology, resources. Ye Qiu: methodology, formal analysis, validation, writing-original draft, writing-review & editing. Xing-Yi Ge: samples collection, resources, conceptualization, funding acquisition, project administration, resources, supervision, validation, writing-review & editing.

CONFLICT OF INTEREST

All authors declare that there are no competing interests.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2025.05.011.

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