



RESEARCH ARTICLE

# Chevrier's Field Mouse (*Apodemus chevrieri*) and Père David's Vole (*Eothenomys melanogaster*) in China Carry Orthohepeviruses that form Two Putative Novel Genotypes Within the Species *Orthohepevirus C*

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## Abstract

Hepatitis E virus (HEV) is the prototype of the family *Hepeviridae* and the causative agent of common acute viral hepatitis. Genetically diverse HEV-related viruses have been detected in a variety of mammals and some of them may have zoonotic potential. In this study, we tested 278 specimens collected from seven wild small mammal species in Yunnan province, China, for the presence and prevalence of orthohepevirus by broad-spectrum reverse transcription (RT)-PCR. HEV-related sequences were detected in two rodent species, including Chevrier's field mouse (*Apodemus chevrieri*, family Muridae) and Père David's vole (*Eothenomys melanogaster*, family Cricetidae), with the infection rates of 29.20% (59/202) and 7.27% (4/55), respectively. Further four representative full-length genomes were generated: two each from Chevrier's field mouse (named RdHEVAc14 and RdHEVAc86) and Père David's vole (RdHEVEm40 and RdHEVEm67). Phylogenetic analyses and pairwise distance comparisons of whole genome sequences and amino acid sequences of the gene coding regions showed that orthohepeviruses identified in Chinese Chevrier's field mouse and Père David's vole belonged to the species *Orthohepevirus C* but were highly divergent from the two assigned genotypes: HEV-C1 derived from rat and shrew, and HEV-C2 derived from ferret and possibly mink. Quantitative real-time RT-PCR demonstrated that these newly discovered orthohepeviruses had hepatic tropism. In summary, our work discovered two putative novel genotypes orthohepeviruses preliminarily named HEV-C3 and HEV-C4 within the species *Orthohepevirus C*, which expands our understanding of orthohepevirus infection in the order Rodentia and gives new insights into the origin, evolution, and host range of orthohepevirus.

**Keywords** Hepatitis E virus (HEV) · Orthohepevirus · Genetic diversity · Complete genome · Chevrier's field mouse · Père David's vole

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## Introduction

Approximately one third of the world's population live in areas endemic for hepatitis E virus (HEV) and are at risk for infection (Perez-Gracia et al. 2013). Although the

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majority of HEV infections is asymptomatic, some of them cause acute and chronic hepatitis E (HE), fulminant liver failure and even extrahepatic symptoms (Kamar et al. 2012). HE may be particularly severe in pregnant women and immunocompromised patients (Kumar et al. 2004; Dalton et al. 2009). HEV is responsible for numerous outbreaks of viral hepatitis in many tropical countries. It is estimated that the number of symptomatic HEV infections in these countries exceeds three million annually, causing up to 70,000 deaths each year (Rein et al. 2012).

The virions of human HEV are icosahedral and spherical particles with a diameter of approximately 27–34 nm (Smith et al. 2014). The viral genomes are positive-sense single-stranded RNA molecules of 6.4–7.2 kb containing three open reading frames (ORFs: ORF1, ORF2 and ORF3), 5'- and 3'-untranslated regions (UTRs), and a poly (A)-tract at the 3'-end. ORF1 encodes the non-structural proteins and functional domains including methyltransferase (Met), Y-domain (Y), papain-like protease (PCP), hypervariable region (HVR), macro-domain (X), RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp) required for RNA replication. ORF2 expresses the capsid protein. ORF3 overlaps with ORF2 and encodes a multifunctional phosphoprotein (Cao and Meng 2012; Kamar et al. 2012; van Tong et al. 2016). An additional ORF4, overlapping the 5' end of ORF1, has been described recently for rat and ferret HEV strains. However, the functional role of ORF4 is not clear (Johns et al. 2014).

According to the latest consensus proposals for classification of the family *Hepeviridae* from the International Committee on Taxonomy of Viruses (ICTV), it is divided into two genera: *Orthohepevirus* including all mammalian and avian HEV strains, and *Piscihepevirus* including cutthroat trout virus. Currently, four species (*Orthohepevirus A*, *B*, *C* and *D*) have been assigned in the genus *Orthohepevirus*. Within the species *Orthohepevirus A*, seven HEV genotypes (HEV-1 to HEV-7) are recognized based on the criterion of amino acid (aa) distances (> 0.088) of concatenated ORF1 and ORF2 (lacking hypervariable regions) (Smith et al. 2014, 2016). HEV-1 to HEV-4 are human pathogens, HEV-1 and HEV-2 infect exclusively humans and are typically associated with large waterborne epidemics in some countries of Asia and Africa as well as in Mexico, while HEV-3 and HEV-4 are detected in humans and a number of other animal species and the main cause of sporadic cases of HE in many industrialized countries (Pavio et al. 2015). HEV-5 and HEV-6 have been identified only in wild boars, and the HEV-7 from dromedary camels has been reported to infect humans and cause chronic HE, as observed in a liver transplant patient (Woo et al. 2014; Lee et al. 2016). Recently, putative HEV-8 is identified in Bactrian camels with an unknown zoonotic potential (Woo et al. 2016; Sridhar et al. 2017). The

species *Orthohepevirus C* contains two genotypes: genotype C1 (HEV-C1), including strains derived from rat and shrew, and HEV-C2, including strains derived from ferret and possibly mink (Smith et al. 2014). Of note, the current newly discovered HEV-related viruses from moose, fox, kestrel and little egret are waiting for further classification (Purdy et al. 2017).

Rodents are highly abundant and diversified in various terrestrial habitats and harbour a variety of viruses closely related to human diseases (Koch et al. 2007). Currently, a number of HEV-related viruses have been detected from Norway rats (*Rattus norvegicus*) and Black rats (*Rattus rattus*) at different sites including China, Vietnam, Indonesia, the USA and eleven European countries (Johns et al. 2010a; Purcell et al. 2011; Li et al. 2013; Mulyanto et al. 2014; Ryll et al. 2017; Wang et al. 2017c). Furthermore, rat HEV sequences have been detected also in the greater bandicoot rat (order: Rodentia) and Asian musk shrew (order: Soricomorpha) (Guan et al. 2013; Li et al. 2013). The zoonotic transmission potential of rat HEV to humans is still controversial (Johns et al. 2014). Sera of rats that are infected with rat HEV are known to cross-react with human HEV antigens (Dong et al. 2011). Moreover, rat HEV was shown to replicate in a human-derived cell line with a reverse genetics system (Li et al. 2015). However, experimental infection of monkeys and domestic pigs with rat HEV failed (Cossaboom et al. 2012).

In this study, we have investigated the presence and prevalence of HEV-related viruses (orthohepeviruses) in 278 small mammal specimens pertaining to 7 species sampled in Yunnan province, China, and report the discovery of novel orthohepeviruses from Chevrier's field mouse (*Apodemus chevrieri*) and Père David's vole (*Eothenomys melanogaster*).

## Materials and Methods

### Sampling and RNA Extraction

278 small mammals (rodents, shrews, and carnivore), which comprised three orders, four families, and seven species, were captured by mouse traps in Luxi city and Lijiang city, Yunnan province, China, from September 2014 to November 2015 (Table 1). All animals live-trapped were anaesthetized to minimize suffering. Different tissues (liver, heart, intestine, spleen, kidney, and lung) were collected separately and used for analysing virus tissue tropism. Samples were transported by nitrogen canister and stored at – 80 °C until thawed for RNA extraction. The animal species were firstly identified by trained field biologists and then confirmed by DNA sequencing of the mitochondrial cytochrome (*CytB*) gene following

**Table 1** Detection of orthohepevirus in small mammals from Yunnan province, China between 2014 and 2015

Order	Family	Species	Positive samples/tested samples (%)		Sampling site (year)
Rodentia	Muridae	<i>Rattus tanezumi</i>	0/2 (0.00)		Lijiang city (2014)
		<i>Apodemus chevrieri</i>	44/82 (53.66)	59/202 (29.20)	Lijiang city (2014)
			0/35 (0.00)		Luxi city (2014)
			15/85 (17.65)		Lijiang city (2015)
	Cricetidae	<i>Mus caroli</i>	0/1 (0.00)		Luxi city (2014)
		<i>Eothenomys chinensis</i>	0/13 (0.00)		Lijiang city (2014)
		<i>Eothenomys melanogaster</i>	4/55 (7.27)		Luxi city (2014)
Soricomorpha	Soricidae	<i>Anourosorex squamipes</i>	0/4 (0.00)		Luxi city (2014)
Carnivora	Mustelidae	<i>Mustela sibirica</i>	0/1 (0.00)		Luxi city (2014)
Total	4	7	63/278 (22.66)		

previously described methods (Ge et al. 2013; Wang et al. 2017b). RNA was extracted from tissue using the High Pure Viral RNA Kit (Roche, Basel, Switzerland) following the manufacturer's instructions, and cDNA was synthesized using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Promega, Madison, WI, USA). The extracted RNA was eluted, aliquoted and stored at  $-80^{\circ}\text{C}$ .

