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Molecular detection and genomic characterization of Samak Micromys paramyxovirus-1 and -2 in *Micromys minutus*, Republic of Korea

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Abstract

Background The discovery of viruses in small mammalian populations, particularly rodents, has expanded the family *Paramyxoviridae*. The overlap in habitats between rodents and humans increases the risk of zoonotic events, underscoring the importance of active surveillance. Rodent species, such as *Apodemus agrarius*, are natural hosts for *Paramyxoviridae* in the Republic of Korea (ROK). However, it is unknown whether *Paramyxoviridae* is present in *Micromys minutus*, another common rodent.

Method Here, we screened *M. minutus* collected from the Gangwon Province in the ROK for paramyxoviruses using nested polymerase chain reaction and confirm positive samples by next-generation metagenomic sequencing. Complete paramyxovirus genomes were further characterized by phylogenetic analysis, amino acid similarity, secondary structure, and cophylogeny.

Result Overall, 57 of 145 (39.3%) *M. minutus* kidney samples tested positive for paramyxoviruses. Among them, four whole genome sequences were identified and clustered within the genus *Jeilongvirus*. One sequence was determined as Samak Micromys paramyxovirus 1 (SMPV-1; 19,911 nucleotides long) and three sequences as Samak Micromys paramyxovirus 2 (SMPV-2; 18,199 nucleotides long). SMPV-1 has a smaller hydrophobic gene and a longer glycoprotein gene than SMPV-2. Cophylogenetic analysis suggests that SMPV-1 evolved through co-divergence, whereas SMPV-2 was inferred to have undergone transfer events.

Conclusion These findings highlight the prevalence of paramyxoviruses in the wild and the potential of *M. minutus* as a natural viral reservoir. The discovery of SMPV-1 and SMPV − 2 also reveals the genetic diversity and evolutionary history of the genus *Jeilongvirus* in the *Paramyxoviridae*.

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Introduction

Paramyxoviruses pose a critical public health threat as emerging infectious diseases (EIDs) [1]. Paramyxoviruses can infect diverse hosts, including large mammals (primate, cattle, canine, and feline), small mammals (bats, shrews, and rodents) and marine species [2]. Hendra and Nipah viruses, which are members of the family *Paramyxoviridae*, have caused outbreaks through spillover events in horses and pigs [3]. Recent studies have reported novel paramyxoviruses of the *Henipavirus* and *Jeilongvirus* genera isolated from small mammals in the Republic of Korea (ROK) [4, 5]. A shrew-borne *Henipavirus*, Langya virus, was reported as a novel etiological agent of respiratory infectious diseases from clinical samples [6]. Investigation of novel paramyxoviruses as potential EIDs is necessary because of the prevalence of zoonotic events within this virus family [7].

Paramyxoviridae are enveloped, negative-sense, nonsegmented RNA genomes classified into nine subfamilies, encompassing 23 genera and 153 species [8]. The virus genomes range from 14,296 to 20,148 nucleotides (nt) and encode six to ten proteins including a nucleoprotein (N), polymerase-related/nonstructural V/C proteins (P/V/C), a matrix protein (M), a fusion protein (F), a receptor binding protein (RBP), and a polymerase protein (large protein L). The receptor glycoprotein genes differ between genera according to the protein function [9].

The *Respirovirus* and *Ferlavirus* genera have a hemagglutinin-neuraminidase protein (HN), the *Henipaviruses* have a glycoprotein (G), and the *Morbilliviruses* have a hemagglutinin (H) [10]. Additional structural proteins, including the small hydrophobic protein (SH) and the transmembrane protein (TM), are limited to certain genera of paramyxoviruses. The *SH* gene is found in *Rubulavirus*, *Pneumovirus*, *Metapneumovirus*, and several members of *Jeilongvirus* whereas the *TM* gene is found only in *Jeilongvirus* [11].

Jeilongvirus is a recently established genus belonging to the *Orthoparamyxovirinae* subfamily [12, 13]. *Jeilongvirus* members have been found in rodents, hedgehogs, bats, and cats [9]. In addition to the *TM* gene, *Jeilongvirus* is distinct from other Paramyxoviruses because of the large size of its G protein and high variance in its C-terminal region [14]. The *Jeilongvirus* includes J virus from *Mus musculus* in Australia, Belerina virus from *Erinaceus europaeus* in Belgium, Ruloma virus from *Lophuromys machangui* in Tanzania, and Paju Apodemus paramyxovirus 1 and 2 (PAPV-1 and −2) from *Apodemus agrarius* in the ROK [5, 11, 15, 16]. Recently, China reported two additional novel *Jeilongvirus* from *Rattus tanezumi* and *A. agrarius* [17]. Another observation on J virus, a *Jeilongvirus* detected in 1972, reported its ability to infect three different species of small wild mammals, specifically *A. agrarius*, *A. peninsulae*, and *Microtus fortis* [18].

In this study, we asked whether Paramyxoviruses were harbored by *Micromys minutus* in the ROK. *M. minutus* is known for its nest-building ability on the stems of wheat or other plants and widely distributed in temperate regions of Asia and Europe [19]. *M. minutus* has been reported as a reservoir of medically relevant pathogens such as *Campylobacter jejuni* [20]. However, the natural virus diversity of *M. minutus* remains unexplored. Wild small mammals are subjected to virus surveillance because of their significance as natural hosts for zoonotic viruses [21]. The increasing size of agriculture in the ROK raises the risk of viral spillover due to more frequent contact between farmers and small wild mammals [22, 23]. Screening and exploration of RNA viruses targeted within specific mammal species could maximize zoonotic virus discovery and aid in preparedness for future pandemics [24].

Materials and methods

Ethics statement

Animal trapping and all animal procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Hallym University (Hallym2016-37, Hallym 2018-6).

Animal trapping and organ sampling

M. minutus was captured from perilla fields in Chuncheon and Hongcheon, Gangwon Province, ROK (Supplementary Fig. 1). The small mammals were sacrificed under carbon dioxide. Individuals were identified using a field guide, and morphometric measurements were taken. A total of 145 rodents were necropsied and various tissues (serum, brain, lung, spleen, kidney, and liver) were collected aseptically. All samples were stored at -80 °C until further analysis.