### Orthohepevirus Detection and Quantification

The extracted RNA was tested by broad-spectrum hemi-nested reverse-transcription (RT)-PCR using degenerate primers based on the conserved domain of the RNA-dependent RNA polymerase (RdRp)-encoding gene of viruses within the genus *Orthohepevirus*. PCR products comprised 291 nucleotides (nt) (Drexler et al. 2012; Wang et al. 2017b). The amplicons underwent gel purification with MinElute Gel Extraction Kit (Qiagen, Hilden; Germany) and they were sequenced with both forward and reverse primers using the 3100 Sequencer (ABI, Waltham, MA, USA) or cloned using the pGEM-T Easy vector system (Promega, Madison, WI, US) for sequencing if the direct sequencing failed.

Based on the sequences obtained in this study, we designed primers and probes that specifically targeted the novel rodent orthohepeviruses detected in China. Probes were labelled at the 5' end with 6-carboxyfluorescein (6-FAM) and at the 3' end with Black Hole Quencher 1 (BHQ1) (Supplementary Table S1). The standard curves of the template RNA were constructed as previously described (Wang et al. 2017b; Yang et al. 2017). Samples were characterized by a well-defined exponential fluorescence curve that crossed the cycle threshold (Ct) within 36 cycles. Specimens with a Ct > 36 were repeated to exclude operational faults. The viral genome copy number was calculated in each sample using the standard curves of the template RNA. Virus loads of 4 representative

orthohepevirus-positive specimens (RdHEVAc18, RdHEVAc53, RdHEVAc55, and RdHEVAc70) of different tissues (liver, heart, intestine, spleen, kidney, and lung) were measured.

### Full-Length Genomic Sequencing

The complete genomic sequences of four rodent orthohepevirus strains were amplified by PCR using degenerate and strain-specific primers (Supplementary Table S1) (Drexler et al. 2012). Genome ends were amplified by using 5'/3'-Full rapid amplification of cDNA ends (RACE) kit (Clontech, Takara Bio, USA). PCR products were gel purified with a MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced with both forward and reverse primers using a 3100 Sequencer (ABI, Waltham, MA, USA). The sequencing chromatograms were inspected carefully for overlapping multicolour peaks, which are an indicator of sequence heterogeneity. The PCR products were cloned using the pGEM-T Easy Vector System (Promega, Germany) and at least three clones for each PCR fragment were sequenced to obtain a consensus sequence.

### Sequence Analysis

Sequence analysis and genome assembly were carried out with Geneious version 10.0.5 (Biomatters Limited, Auckland, New Zealand) as previously described (Wang et al. 2017a; b). Pairwise distances of complete genome nt and concatenated ORF1/ORF2 aa sequences were analysed using SSE as recommended by ICTV *Hepeviridae* Study Group (Simmonds 2012; Smith et al. 2014). Sequence similarity comparisons were implemented using the BLASTn search engine (<https://blast.ncbi.nlm.nih.gov>). Homology to known protein domains was identified using Motif Scan (<http://myhits.isb-sib.ch>). To identify possible recombination, bootscanning analyses of full-length sequences were performed in the SimPlot software

program, version 3.5.1 with a sliding window size of 200 and a step size of 20 aa increment (Lole et al. 1999; Drexler et al. 2012). All orthohepevirus sequences reported in this study were submitted to GenBank under Accession Numbers: MG019963–MG020021 for orthohepevirus partial RdRp gene sequences and MG020022–MG020025 for orthohepevirus full-length genome sequences.

## Phylogenetic Analysis

The preliminary sequence alignment and editing were performed using MAFFT (Kato et al. 2002). Phylogenetic reconstructions were performed using MEGA version 7 with 1000 bootstrap replicates (Kumar et al. 2016). The neighbour-joining trees of p-distances among aligned partial RdRp-coding gene sequences comprised 291 nt corresponding to positions 4300–4590 within *Orthohepevirus A* reference strain (GenBank Accession No. M73218) and aligned complete genome sequences of the newly detected rodent orthohepeviruses were built, respectively. The maximum-likelihood trees were reconstructed from aa sequences for ORF1, ORF2, ORF3, and putative ORF4-encoding proteins respectively, using the Jones–Taylor–Thornton model with frequencies, and gamma distribution with invariant sites according to the previous study (Smith et al. 2014). The reference HEV strains within the family *Hepeviridae* and complete genome sequences derived from rat and ferret within the species *Orthohepevirus C* were used for phylogenetic analyses. The branches are labelled with the strain designation, the host species, and the GenBank accession number. The classification of the respective species or genotypes is indicated on the right.

## Statistical Analysis

GraphPad Prism Software version 7.03 (GraphPad Software, San Diego, CA) was used for data analysis using a Student *t* test. All results were presented as mean  $\pm$  standard errors of the means (SEM). *P* values of less than 0.05 (single asterisks in figures) were considered statistically significant; whereas *P* values less than 0.01 (double asterisks) and 0.001 (triple asterisks) were considered highly significant.

## Results

### Presence and Prevalence of Orthohepeviruses in Rodents

A total of 278 small mammals, belonging to three orders, four families, and seven species, were captured between 2014 and 2015 from two geographical locations: Lijiang

city and Luxi city in Yunnan province, China. Blood and tissue samples harvested from these animals were examined for the presence and prevalence of orthohepevirus using broad-spectrum hemi-nested RT-PCR as previously described (Drexler et al. 2012; Wang et al. 2017b). As illustrated in Table 1, 63 samples produced fragments of the expected size and were confirmed to be positive for orthohepevirus RNA by direct sequencing or clone sequencing. The orthohepevirus-positive samples originated from two species: Chevrier's field mouse (*Apodemus chevrieri*) belonging to the family Muridae from Lijiang city and Père David's vole (*Eothenomys melanogaster*) belonging to the family Cricetidae from Luxi city, with the infection rates of 29.20% (59/202) and 7.27% (4/55), respectively (Table 1). Based on 291 nt of partial RdRp-encoding gene, the sequences of the samples had nt identity of 73%–84% with *Orthohepevirus C* strains. The 59 orthohepevirus strains from Chevrier's field mouse from Lijiang city reflected remarkable diversity within the group (71%–99% nt identity). Above all, 56 strains had the highest nt identity of 74% to ferret HEV strain F63 (GenBank Accession No. LC177792) and three strains had the highest nt identity of 86% to rat HEV strain rat/Mu09/0434/DEU/2010 (GenBank Accession No. JN167538). The four orthohepevirus strains from Père David's vole from Luxi city shared nt identity of 77%–99% within the group and had the highest nt identity of 72% to kestrel HEV strain kestrel/MR16/2014/HUN (GenBank Accession No. KU670941). While other small mammal samples, including *Rattus tanezumi*, *Mus caroli*, and *Eothenomys chinensis* (order: Rodentia), *Anourosorex squamipes* (order: Soricomorpha), and *Mustela sibirica* (order: Carnivora) were negative for orthohepevirus RNA detection (Table 1).

### Complete Genome Characterization of Newly Discovered Orthohepeviruses

Since complete genome sequences are required for new orthohepevirus assignment (Smith et al. 2014, 2016), and in order to further characterize the novel orthohepevirus strains from the different wild rodent samples, we generated four full-length genome sequences from selected samples, two each from Chevrier's field mouse (designated RdHEVAc: RdHEVAc14 and RdHEVAc86) and Père David's vole (designated RdHEVEm: RdHEVEm40 and RdHEVEm67). The lengths of the whole-genome sequences were 6967 nt for RdHEVAc14; 6966 nt for RdHEVAc86; 6961 nt for RdHEVEm40; and 6964 nt for RdHEVEm67, respectively, excluding the poly(A)-tail. The G + C contents of RdHEVAc14 and RdHEVAc86 were 55.0 and 54.7%, but only 50.5 and 50.3% for RdHEVEm40 and RdHEVEm67. The genomic features and putative domains of these newly detected rodent

**Table 2** Genomic features and putative domains of novel rodent orthohepevirus strains

Novel rodent orthohepeviruses genomic region and putative domains	Start site/end site				Functional description (Cao and Meng 2012; Kamar et al. 2012; van Tong et al. 2016)
	RdHEVAc14	RdHEVAc86	RdHEVEm40	RdHEVEm67	
5'-UTR	nt 1/18	nt 1/18	nt 1/41	nt 1/41	
ORF1	nt 19/4935 aa 1/1639	nt 19/4935 aa 1/1639	nt 42/4907 aa 1/1622	nt 42/4907 aa 1/1622	Non-structural protein
Met	nt 78/1275 aa 26/425	nt 78/1275 aa 26/425	nt 72/1269 aa 26/423	nt 72/1269 aa 26/423	Catalyze the capping of viral RNA, guanine-7-methyltransferase and guanyltransferase activities
X	nt 2076/2373 aa 692/791	nt 2076/2373 aa 692/791	nt 2187/2478 aa 729/826	nt 2190/2481 aa 730/827	ADP-ribose metabolism and post-translational modifications
Hel	nt 2763/3405 aa 921/1135	nt 2763/3405 aa 921/1135	nt 2697/3345 aa 899/1115	nt 2697/3345 aa 900/1116	NTPase and RNA unwinding activities
RdRp	nt 3666/4872 aa 1222/1624	nt 3555/4872 aa 1185/1624	nt 3465/4821 aa 1155/1607	nt 3468/4824 aa 1156/1608	Replicate the genomic RNA through an anti-genomic RNA intermediate
ORF2	nt 4962/6902 aa 1/647	nt 4962/6902 aa 1/647	nt 4888/6888 aa 1/667	nt 4891/6891 aa 1/667	Capsid protein
ORF3	nt 4951/5268 aa 1/106	nt 4951/5268 aa 1/106	nt 4934/5269 aa 1/112	nt 4937/5272 aa 1/112	Multifunctional phosphoprotein that can modulate cellular signaling and is related to particle secretion
ORF4	nt 35/553 aa 1/173	nt 35/553 aa 1/173	NA	NA	Unknown
3'-UTR	nt 6903/6967	nt 6903/6966	nt 6889/6961	nt 6892/6964	

Homology to known protein domains were identified using Motif Scan (<http://myhits.isb-sib.ch>).

Nucleotide for nt; amino acid for aa; and not available for NA. Viral methyltransferase for Met; Marco domain for X; Viral RNA helicase for Hel; and RNA-dependent RNA polymerase for RdRp.

orthohepevirus strains were characterized in Table 2. A similar genomic organization including 3 ORFs of the family *Hepeviridae* was observed for these novel rodent orthohepevirus strains, 4 putative functional domains including Met, X, Hel, and RdRp could be predicted in the ORF1 (Table 2). Surprisingly, the putative ORF4 overlapping ORF1 as postulated to exist in the HEV-C1 and HEV-C2 was found at genomic position 35–553 of RdHEVAc, but was absent in RdHEVEm (Johne et al. 2010b, 2014). No putative ORF5 and ORF6 were observed for these novel strains (Johne et al. 2010b; Raj et al. 2012).

### Comparative Analysis of RdHEVAc and RdHEVEm

Complete genome nt identity between RdHEVAc14 and RdHEVAc86 was 92.3, and 89.8% between RdHEVEm40 and RdHEVEm67. However, RdHEVAc and RdHEVEm were highly divergent with only 56.4%–56.8% nt identity in the genomic sequences. Comparisons of the whole-genome nt sequences and aa sequences of deduced ORFs of newly detected rodent orthohepevirus strains with representative orthohepevirus reference strains are shown in Tables 3 and 4. All the novel rodent orthohepevirus strains

in this study had a highest nt identity to the strains within the species *Orthohepevirus*, HEV-C1 (65.5%) and HEV-C2 (62.8%) for RdHEVAc, and HEV-C1 (56.8%) and HEV-C2 (56.1%) for RdHEVEm. Similar results were obtained using the coding regions for comparison: RdHEVAc strains shared higher percentage of aa sequence similarity to HEV-C1 or HEV-C2 than other species and genotypes within the family *Hepeviridae*, HEV-C1 (ORF1, 68.0%; ORF2, 74%; ORF3, 46.2%; and ORF4, 50.8%) and HEV-C2 (ORF1, 68.6%; ORF2, 71.6%; ORF3, 36.3%; and ORF4, 54.1%). Likewise, identities of three ORFs from RdHEVAc to HEV-C1 (ORF1, 53.4%; ORF2, 62.8%; ORF3, 24.8%; and ORF4, 50.8%) to HEV-C2 (ORF1, 54.3%; ORF2, 58.8%; and ORF3, 26.3%) (Tables 3, 4). Compared to other orthohepevirus strains within the species *Orthohepevirus C*, the genomic organization of RdHEVAc and RdHEVEm also demonstrated genetic variability. As shown in Fig. 1, HEV-C1 had the two distinct translational frames for ORF1 and ORF2, and ORF3 and ORF4, respectively. However, HEV-C2 and RdHEVAc implemented three different translational frames for ORF1 and ORF3, ORF2, and ORF4, respectively. Importantly, RdHEVEm used three translational

**Table 3** Identities of complete genome and ORFs between RdHEVAC and representative orthohepevirus reference strains

<i>Hepeviridae</i>		Degree of identity (%)								
		Full genome	ORF1		ORF2		ORF3		ORF4	
			nt	nt	aa	nt	aa	nt	aa	nt
<i>Orthohepevirus A</i>	HEV-1	51.2–51.6	49.3–49.4	48.3–48.6	55.6–56.3	55.4–55.8	39.6–40.9	29.4	NA	NA
	HEV-2	50.7–51.0	48.4–49.0	47.6–48.0	55.4	55.1–55.4	39.0–40.1	26.1–27.7	NA	NA
	HEV-3	50.9–51.0	49.2–49.3	48.9–49.0	56.2–56.9	55.7–56.0	40.7–41.6	28.8	NA	NA
	HEV-4	50.4–50.6	48.5–48.8	48.9–49.2	54.7–55.1	53.8–54.2	36.8–38.4	26.1–26.9	NA	NA
	HEV-5	51.4–51.7	49.3–49.5	48.8–49.2	56.2–56.5	55.1–55.4	38.8–39.9	27.4–30.0	NA	NA
	HEV-6	51.1–51.7	49.3–49.4	49.1–49.5	56.4–57.4	54.7–55.3	38.5–39.7	27.4–30.8	NA	NA
	HEV-7	51.1–51.5	49.3	48.1–48.3	55.6–57.0	56.0	39.9–42.1	28.8	NA	NA
<i>Orthohepevirus B</i>		45.6–45.8	46.4–46.8	43.2–43.3	45.7–45.8	40.6–40.9	25.5–26.1	16.4	NA	NA
<i>Orthohepevirus C</i>	HEV-C1	64.3–65.5	61.5–63.8	66.0–68.0	67.7–69.6	71.9–74.0	60.1–62.6	41.5–46.2	70.5–72.6	47.5–54.1
	HEV-C2	62.0–62.8	61.2–62.1	67.8–68.6	64.7–65.5	70.9–71.6	54.9–56.7	35.5–36.3	69.7–70.7	49.2–50.8
	RdHEVEm	56.4–56.8	52.4–52.7	51.6–53.4	61.2–62.0	62.2–62.8	45.5–45.8	23.5–25.2	NA	NA
<i>Orthohepevirus D</i>		47.2–47.4	45.6–45.8	41.5–41.8	48.5–49.3	45.0–45.2	27.5–28.2	10.6–13.0	NA	NA
<i>Piscihepevirus A</i>		36.1–36.3	36.8	24.2–24.6	31.3–31.5	15.6–15.9	18.3–19.1	11.4–12	NA	NA

The sequences were aligned using MAFFT. The evolutionary analyses were conducted using MEGA version 7. The GenBank Accession numbers are as follows: M73218 for HEV-1; M74506 for HEV-2; AF082843 for HEV-3; AJ272108 for HEV-4; AB573435 for HEV-5; AB602441 for HEV-6; KJ496143 for HEV-7; AY535004 for *Orthohepevirus B*; AB847309, AB847305, AB847306, AB847308, AB847307, GU345043, GU345042, JN167537, and JN167538 for HEV-C1; AB890001, JN998607, and JN998606 for HEV-C2; JQ001749 for *Orthohepevirus D*; and HQ731075 for *Piscihepevirus A*. RdHEVAC14 and RdHEVAC86 for RdHEVAC; RdHEVEM40 and RdHEVEM67 for RdHEVEm. Nucleotide for nt; amino acid for aa; and not available for NA.