Genetic identification of hosts by mitochondrial DNA (mtDNA) analysis

M. minutus species were identified based on field morphological characteristics and confirmed by sequencing of the mitochondrial cytochrome *b* (mtDNA cyt *b*) gene. Total DNA was extracted from kidney tissues using the TRIzol™ reagent. The full-length (1,140 nt) mtDNA cyt *b* was amplified as described in our previous study, and the amplicon was sequenced using the Sanger method [4]. The collected sequences were aligned using MUSCLE, and the results were analyzed using the IQ-TREE web server [25].

Molecular screening of paramyxovirus from *M. minutus* samples

Total RNA was extracted from *M. minutus* kidney tissues using the TRIzol™ reagent (Invitrogen, USA) according to the manufacturer's instructions. Using random

hexamers, cDNA was synthesized using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Paramyxovirus screening was performed using nested PCR reaction. The primers targeted the RNA-dependent RNA polymerase region of paramyxoviruses [26]. First and second PCRs were performed in a 25-µL reaction mixture containing 2.5 U of Supertherm DNA polymerase (JMR Holdings, Kent, UK), 2 µg of cDNA, 10 pM of each primer. Oligonucleotide primer sequences for the PCR were PAR-F1 (outer): 5′-GAA GGI TAT TGT CAI AAR NTN TGG AC-3′, PAR-F2 (inner): 5′-GTT GCT TCA ATG GTT CAR GGN GAY AA-3, PAR-R (outer and inner): 5′-GCT GAA GTT ACI GGI TCI CCD ATR TTN C-3′. Initial denaturation was performed at 95℃ for 5 min, followed by six cycles of denaturation at 94℃ for 40 s, annealing at 37℃ for 40 s and elongation at 72℃ for 1 min, then thirty-two cycles of denaturation at 94℃ for 40 s, annealing at 42℃ for 40 s and elongation at 72℃ for 1 min (ProFlex PCR System, Life Technology, CA, USA).

PCR products were purified by MinElute® PCR purification kit (Qiagen, Hilden, Germany), and sequencing was performed in both directions of each PCR product using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, USA) on an automated sequencer (ABI 3730XL DNA Analyzer, Applied Biosystems). The forward and reverse sequences were assembled and manually checked using SeqMan of the DNAStar Lasergene (DNAStar Inc.). Sequence consensus was identified using the nucleotide-basic Local Alignment Search Tool (BLASTn) in the National Center for Biotechnology Information (NCBI) RefSeq database [27].

Metagenomic sequencing and *de novo* assembly for novel paramyxovirus identification

Libraries were prepared from total RNA using previously described protocols [5]. High-throughput sequencing was performed as paired-end 100 sequencing using HiSeq X10 (Illumina, USA). In this metagenomic approach, the reads were trimmed using Trimmomatic (v.0.36) to remove adapter sequences [28]. Additional host-sequence removal was done using Bowtie (v.2.2.6) [29]. The complete genome sequence of the species from the NCBI RefSeq database was used as the host reference [30]. The remaining reads were filtered for quality using FaQCs (v0.11.5), and de novo assembly was conducted using SPAdes (v3.15.5) [31, 32]. The assembled contigs were subsequently analyzed in the NCBI RefSeq database consisting of complete viral genomes by BLASTn (v2.6.0) [27, 30]. The contigs matched with paramyxovirus species were extracted and evaluated for coding sequences. For the reference mapping strategy, raw reads were trimmed, filtered, and mapped using CLC Genomics Workbench v.24.0.1 (CLC Bio, Cambridge, MA, USA). The *Paramyxoviridae* species reference was used as the mapping framework. Consensus genomic sequences of the novel virus were determined by combining viral contigs extracted from de novo assembly and reference mapping analysis, with an average depth cutoff of 10-fold.

Genomic characterization and analysis for N-glycosylation potential at glycoprotein gene

Viral genomic sequences were annotated using Geneious Prime (v. 2023.2.1). The open reading frames (ORF) extracted from the whole genome were translated and submitted to BLASTP to check for protein sequence homology. The complete genome of the virus species identified through BLASTP was subsequently collected to serve as the reference backbone for phylogenetic analysis.

For protein similarity analysis, the translated ORF of the novel virus was aligned with the coding sequence (CDS) translation of the curated viral sequence using CLUSTAL Omega 1.2.2, and the similarity percentage was evaluated based on the BLOSUM62 matrix [33, 34]. The translated glycoprotein gene of the novel paramyxovirus sequence was then analyzed using NetNGlyc-1.0, to predict the N-glycosylation sites [35]. The secondary structure prediction was performed using web-based Network Protein Sequence Analysis (NPSA), with Multivariate Linear Regression Combination (MLRC), Discrimination of Secondary Structure Class (DSC), and Profile Network from HeiDelberg (PHD) chosen as the default tools [36].

Phylogenetic and cophylogenetic analysis

Whole genome sequences were aligned and trimmed using MUSCLE 5.1 [37]. Phylogenetic trees were generated using maximum likelihood methods according to the best-fit substitution model in the IQ-TREE web server, and the tree was visualized using FigTree (v.1.4.4.; https://github.com/rambaut/figtree) software. The topologies were supported by 1,000 bootstrapping iterations.

Cophylogenetic analysis was performed using the systematic cophylogeny reconciliation tool called eMPRess [38]. This software reconciles symbiont and host trees using the duplication transfer-loss model with the assumption that a smaller cost increases the likelihood of the event [39]. The input was the Maximum Likelihood phylogenetic trees of cytochrome *b* from the host mitochondria and the *L* gene of the virus. The analysis was conducted using the following eMPRess parameters: duplication cost = 1, transfer cost = 1, and loss cost = 1. The original cost of reconciliation was lower than expected by chance ($p < 0.01$).