**Table 4** Identities of complete genome and ORFs between RdHEVEm and representative orthohepevirus reference strains

<i>Hepeviridae</i>		Degree of identity (%)						
		Full genome	ORF1		ORF2		ORF3	
			nt	nt	aa	nt	aa	nt
<i>Orthohepevirus A</i>	HEV-1	50.7–51.0	49.9–50.1	46.9–47.6	55.2–55.3	53.6	40.2–40.7	20.9–23.3
	HEV-2	50.3–50.7	50.0–50.2	47.2–48.2	54.0–54.2	52.6	38.3–38.8	19.4–20.9
	HEV-3	50.4	50.1	47.2–48.2	55.4	52.6–52.7	40.5–40.8	20.3–22.7
	HEV-4	50.0	49.9–50.0	47.2–48.1	54.5–54.9	51.2–51.4	38.8–39.4	23.3–24.0
	HEV-5	50.8–51.0	50.3	47.0–47.8	55.0	52.8–52.9	40.3–40.5	19.7–20.5
	HEV-6	50.0–50.5	49.9–50.0	47.7–48.5	54.0–54.5	53.5	41.1	20.5–22.0
	HEV-7	50.8–51.2	50.4–51.0	47.6–48.4	54.5–54.8	54.5–54.7	41.6–42.7	20.3–21.1
<i>Orthohepevirus B</i>		44.8–45.0	46.5–46.6	40.7–41.5	45.4–45.5	40.1	23.2–23.8	11.6–12.4
<i>Orthohepevirus C</i>	HEV-C1	55.9–56.8	52.4–54.1	52.4–53.5	60.5–61.8	61.1–62.8	42.3–47.9	22.3–24.8
	HEV-C2	55.1–56.1	53.5–54.2	52.8–54.3	57.8–59.7	58.4–58.8	45.7–46.3	22.0–26.3
	RdHEVAc	56.4–56.8	52.4–52.7	51.6–53.4	61.2–62.0	62.2–62.8	45.5–45.8	23.5–25.2
<i>Orthohepevirus D</i>		46.7–46.9	47.7–47.9	41.2–41.8	48.0–48.2	44.1–44.2	27.7–27.9	17.1
<i>Piscihepevirus A</i>		34.6–35.0	36.1–36.4	24.5–24.9	30.7–30.9	16.7	17.5–17.9	11.4

The sequences were aligned using MAFFT. The evolutionary analyses were conducted using MEGA version 7. The GenBank Accession numbers are as follows: M73218 for HEV-1; M74506 for HEV-2; AF082843 for HEV-3; AJ272108 for HEV-4; AB573435 for HEV-5; AB602441 for HEV-6; KJ496143 for HEV-7; AY535004 for *Orthohepevirus B*; AB847309, AB847305, AB847306, AB847308, AB847307, GU345043, GU345042, JN167537, and JN167538 for HEV-C1; AB890001, JN998607, and JN998606 for HEV-C2; JQ001749 for *Orthohepevirus D*; and HQ731075 for *Piscihepevirus A*. RdHEVAC14 and RdHEVAC86 for RdHEVAC; RdHEVEM40 and RdHEVEM67 for RdHEVEm. Nucleotide for nt; amino acid for aa; and not available for NA.

frames for ORF1, ORF2, and ORF3, which is the same as for HEV-1 to HEV-3 (Sridhar et al. 2017). Moreover, the overlaps of ORF1, ORF2, and ORF3 for HEV-C1, HEV-C2, RdHEVAc, and RdHEVEEm vary length and location (Fig. 1). In addition, the 5'-UTR region of RdHEVAc carried a 10 nt motif GCAACCCCG reported to be uniquely present in the rat HEV strains, yet RdHEVEEm lacked this motif despite having a longer 5'-UTR region (Mulyanto et al. 2014).

### Phylogenetic Relationship and Pairwise Distance Analysis of RdHEVAc and RdHEVEEm

A neighbour-joining tree of pairwise distances among alignments of partial RdRp-coding sequences showed that the detected orthohepevirus strains from the two rodent species in this study were highly diverse within the genus *Orthohepevirus* (Fig. 2A). Intriguingly, three orthohepevirus sequences derived from Chevrier's field mouse clustered into the HEV-C1 cluster. Conversely, the other 56 orthohepevirus sequences derived from the same species formed a separate group (RdHEVAc) (Fig. 2B). Meanwhile, four orthohepevirus strains derived from Pèrè David's vole formed a separate clade (RdHEVEEm) and seemed to be more divergent within the family *Hepeviridae*. Phylogenetic analysis of the complete genome sequences indicated that the RdHEVAc and RdHEVEEm from the order Rodentia formed two independent branches within the clade of the species *Orthohepevirus C*, which reflected even higher divergence from HEV-C1 (order:

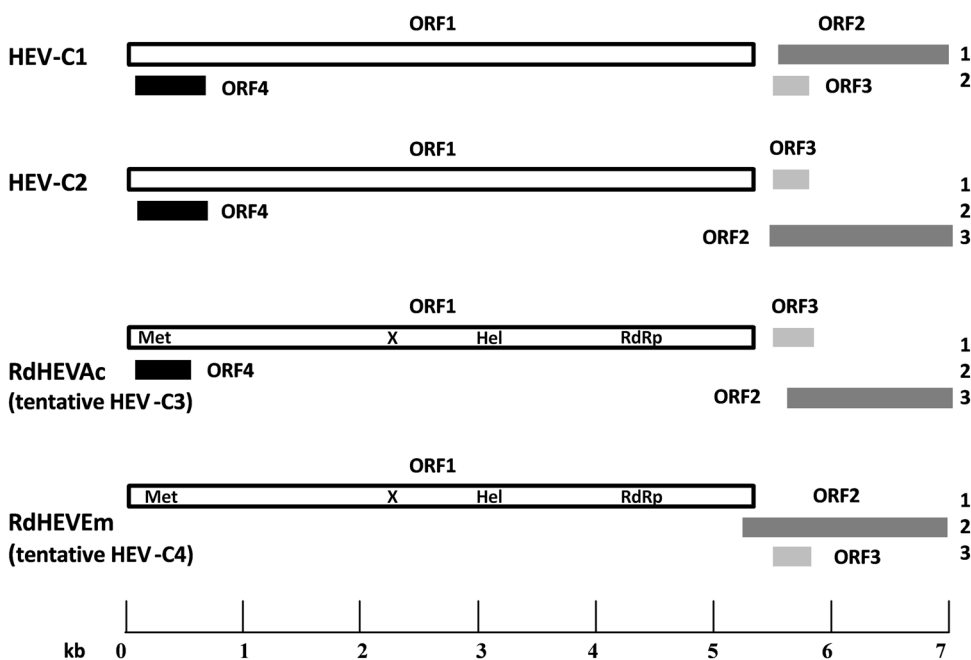
Rodentia) than HEV-C2 (order: Carnivora) (Fig. 3A). Similar phylogenetic topologies were observed when the deduced ORF1 and ORF2 were analysed separately (Fig. 3B, C). Although the phylogenetic topologies of ORF3 and ORF4 seemed to be more complex, RdHEVAc and RdHEVEEm were remarkably divergent from other assigned species or genotypes within the family *Hepeviridae* (Fig. 3D, E),

The aa identity plot from bootscan analyses with Simplot V3.5 (Lole et al. 1999) demonstrated that the novel rodent orthohepevirus strains had no evidence of recombination between ORF1- and ORF2-encoded aa sequences compared to reference strains of *Orthohepevirus A* to *Orthohepevirus D* (Supplementary Figure S1). Pairwise distances of complete genome nt and concatenated ORF1/ORF2 aa sequences were analysed using SSE (Simmonds 2012) (Supplementary Table S2). The mean pairwise distance results were indicative that HEV-C1 and HEV-C2 differed from each other by a distance of 0.311 (nt) or 0.229 (aa). However, the pairwise distances between RdHEVAc and HEV-C1 was 0.340 (nt) or 0.298 (aa), and between RdHEVAc and HEV-C2 was 0.351 (nt) or 0.286 (aa). Similarly, the pairwise distance between RdHEVEEm and HEV-C1 was 0.405 (nt) or 0.411 (aa), and between RdHEVEEm and HEV-C2 was 0.409 (nt) or 0.412 (aa). Lastly, the pairwise distance between RdHEVAc and RdHEVEEm was 0.405 (nt) or 0.416 (aa). Thus, the divergences between RdHEVAc, RdHEVEEm, HEV-C1 and HEV-C2 were comparable to or even higher than the divergence between HEV-C1 and HCV-C2.