Results

Epidemiological survey of *M. minutus* and molecular prevalence of paramyxovirus

A total of 145 rodents were collected from two cities in Gangwon province (Hongcheon and Chuncheon) from March to April between 2016 and 2020. The rodents were categorized by year, trapping site, weight, and sex. Body weight was used to estimate rodent maturity. The collected animals comprised 81 males and 64 females. The animals were categorized as <3 g for babies, 3–6 g for adolescents, 6–9 g for adults, and 9 g or more for the late-adults (Table 1). *M. minutus* was identified through mitochondrial *Cytb* gene sequencing (Supplementary Fig. 2). Phylogenetic analysis revealed that the animals in the present study shared a common ancestor with *M. minutus* from European countries.

A total of 57 (39.3%) *M. minutus* isolates tested positive for paramyxovirus using pan-paramyxovirus primers. The positivity rate was highest in late-adult animals, followed by adolescent and adult animals. In terms of sex, the positive result was higher in males than in females *M. minutus* in Gangwon Province, ROK (Table 1).

Metagenomic sequencing and genomic characterization of Samak Micromys paramyxovirus from *M. Minutus*

The de novo assembly of raw reads from samples Mm16-29, Mm16-32, Mm17-42, and Mm19-42 constructed long contigs of more than 18,000 base pairs. These contigs matched *Jeilongvirus* species with a percent identity below 85% and contained ORFs that matched the CDS of *Jeilongvirus*. After evaluating the coverage to generate consensus and re-mapping the raw reads to validate the sequence, we identified four complete novel paramyxovirus genome sequences from the *M. minutus* samples. The whole genome obtained from sample Mm17-42 had distinct characteristics compared to those obtained from samples Mm16-29, Mm16-32, and Mm19-42.

The length of the genome from Mm17-42, designated as Samak *Micromys* paramyxovirus (SMPV) 1, was 19,911 nt with a GC content of 40.8%. The genome structure of SMPV-1 consisted of eight genes in the order 3′-N-P-M-F-SH-TM-G-L-5′ (Fig. 1). The whole genomes identified from Mm16-29, Mm16-32, and Mm19-42 shared similar genomic characteristics and were referred to as SMPV-2. The full-length genome of SMPV-2 was 18,199 nt with a GC content of 40.18% in Mm16-29, 40.15% in Mm16-32, and 40.19% in Mm19-42. The genome structure of SMPV-2 consisted of seven genes in the order 3′-N-P-M-F-TM-G-L-5′ (Fig. 1). Each gene and their translation shared similarities with genes from other *Paramyxoviridae*(Fig. 2, Supplementary Tables 1 to 3). SMPV-1 and SMPV-2 had attachment glycoproteins of different sizes, although both infect the same host species. We attempted to observe their host immune-evasion potential through the proportion of amino acids and the glycosylation potential of the glycoprotein domain. Threonine, proline, and serine were the most abundant amino acids within the G domain (Supplementary Table 4). The glycoprotein of SMPV-1 contained 11.6% threonine, 9.6% serine, and 6.7% proline, whereas SMPV-2 consisted of 9.3% threonine, 8.8% proline, and 8.0% serine. N-glycosylation analysis of glycoprotein translation revealed seven potential sites on SMPV-1 and five on SMPV-2 (Supplementary Fig. 3). SMPV-1 exhibited recurring secondary structure patterns and potential glycosylation sites with PAPV-1 and Ninomys virus, whereas SMPV-2 did with PAPV-2 and Mount Mabu Lophuromys virus 1 (MMLPV-1). The start, stop, and intergenic region sequences of SMPV-1 and −2 are listed in Table 2.

Phylogenetic and cophylogenetic analysis of SMPV

The complete and partial L genome sequences of these novel paramyxoviruses clustered with viruses of the *Jeilongvirus* genus and formed two distinct genetic lineages, SMPV-1 and SMPV-2 (Fig. 3 and Supplementary Fig. 4). The SMPV-1 cluster shared a common ancestor with Ninomys virus and Longquan Niviventer fulvescens jeilongvirus 2. The SMPV-2 cluster was closer to MMLPV-1 and PAPV-2. The SMPV-1 and −2 clusters consisted of thirty-seven and twenty partial L-genome sequences, respectively. Cophylogenetic analysis resulted in eight co-divergence and nine transfer events (Fig. 4). According to the Maximum Parsimony Reconciliation (MPR) analysis, SMPV-1 and Ninomys viruses acquired

Table 1 The epidemiological characteristics of Paramyxovirus infection in *Micromys minutus*

Year	City	Total samples	Total RNA Positivity (%)[#]	RNA Positivity by weight (%)[#]				RNA Positivity by gender (%)[#]	
				< 3 g	3–6 g	6–9 g	9 g <	Male	Female
2016	Chuncheon	33	6/33 (18.2)	0/0	4/21 (19.0)	2/12 (16.7)	0/0	4/18 (22.2)	2/15 (13.3)
2017	Chuncheon	22	10/22 (45.5)	0/0	3/5 (60.0)	3/13 (23.0)	4/4 (100)	7/14 (50.0)	3/8 (37.5)
	Hongcheon	7	0/7 (0)	0/0	0/3 (0)	0/4 (0)	0/0	0/3 (0)	0/4 (0)
2019	Chuncheon	35	19/35 (54.3)	0/0	8/20 (40.0)	9/12 (75.0)	2/3 (66.7)	13/20 (65.0)	6/15 (40.0)
2020	Chuncheon	48	22/48 (45.8)	0/2 (0)	11/20 (55.0)	10/24 (41.7)	1/2 (50.0)	16/26 (61.5)	6/22 (27.3)
Total		**145**	**57/145 (39.3)**	**0/2 (0)**	**26/69 (37.7)**	**24/65 (36.9)**	**7/9 (77.8)**	**40/81 (49.4)**	**17/64 (26.6)**

[#]The positive rate of SMPV-1 and SMPV-2 RNA indicated by the detection of the partial L gene (targeting pan-*Orthoparamyxovirinae*) using RT-PCR and Sanger sequencing

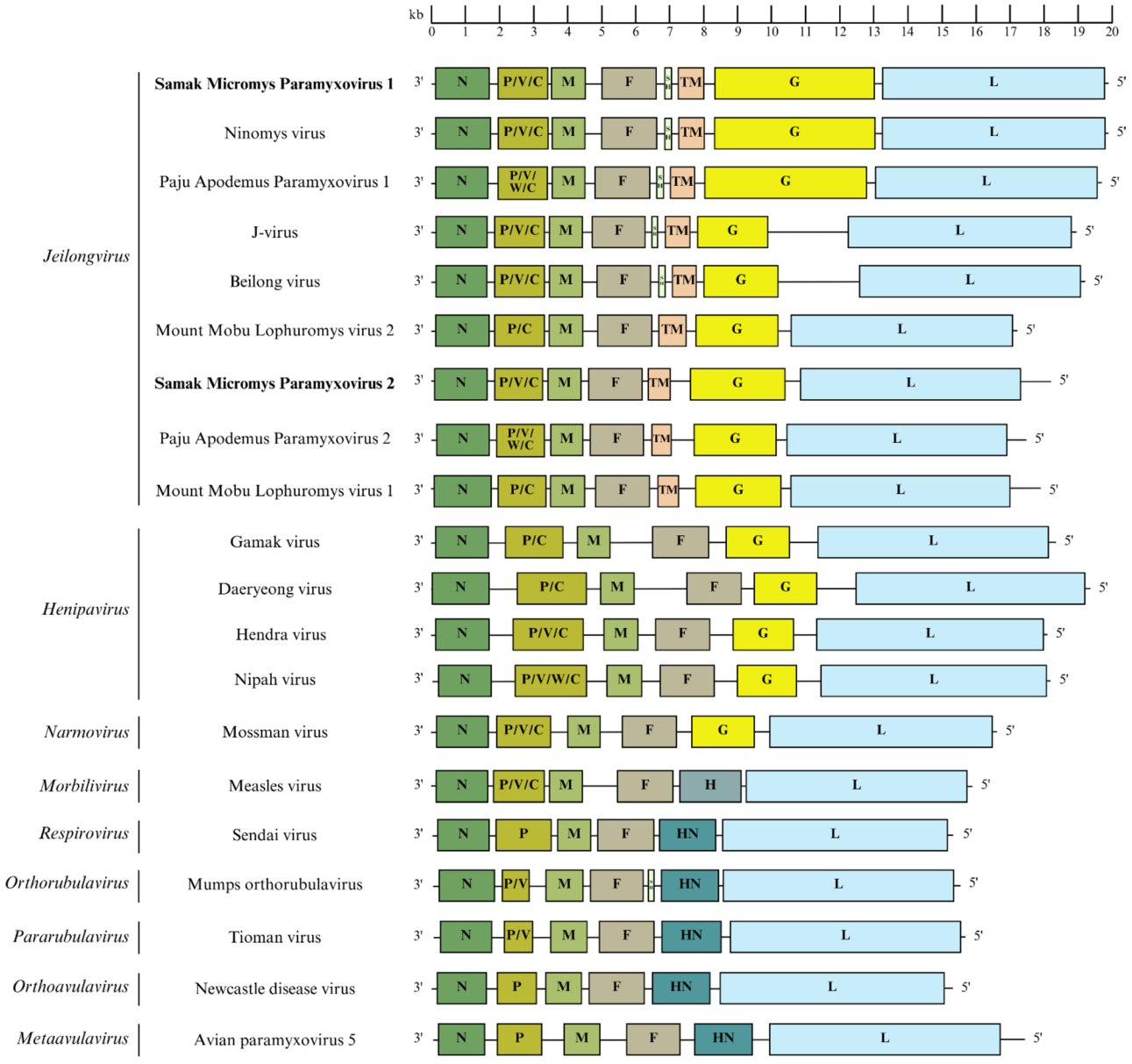

Fig. 1 Comparative genome organization of Samak Micromys paramyxovirus 1 and 2 with other Paramyxoviruses. The genome organization of Samak Micromys parayxovirus 1 and 2 (SMPV-1 and −2) compared with *Jeilongvirus* members and different genera within *Paramyxoviridae* family. SMPV-1 had longer whole genome sequence and G protein domain compared to SMPV-2. Abbreviations: N, nucleocapsid protein; P, phosphoprotein; M, matrix protein; F, fusion protein; SH, small hydrophobic protein; TM, transmembrane protein; G, glycoprotein; H, hemagglutinin protein; HN, hemagglutinin-neuraminidase protein; L, large protein

their hosts through co-divergence, while SMPV-2 acquired its host through a transfer event. The co-divergence event was detected between Ninomys virus and SMPV-1, harbored by *M. minutus* from Belgium and Korea, with 100% probability of occurrence. When we traced back to the nearest common ancestor, a transfer event from PAPV-1, harbored by *A. agrarius*, was followed by a co-divergence event between viruses harbored by *Rattus* sp. (Beilong virus and Tailam virus) and *Micromys* sp. (Ninomys virus and SMPV-1). Another transfer event (with a probability of occurrence 50%) was detected from MMLPV-1, harbored by *Lophuromys machangui*, which became SMPV-2 in *M. minutus*. SMPV-2 is also indicated to have undergone a transfer event, resulting in PAPV-2, harbored by *A. agrarius* (50% probability of occurrence).