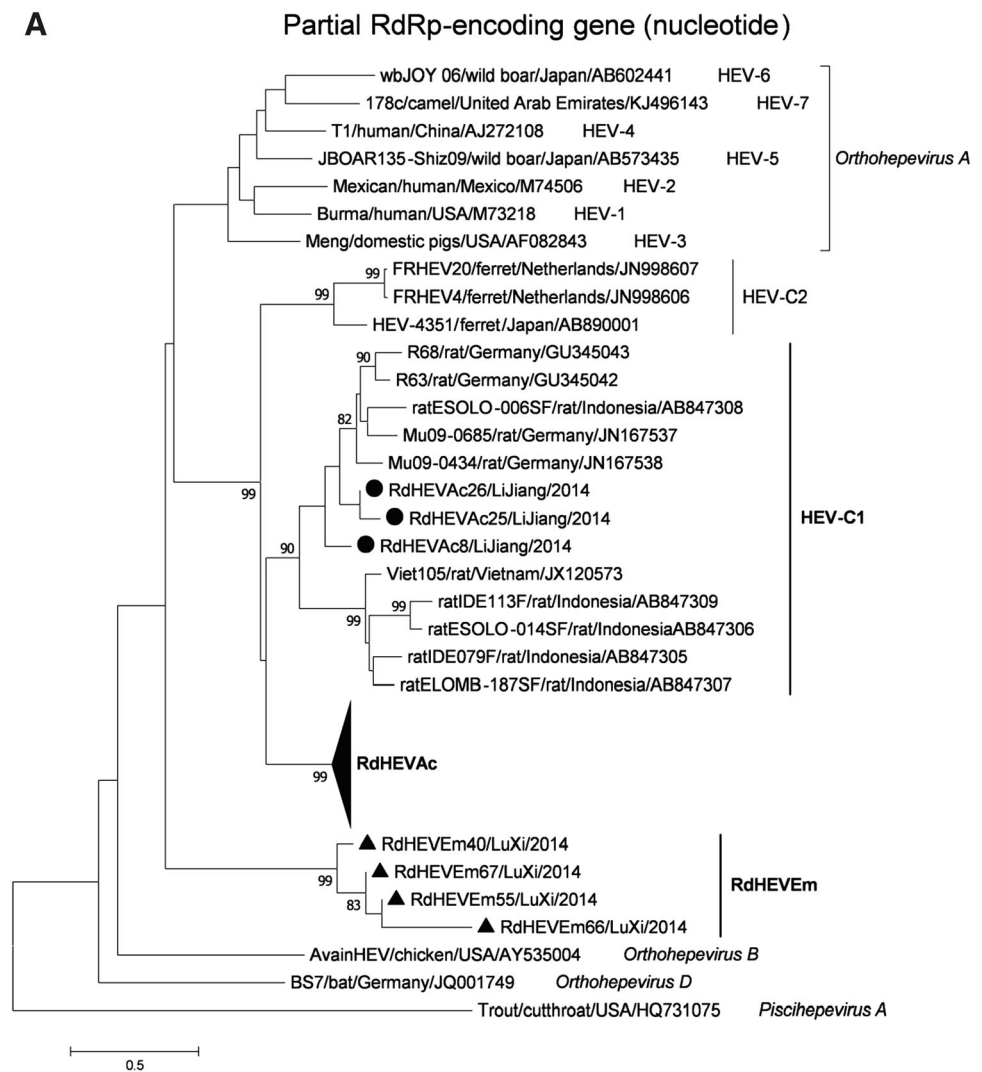
**Fig. 1** Schematic description of the species *Orthohepevirus C* genomes and viral proteins.

Different reading frames are denoted on the right, ORF1 is considered as frame 1. HEV-C1 is relative to the reference strain R63 (GenBank Accession No. GU345042); HEV-C2 is relative to the reference strain FRHEV4 (GenBank Accession No. JN998606). ORF1 encodes the non-structural polyprotein, and putative domains of RdHEVAc and RdHEVEEm including methyltransferase (Met), macrodomain (X), RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp) are indicated. ORF2 encodes the capsid protein. ORF3 encodes a small multifunctional protein. The function of particular ORF4 is still unknown (adapted from Sridhar et al. 2017).

### *Orthohepevirus C*



**Fig. 2** Phylogenetic analysis of partial RdRp gene sequences. Branches supported by > 75% of bootstrap replicates are indicated. The branches are labelled with the strain designation, the host species, location, and the GenBank accession number. The classification of the respective species or genotypes is indicated on the right. Newly detected orthohepevirus sequences in this study are highlighted in bold. **A** Partial RdRp gene alignment comprising 291 nucleotides corresponding to positions 4300–4590 within the *Orthohepevirus A* reference strain (GenBank Accession No. M73218). To allow better legibility, RdHEVAc is collapsed. **B** Expansion of RdHEVAc from **A**.



## Quantification of Novel Orthohepeviruses

Viral load detected by qPCR in different rodent tissues are presented in Fig. 4. The overall viral titres were significantly higher in liver than in other tissues (heart, intestine, spleen, kidney, and lung) ( $P < 0.01$ ). Specifically, the viral concentration for two specimens of the liver reached  $5.4 \times 10^6$  and  $1.8 \times 10^6$  genomic RNA copies per gram of tissue, respectively. In contrast, viral titres from other tissues were lower than  $4.3 \times 10^5$  copies per gram. Moreover, several samples from intestine, spleen, heart, kidney, and lung were under detection limit.

## Discussion

In this study, we discovered diverse novel orthohepevirus strains in two rodent species from Yunnan province, China, including Chevriér's field mouse (*Apodemus chevriéri*)

belonging to the family Muridae from Lijiang city and Père David's vole (*Eothenomys melanogaster*) belonging to the family Cricetidae from Luxi city, with the infection rates of 29.20% (59/202) and 7.27% (4/55), respectively. These novel orthohepeviruses shared limited nt and aa sequence identities to known orthohepevirus strains within the family *Hepeviridae*. In the previous studies, HEV-related viruses in rodents have been molecularly detected in the species *R. norvegicus*, *R. rattus*, *R. flavipectus*, *R. rattoides losea*, and *R. exulans*, which exclusively belong to the family Muridae and then formed HEV-C1 (Johne et al. 2010a, b; 2012; Lack et al. 2012; Wang et al. 2017c). Therefore, this is, to the best of our knowledge, the first report of divergent HEV-like viruses in another two rodent species: *A. chevriéri* (Family: Muridae) and *E. melanogaster* (family: Cricetidae). It is expected that there are many more orthohepeviruses circulating in other rodent species and in various geographic regions.



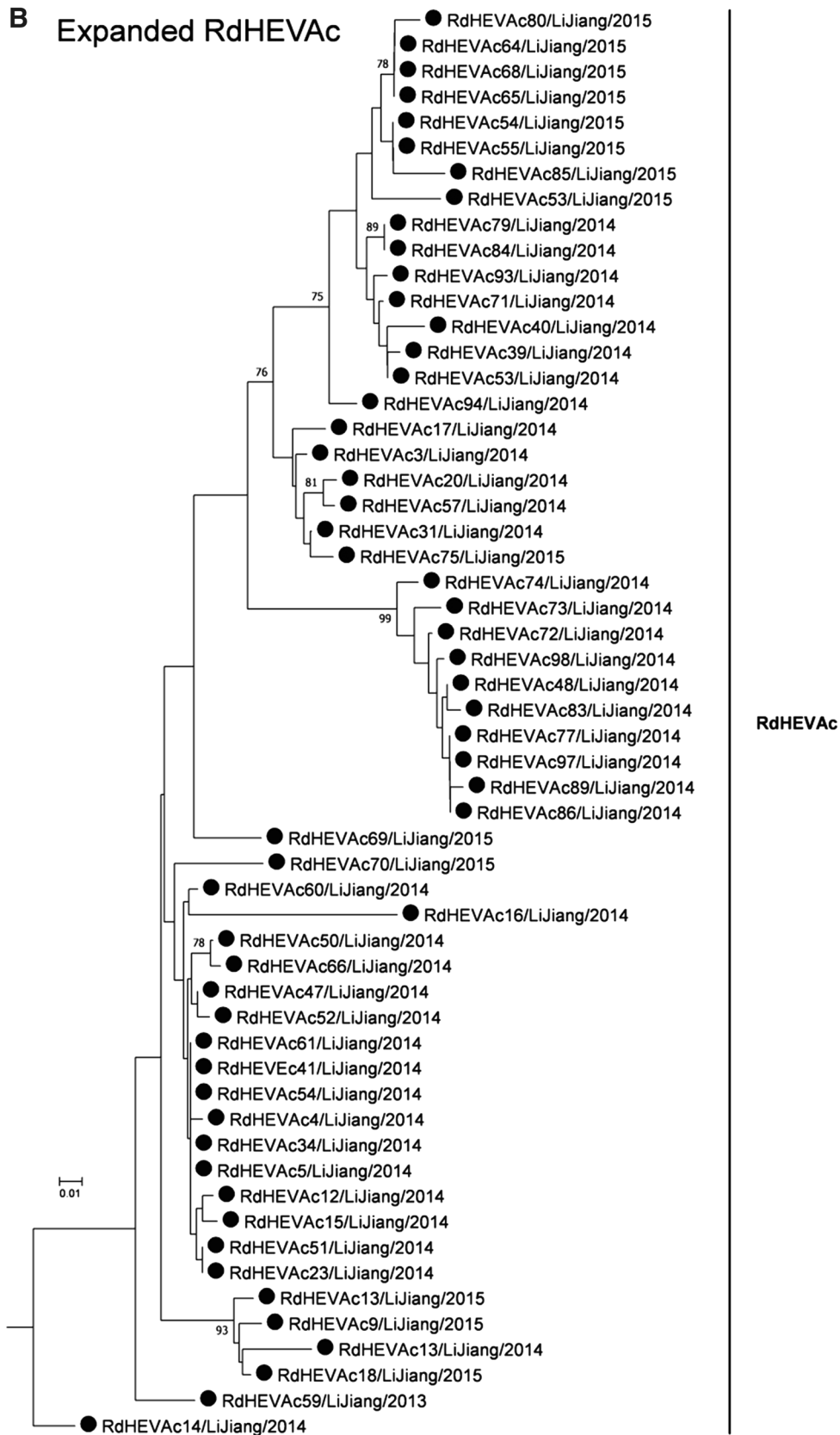
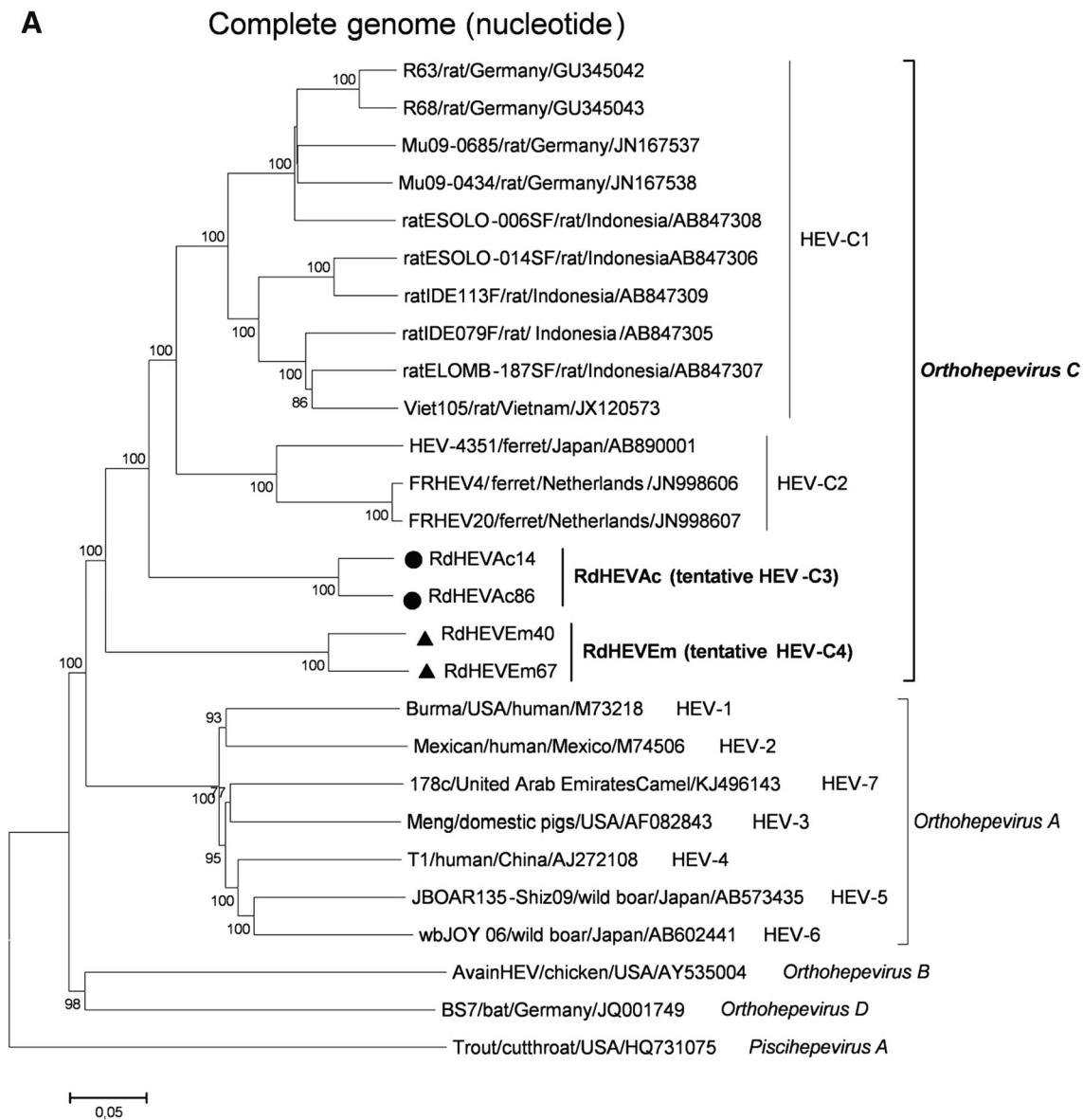


Fig. 2 continued



**Fig. 3** Phylogenetic analysis of RdHEVAc and RdHEVEm. Branches supported by > 75% of bootstrap replicates are indicated. The branches are labelled with the strain designation, the host species, location, and the GenBank accession number. The classification of the respective species or genotypes is indicated on the right. RdHEVAc

and RdHEVEm are highlighted in bold. **A** Complete genome nucleotide sequences. **B** Amino acid sequences of ORF1-encoded proteins. **C** Amino acid sequences of ORF2-encoded proteins. **D** Amino acid sequences of ORF3-encoded proteins. **E** Amino acid sequences of ORF4-encoded proteins.

Sequence and phylogenetic analysis of partial nt sequences of the RdRp-encoding gene showed that novel orthohepevirus strains in this study were highly diverse within the genus *Orthohepevirus*, 56 orthohepevirus strains from Chevrier’s field mouse and four orthohepevirus strains from Père David’s vole were classified into two separated clusters, namely RdHEVAc and RdHEVEm, notably, three orthohepevirus strains from Chevrier’s field mouse clustered into HEV-C1 clade. Whether they are

spillover infections from rat HEV strains needs to be further determined. Although rats were found to be infected with human pathogenic HEV-3 or rabbit HEV-3 in other studies, no zoonotic mammalian orthohepeviruses were detected in Chevrier’s field mouse or Père David’s vole (Kanai *et al.* 2012; Lack *et al.* 2012; Ryll *et al.* 2017). Phylogenetic analyses of complete genome nt sequences and aa sequences of deduced separate ORF1 and ORF2 reflected that RdHEVAc and RdHEVEm fell within the