Discussion

The virus surveillance focusing on small mammals continuously discovers novel paramyxoviruses [7]. Our study identifies two novel viruses within the *M. minutus* population in the Korean Peninsula. We obtained four

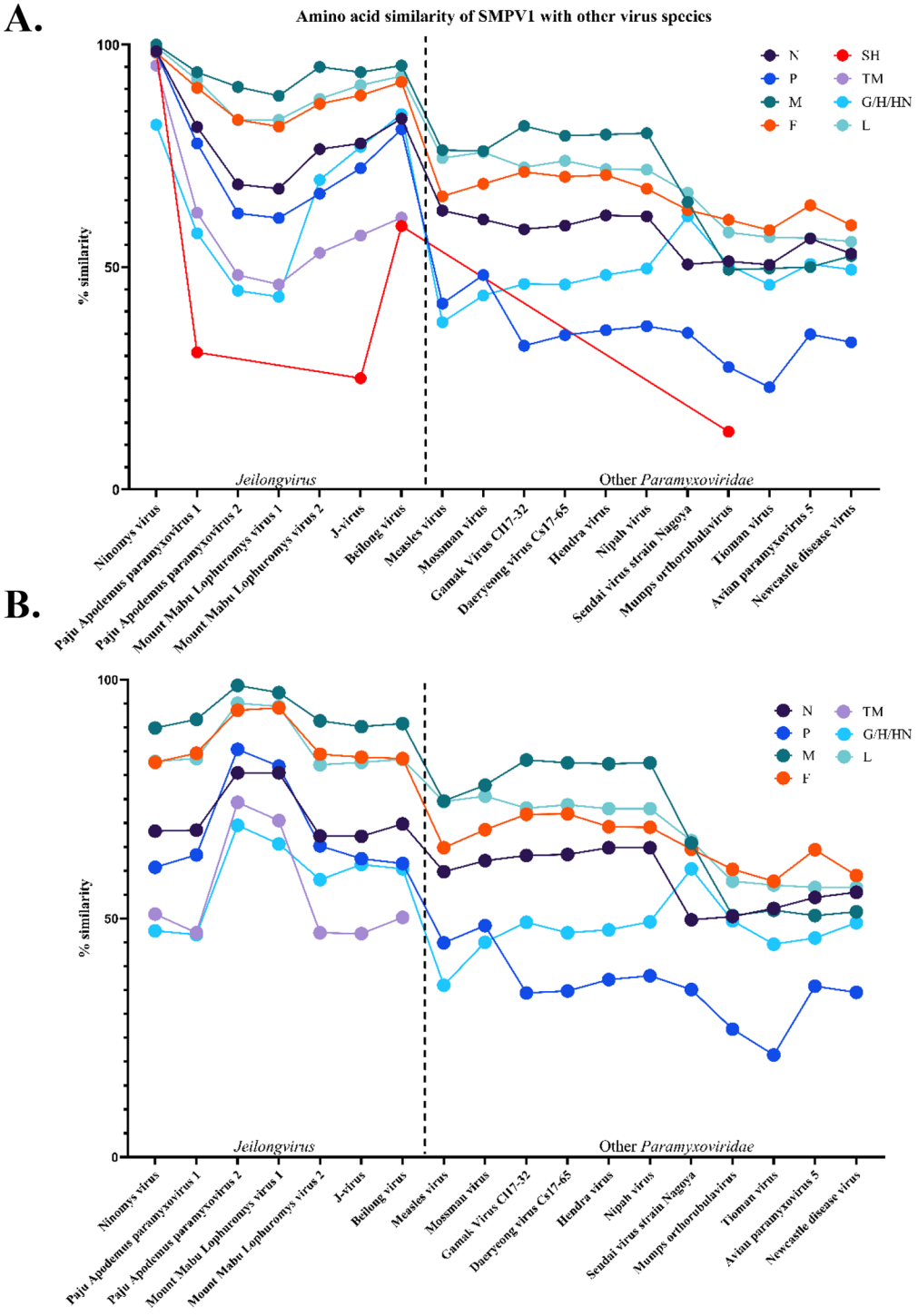

Fig. 2 Amino acid similarity of Samak Micromys paramyxovirus 1 and 2 with other species of *Paramyxoviridae*. The amino acid similarity percentage of each gene was based on BLOSUM62 matrix after multiple sequence alignment. Each gene was presented with a different color according to the legend. (**A**) Amino acid similarity percentage with SMPV-1 as the query. Ninomys virus showed high similarity with SMPV-1 (**B**) Amino acid similarity percentage with SMPV-2 as the query. PAPV-2 and MMLV-1 displayed high similarity with SMPV-2. Abbreviations: N, nucleocapsid protein; P, phosphoprotein; M, matrix protein; F, fusion protein; SH, small hydrophobic protein; TM, transmembrane protein; G, glycoprotein; H, hemagglutinin protein; HN, hemagglutinin-neuraminidase protein; L, large protein; SMPV-1: Samak Micromys paramyxovirus 1; SMPV-2: Samak Micromys paramyxovirus 1; PAPV-2: Paju Apodemus paramyxovirus 2; MMLV-1: Mount Mabu Lophuromys virus 1

Table 2 Sequence of intergenic regions (IGR) and transcriptional start and stop signals of Samak Micromys paramyxovirus 1 and 2

	Genes	Gene stop	IGR	Gene Start
SMPV-1	/N	-	CTT	AGGAGCAAAG
	N/P	TTAAGAAAAA	CTT	AGGAACAAGG
	P/M	TTATAAAAAA	CTT	AGGAGTAAGG
	M/F	TTAAGAAAAA	CTT	AGGCACAAAG
	F/SH	ATACAAAAAA	CTT	AGGGAAAAAG
	SH/TM	CTAAGAAAAA	CTT	AGGGCAAATG
	TM/G	CTAAGAAAAA	CTT	AGGAGTAAAG
	G/L	TTAAGAAAAA	CTT	AGGATCAAAG
	L/	TTAAGAAAAA	-	-
Consensus		HTAHRAAAAA	CTT	AGGVNHAADG
SMPV-2	/N	-	CTT	AGGGACAAAG
	N/P	TTAAGAAAAA	CTT	AGGATTAAAG
	P/M	TTAAGAAAAA	CTT	AGGAAGAAAG
	M/F	TTAGAAAAAA	CTT	AGGAGCAAAG
	F/TM	TAATAAAAAA	CTT	AGGGTCAATG
	TM/G	TTAAGAAAAA	CTT	AGGACTAATG
	G/L	TTATAAAAAA	CTT	AGGGACTAAC
	L/	TTAAGAAAAA	-	-
Consensus	-	TWADRAAAAA	CTT	AGGRNBWAWS

Abbreviation SMPV-1, Samak Micromys paramyxovirus 1; SMPV-2, Samak Micromys paramyxovirus 2; N, nucleocapsid protein; P, phosphoprotein; M, matrix protein; F, fusion protein; SH, small hydrophobic protein; TM, transmembrane protein; G, glycoprotein; H, hemagglutinin protein; HN, hemagglutinin-neuraminidase protein; L, large protein

whole-genome sequences, consisting of one SMPV-1 and three SMPV-2 sequences, using NGS. The genomes of SMPV-1 and −2 follow the Paramyxoviridae genome organization criteria 3′-N-P-M-F-(SH)-TM-G-L-5′, with variations in the P gene attributed to an additional ORF. SMPV-1 has the SH gene and a larger G gene than SMPV-2. The presence of the SH gene separated *Jeilongvirus* into two groups. J virus, Beilongvirus, PAPV-1, and Ninomys virus harbor the *SH* gene, while PAPV-2, MMLPV-1, and MMLPV-2 lack it. However, the *SH* gene is not exclusive to *Jeilongvirus*, as Mumps virus (MuV) from the genus *Orthorubulavirus* carries this gene. Notably, SMPV-1 and −2 exhibit different sizes and secondary structures of the G protein despite infecting the same species. The G protein is involved in the binding of viral particles to target cells [11]. The G proteins within *Jeilongvirus* species were varied, and the C-terminal region was enriched with proline (P), threonine (T), and serine (S) residues [14]. SMPV-1 and −2 had dominant P/T/S residues in their glycoprotein amino acid composition and potential N-glycosylation sites. In addition, other rodent species similarly exhibit host sharing of phylogenetically distant paramyxoviruses, including *Lophuromys machangui* with MMLPV-1 and −2, *A. agrarius* with PAPV-1 and −2, and *Myodes glareolus* with Pohorje Myodes Paramyxovirus 1 (PMPV-1) and bank vole virus [5, 14, 42]. Our initial analysis of *Jeilongvirus* glycoprotein domain implied that the glycoprotein might elicit different host immune responses. Since co-infection with SMPV-1 and SMPV-2 was not observed in the specimens, each virus might selectively infect *M. minutus* individuals with distinct immune profiles. Further study characterizing the SMPV-1/-2 infected *M. minutus* immune response will improve our understanding of the host-virus interaction.

Mutations on RNA virus genomes are often dominated by synonymous substitutions in CDS maintaining the amino acid sequence of the protein [43]. The analysis on the CDS of SMPV-1 revealed that its genetic diversity did not translate to significant variation at the amino acid level. The amino acid sequence of SMPV-1 showed high similarity percentage with Ninomys virus, a *Jeilongvirus* found in *M. minutus* from Belgium, despite substantial phylogenetic divergence in complete and partial genome sequences [44]. RNA viruses utilize host cellular machinery for its replication, driving a coevolutionary process between the virus and the host [45]. Cophylogenetic reconstruction indicated that co-divergence events influenced the evolution of SMPV-1 and Ninomys viruses within the host population. *M. minutus* presumed to have low diversity owing to bottlenecking and lineage sorting during the Quaternary glacial cycles [46]. It is possible that speciation events of SMPV-1 and Ninomys virus coincided with the geographic genetic differentiation of *M. minutus* across Europe and Asia, with their evolution potentially constrained by the low genetic variability of the host. Observations in the *Flaviviridae* have shown that genetic constraints imposed by hosts and vectors pressure the viral coding region to evolve in adaptation to specific host-vector lineages [47]. Purifying selection during inter-host transmission might have eliminated mutations from intra-host pressure, leading to the fixation of advantageous mutations that efficiently spread the virus in larger populations [48]. This phenomenon might account for the observed disparity between genetic divergence and amino acid similarity in SMPV-1 and Ninomys virus, where amino acid compositions were likely conserved to preserve essential protein functions.

While SMPV-1 showed a phylogenetic relationship to the Ninomys virus, SMPV-2 shared a common ancestor with PAPV-2 and MMLV-1 hosted by other rodent species. Cophylogenetic analysis revealed that the cross-species transfer event from *Lophuromys sp.* preceded the speciation of SMPV-2. Since *Lophuromys sp.* is endemic to Africa, the transfer event likely occurred following their interaction with *M. minutus* populations that migrated from Europe to Asia during the Pliocene/Pleistocene.[19, 49] However, the current phylogenetic tree might not be fully comprehensive, as the transfer node only showed a 50% occurrence probability of the event; thus, there could be additional virus and host species serving as intermediaries bridging the transfer event from *Lophuromys* to *Micromys*. A transfer event detected