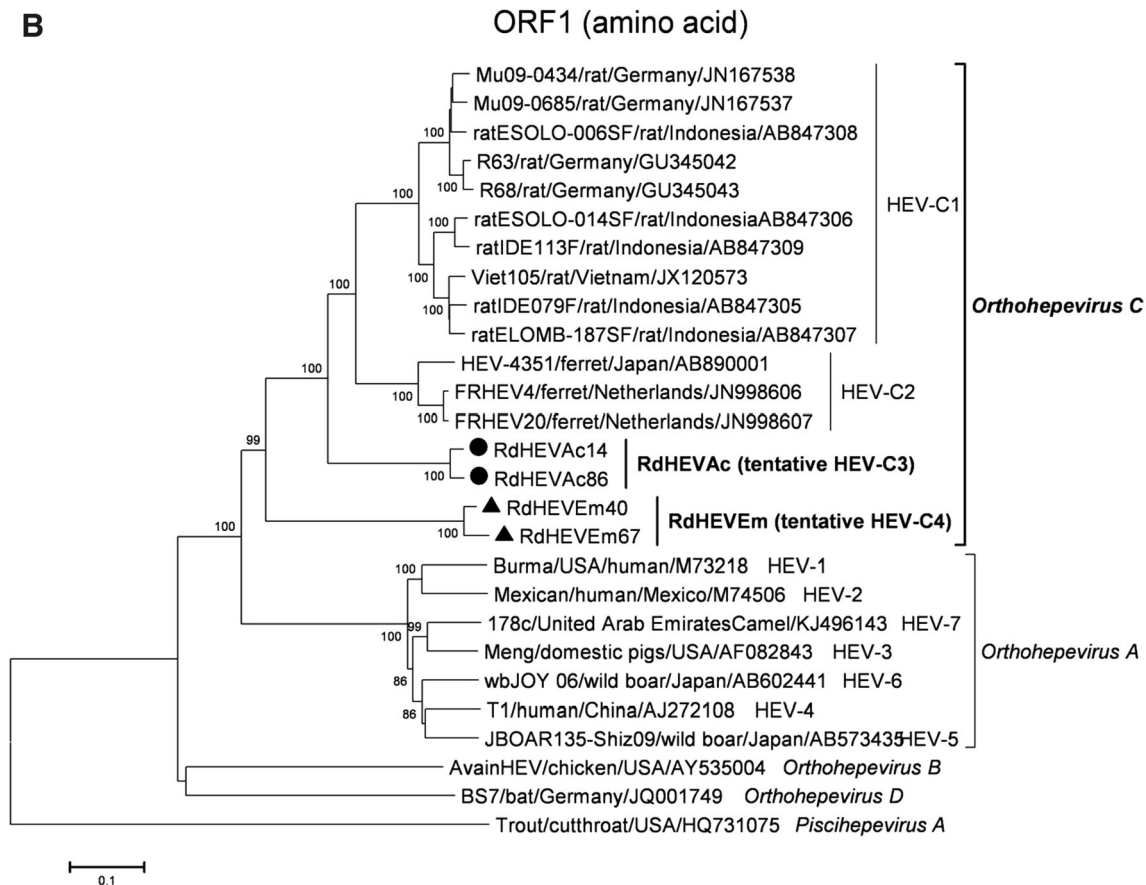


Fig. 3 continued

cluster of the species *Orthohepevirus C*. However, RdHEVAc and RdHEVEm clustered into two separate monophyletic branches among the *Orthohepevirus C* strains, which were divergent from HEV-C1 and HEV-C2. Notably, RdHEVAc and RdHEVEm from the order Rodentia reflected even higher divergence from HEV-C1 (order: Rodentia) than HEV-C2 (order: Carnivora), which supports the significance of host switches evolution while opposing the co-evolutionary theory associated of the species *Orthohepevirus C*.

Putative ORFs and functional domains were predicted to exist in RdHEVAc and RdHEVEm, however with slight differences in the length and location. Comparative analysis of RdHEVAc and RdHEVEm with other *Orthohepevirus C* strains indicate that there are some unique features: firstly, a putative ORF4 overlapped with ORF1 as postulated to exist in the species *Orthohepevirus C* was also observed for both RdHEVAc, whereas it was absent in RdHEVEm (John et al. 2010b; Raj et al. 2012); secondly, the G + C content of RdHEVAc14 and RdHEVAc86 were

55.0% and 54.7%, a little less than other rat HEV strains (56.0%–57.8%), whereas those for RdHEVEm40 and RdHEVEm67 were only 50.5% and 50.3%, comparable with lowest mammalian HEV G + C content from bat HEV strains BS7 (51.6%) and BtHEVMd2350 (50.4%) within the species *Orthohepevirus D* (Drexler et al. 2012; Wang et al. 2017b); thirdly, RdHEVAc and RdHEVEm, HEV-C1, and HEV-C2 used distinct translational frames and the overlaps of ORF1, ORF2, and ORF3 are irregular; finally, the 5'-UTR region of RdHEVAc carried a 10 nt motif GCAACCCCG reported to be uniquely present in the rat HEV strains (Mulyanto et al. 2014), yet RdHEVEm despite having a longer 5'-UTR region lacked this motif (Mulyanto et al. 2014).

As expected, novel rodent orthohepevirus strains in this study have considerable diversity compared to other known orthohepevirus strains, complete genome sequence identities range from 34.6% (cutthroat trout virus) to 65.5% (HEV-C1). Similar results were obtained using the coding regions, which indicated RdHEVAc and RdHEVEm had

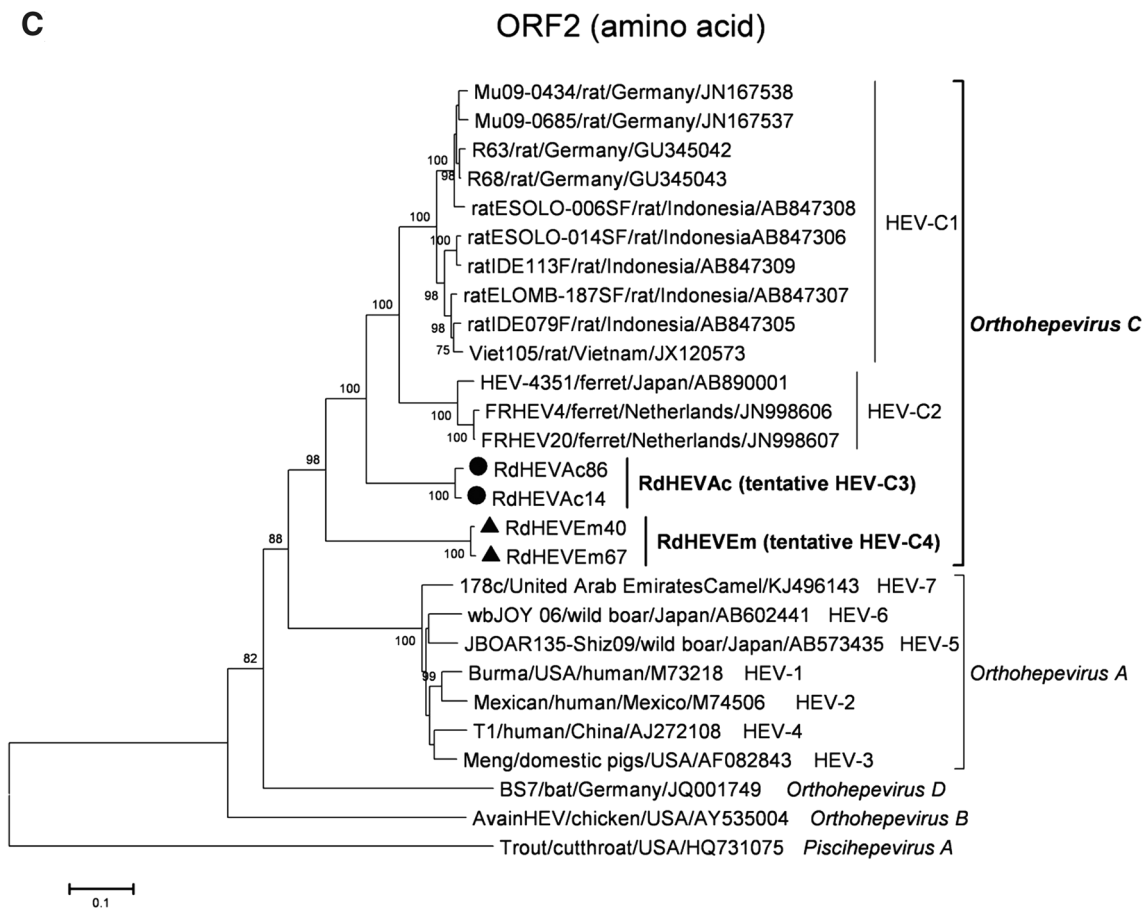


Fig. 3 continued

the highest aa sequence identity to HEV-C1 or HEV-C2. Pairwise distances of complete genome nt sequences and concatenated ORF1/ORF2 aa sequences between representative HEV prototype strains within the genus *Orthohepevirus* were calculated as recommended in recent studies (Smith et al. 2013, 2014, 2016). The divergences between RdHEVAc, RdHEVEm, HEV-C1 and HEV-C2 are comparable to or even higher than the divergence between HEV-C1 and HEV-C2. Above all, in accordance to the guidelines provided by the latest consensus proposals for classification of the family *Hepeviridae* for assigning new HEV strains, phylogeny and pairwise distance based taxonomy consistently suggested that two putative novel genotypes could be designated, preliminarily named HEV-C3 and HEV-C4, within the species *Orthohepevirus C*.

HEV causes a systemic disease affecting the liver predominantly. According to the results of in-house orthohepevirus-specific qRT-PCR, the concentration of viral genome copies in orthohepevirus RNA-positive rodent are

distinct in different tissues, which may be related to different infection stages. Importantly, virus titres in liver samples are significantly higher than other tissues, demonstrating the hepatotropism of these viruses, which is analogous to rat HEV strains (Johns et al. 2010b, 2012).