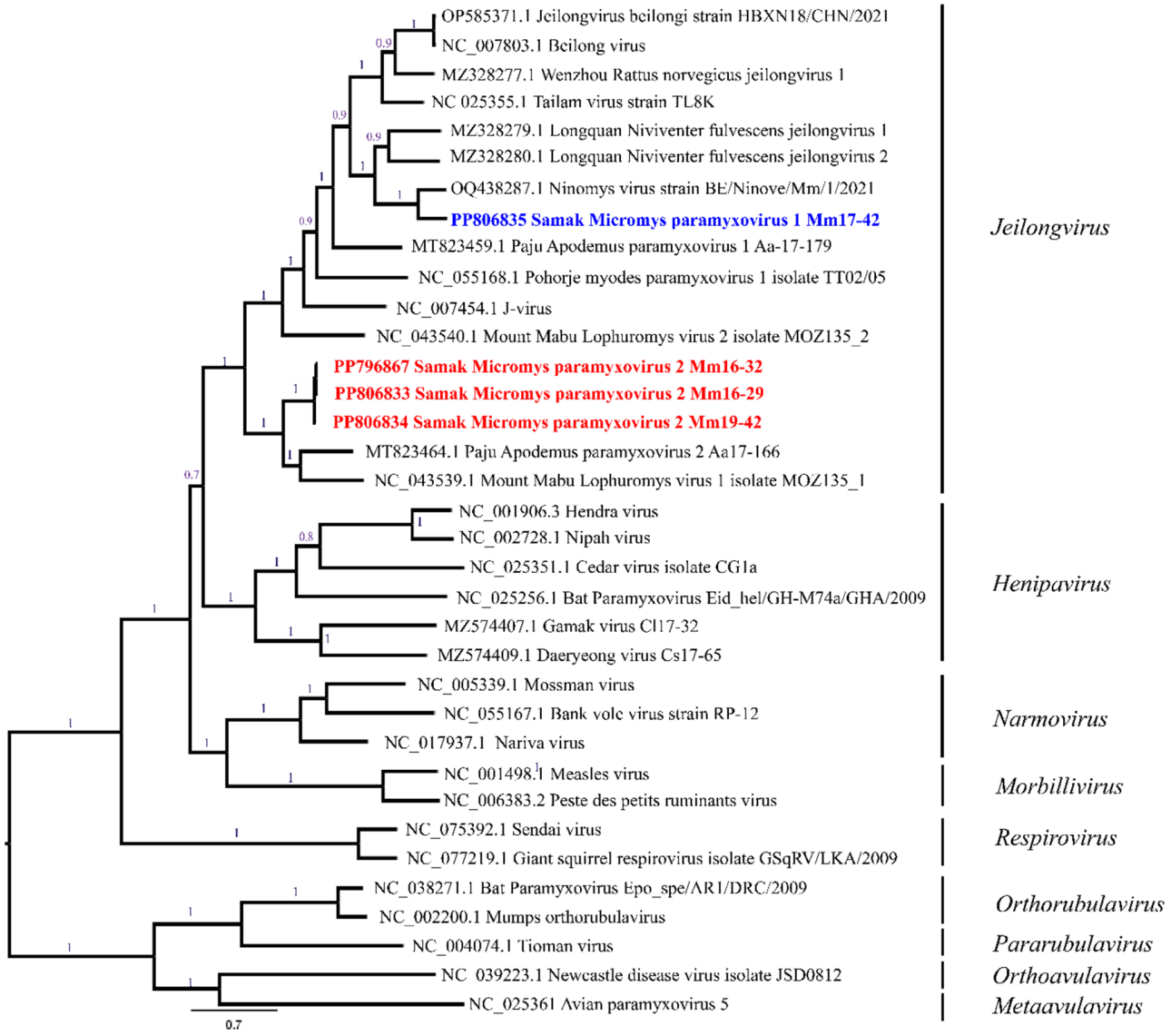

Fig. 3 Phylogenetic tree of Samak Micromys paramyxovirus 1 and 2 within *Paramyxoviridae* members. The phylogenetic tree was constructed using maximum likelihood analysis by IQTREE web server, with GTR + F + I + G4 model chosen according to BIC and 1000 bootstrapping. The blue-colored label corresponds to Samak Micromys paramyxovirus 1 (SMPV-1), while the red-colored label represents Samak Micromys paramyxovirus 2 (SMPV-2). These two viruses cluster within the genus *Jeilongvirus*. SMPV-1 shared common ancestor with Ninomys virus, while SMPV-2 shared common ancestor with Paju Apodemus Paramyxovirus 2 and Mount Mabu Lophuromys virus 1

from *M. minutus* to *A. agrarius* in the ROK antecedes PAPV-2. While direct interactions between the two host species on the Korean Peninsula might facilitate host-switching events, the potential for involvement of intermediate hosts or viruses in the evolution requires further investigation.

Compared with other rodent species, *M. minutus* rarely subjected to zoonosis surveillance. Being the smallest rodent in the family Muridae, *M. minutus* is difficult to collect [19]. Studies on other rodent and shrew genera in the ROK revealed their capacity as natural hosts of novel paramyxoviruses. Herein, SMPV detection within *M. minutus* population demonstrated its capacity to harbor and transmit rodent-borne paramyxoviruses. In particular, the SMPV positivity rate was higher in male compared to that in female *M. minutus*. This gender-specific prevalence of SMPV-1 and −2 is similar to that of *Orthohantavirus puumalaense* in the bank vole of the Ural Mountains, *Orthohantavirus hantanense*, and *Thottimvirus imjinense* in small mammals of the Korean Peninsula [50–52]. Aggression is believed to influence the positivity rate, as a study on *Orthohantavirus seoulense* among *R. norvegicus* showed a correlation between wound severity and viral loads in male [53]. In addition to the behavioral aspect, sex dimorphism, hormones, and disparity in immune response between genders could be attributed to