In summary, here we have described the detection of diverse orthohepeviruses in Chevrier's field mouse (*Apodemus chevrieri*) and Père David's vole (*Eothenomys melanogaster*), which expands the knowledge of orthohepevirus infection in the order Rodentia. We hypothesize that RdHEVAc and RdHEVEm may represent two putative novel genotypes within the species *Orthohepevirus C*. The present work supports data for the epidemiology and genetic diversity of orthohepevirus in Chinese wild rodents, and gives new insights into the origin, evolution, and host range of HEV. Future investigations on field samples, experimental infection trials and epidemiological studies are essential to clarify the distinct host range and zoonotic potential of these novel viruses.

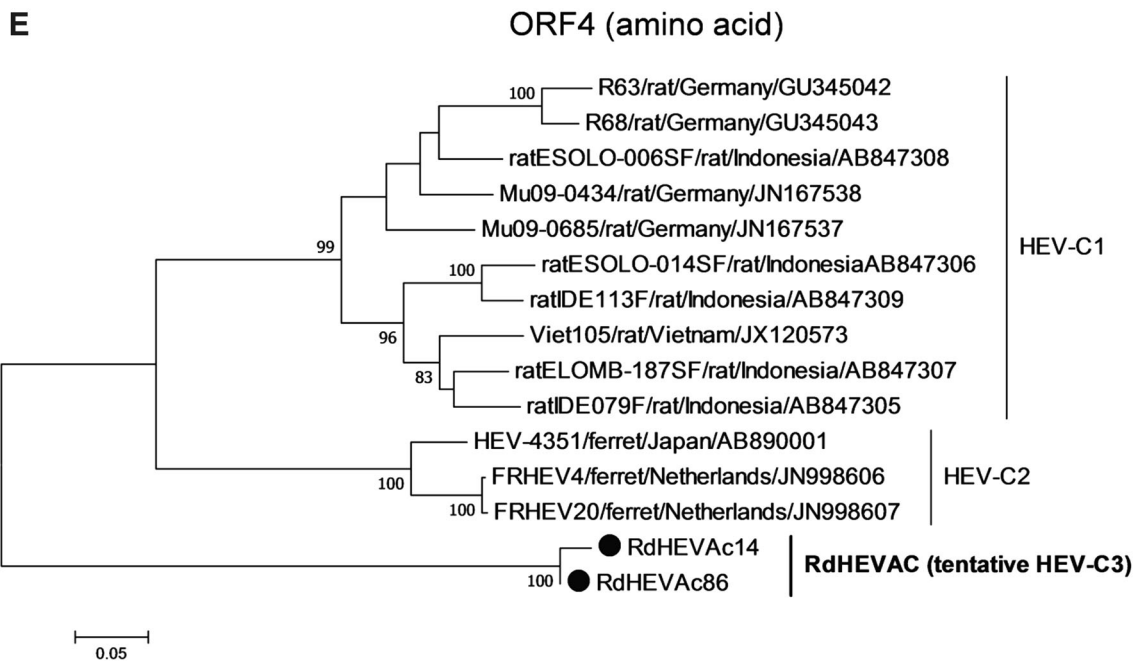
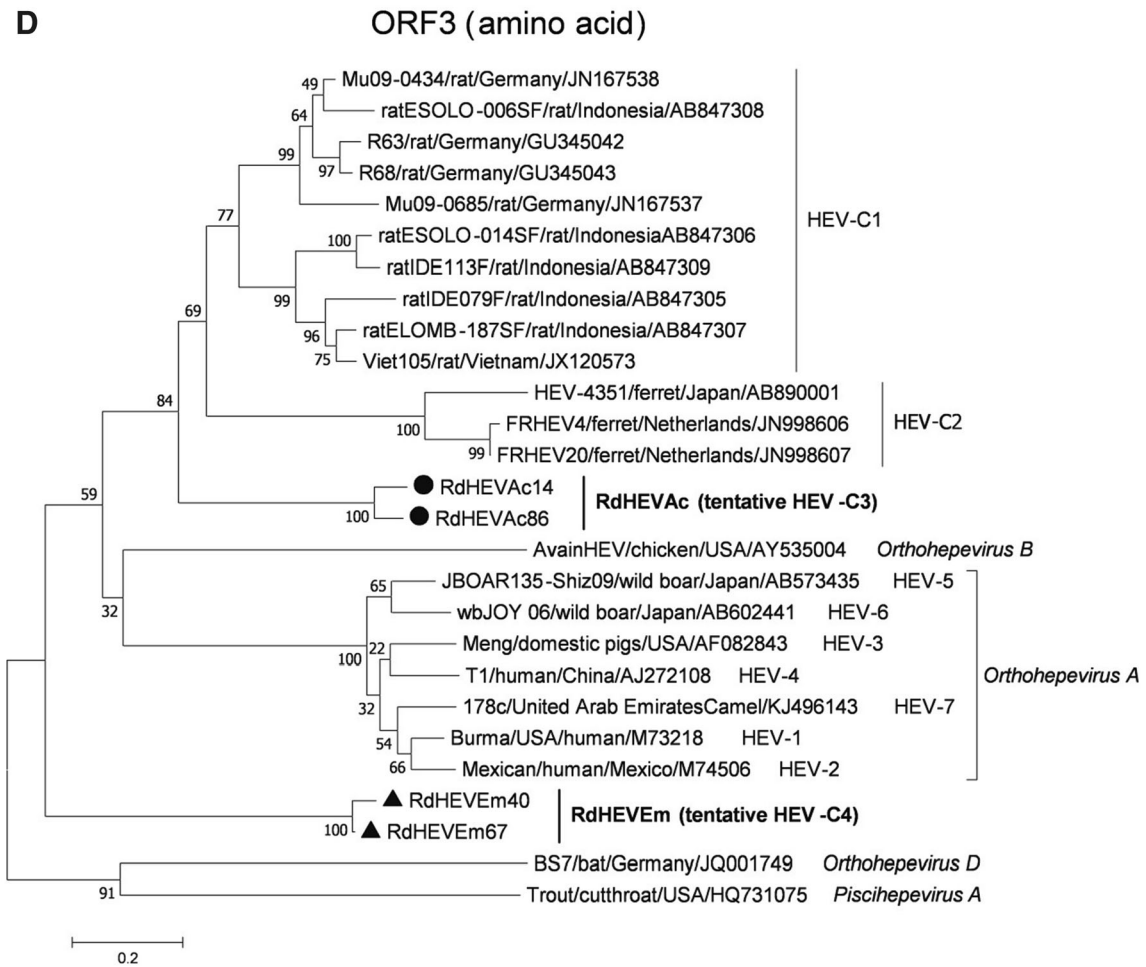
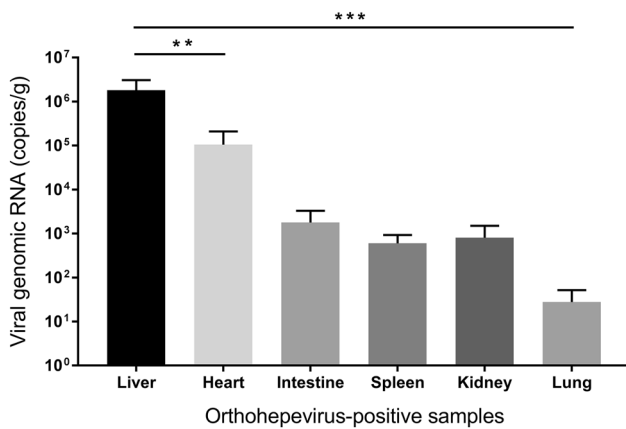


Fig. 3 continued



**Fig. 4** Quantification of genomic RNA of 4 orthohepevirus-positive specimens from liver, heart, intestine, spleen, kidney, and lung (mean  $\pm$  standard error of the mean). Units were copies per gram. Significance were tested with the Student *t* test (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

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**Author Contributions** YZZ and XLY designed and coordinated the study. YZZ, JHZ, WHY, HP and LXW collected samples. XLY, BW, LW, BL and WZ performed molecular studies. BW and XLY analysed the data. XLY, BW, ZLS, YZZ and CTB drafted the manuscript. All authors read and approved the final manuscript.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Animal and Human Rights Statement** The study was approved by the Ethics Committee of Yunnan Institute of Endemic Diseases Control and Prevention. Sampling procedures were performed following the guidelines and protocols for the Laboratory Animal Use and Care from the Chinese Centres for Disease Control and the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China.

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