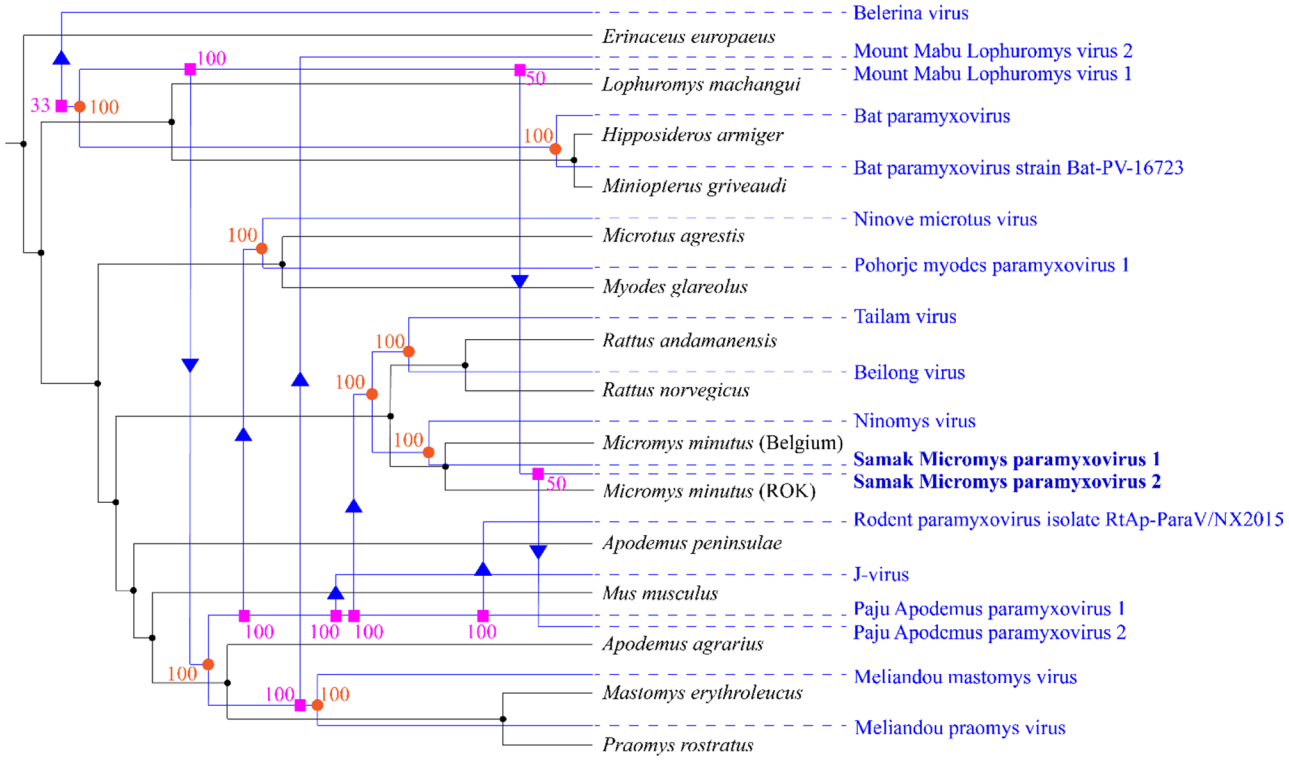

Fig. 4 Cophylogenetic analysis of Samak Micromys paramyxovirus 1 and 2 with members of *Jeilongvirus* and their respective host. The SMPV-1 and-2 are labelled with bold blue font. The black colored phylogenetic tree is the host tree based on mtDNA cyt*b* sequences. The blue colored phylogenetic tree is the *Jeilongvirus* tree based on *L* gene of respective virus. The orange circle and number are the nodes for co-divergence and the probability of occurrence. The blue arrow showed the direction of the transfer event. The pink square and number are the nodes for transfer event, followed by the probability of occurrence in percent (%)

the higher SMPV prevalence in male specimens [54–56]. Since our study did not observe the wounding, behavior, or any physiological information of the animals, the transmission routes and risk factors for SMPV infection in *M. minutus* remain unclear.

The limitation of this study is the absence of SMPV-1 and-2 virus isolation and RACE PCR results owing to the small sample size. We were unable to provide additional information on SMPV pathogenesis or virulence factors. Moreover, the immune profile of *M. minutus* remains unexplored. Further investigations of *M. minutus* populations are required to address this issue in the ROK.

In conclusion, our study identified both SMPV-1 and −2 from *M. minutus* population in the ROK through metagenomic analysis by next-generation sequencing. Although current evidence has not detected zoonotic transmission from the genus *Jeilongvirus*, the inclusion of SMPV-1 and −2 sequences in paramyxoviruses is crucial for the surveillance of emerging infectious diseases. This study provides insights into the genetic diversity and evolutionary history of paramyxoviruses in small wild mammals.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-024-02532-6.

Supplementary Material 1: Supplementary Figure 1. Distribution of Paramyxovirus-positive samples in the city of Chuncheon and Hongcheon, Gangwon Province. (A) The map shows the trapping sites in Chuncheon and Hongcheon, two cities located in Gangwon province. The red dot in Chuncheon represents the area where the captured M. minutus tested positive for paramyxovirus screening. Conversely, the blue dot in Hongcheon represents the area with negative screening results. (B) The picture shows a Micromys minutus specimen captured in this studySupplementary Figure 2. Mitochondrial cytb sequences result and phylogenetic tree of Micromys minutus. The phylogenetic tree was constructed using maximum likelihood analysis by IQTREE web server, with TPM3u+F+G4 model chosen according to BIC and 1000 bootstrapping. The blue colored label is mtDNA cytb sequences of the M. minutus in this study. The red colored label is the cytb sequences of M. minutus infected by Jeilongvirus from Horemans et al. study (2023). The sample from the Republic of Korea shared common ancestor with M. minutus from EuropeSupplementary Figure 3. The predicted N-glycosylation site of Samak Micromys paramyxovirus 1 and 2, in comparison with members of Paramyxoviridae. The potential N-glycosylation site marked by green bar crossing the purple threshold line. The secondary structure of the glycoprotein was added beneath the N-glycosylation graphics for additional visualization. Seven viruses are members of Jeilongvirus while Nipah virus from Henipavirus is added as control. Abbreviations: SMPV-1: Samak Micromys paramyxovirus 1; SMPV-2: Samak Micromys paramyxovirus 1; PAPV-1: Paju Apodemus paramyxovirus 1; PAPV-2: Paju Apodemus paramyxovirus 2; MMLV-1: Mount Mabu Lophuromys virus 1Supplementary Figure 4. Phylogenetic tree of SMPV-1 and -2 partial genome with other paramyxovirus. The partial sequences were generated from amplicon sequencing of the pan-paramyxovirus

nested PCR product. The phylogenetic tree was constructed using maximum likelihood analysis by IQTREE web server, with TPM3u+F+G4 model chosen according to BIC and 1000 bootstrapping. The partial sequences generated two distinct clusters, in concordance with the phylogenetic tree generated from the whole genome sequences. The blue-colored label represents Samak Micromys paramyxovirus 1, and the red-colored label represents Samak Micromys paramyxovirus 2. The dot represents the samples from which the whole genome sequences originated

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Author contributions
A.N. and W-K.K. contributed to conceptualization, investigation, writing of the original draft, review, and editing. A.N. and S.E.P. contributed to data analysis and interpretation. S.H.C, H.S.P., J.P., K.P., S.P.P., B.K., J.K.,S.B., contributed equally to methodology, sample processing, data curation, and figures preparation. J.G.S. and J.S.L. contributed to specimen collection, figure preparation, and rodent data analysis. C.B.L, J.W.S, and Y.O. contributed equally to review and editing. All authors have reviewed and approved the final manuscript.

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Data availability
All the viral and Ctyb sequences can be accessed at Genbank with accession number listed in supplementary Table 5. The raw NGS data can be provided upon request from the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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