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ORIGINAL ARTICLE

Genetic diversity and phylogenetic analysis of newly discovered bat astroviruses in Korea

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Abstract

Bats have been identified as a natural reservoir for several potentially zoonotic viruses. Recently, astroviruses have been reported in bats in many countries, but not Korea. We collected 363 bat samples from thirteen species at twenty-nine sites in Korea across 2016 and tested them for astrovirus. The detection of the *RNA-dependent RNA polymerase* (*RdRp*) gene in bat astroviruses was confirmed in thirty-four bats across four bat species in Korea: twenty-five from *Miniopterus fuliginosusi*, one from *Myotis macrodactylus*, four from *M. petax*, and four from *Rhinolophus ferrumequinum.* The highest detection rates for astrovirus were found in Sunchang (61.5%, 8/13 bats), and in the samples collected in April (63.2%, 12/19 bats). The amino acid identity of astroviral sequences identified from bat samples was ≥ 46.6%. More specifically, the amino acid identity within multiple clones from individual bats was ≥ 50.8%. Additionally, the phylogenetic topology between astroviruses from different bat families showed a close relationship. Furthermore, phylogenetic analysis of the partial ORF2 sequence of bat astroviruses was found to have a maximum similarity of 73.3–74.8% with available bat astrovirus sequences. These results indicate potential multiple-infection by several bat astrovirus species in individual bats, or hyperpolymorphism in the astrovirus strains, as well as the transmission of astroviruses across bat families; furthermore, our phylogenetic analysis of the partial ORF2 implied that a novel astrovirus may exist. However, the wide diversity of astroviral sequences appeared to have no significant correlation with bat species or the spatiotemporal distribution of Korean bat astroviruses.

Introduction

Bats (order *Chiroptera*) comprise more than 20% of all living mammalian species; their high diversity and broad geographic distribution distinguishes them from other mammals, after rodents. Bats have been identified as a natural reservoir of several potentially zoonotic viruses, including DNA viruses (such as adenovirus, herpesvirus, and parvovirus) and RNA viruses (such as coronavirus, picornavirus, astrovirus, retrovirus, and reovirus) [1].

Astroviruses (AstVs) are non-enveloped, positive-sense, single-stranded RNA viruses with a genome ranging from approximately 6.2 to 7.7 kb in size [2]. The *Astroviridae* family is made up of two genera: *Mamastrovirus* and *Avastrovirus* [3]. *Mamastrovirus* viruses have been identified in numerous mammalian species such as human, pig, mink, sheep, and bat [4, 5]. *Avastrovirus* viruses have been isolated from avian species including turkey, duck, chicken, fowl, goose, and many others [3, 6]. Astrovirus infections cause gastroenteritis in humans and other mammals [7]. Recently, astrovirus strains associated with encephalitis and ganglionitis were reported in humans, pigs, cattle, and sheep [8–13].

Bat astroviruses (BtAstVs) have been discovered in several locations in many countries across Europe, China, South-East Asia, and Africa [14, 15]. In recent studies, diverse coronaviruses and novel rotaviruses were discovered in the feces of Korean bats [16, 17]; bat astrovirus, however, has not been identified in Korea. Current surveillance studies on bats are insufficient in Korea. Therefore, in this study, we performed a molecular and epidemiological investigation

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of astroviruses from several bat species inhabiting several regions of Korea in 2016.

Materials and methods

Sample collection

A total of 363 bat samples (sixty-one oral swabs, 244 fecal samples, and fifty-eight bat carcasses) were collected at twenty-nine sites across sixteen provinces in Korea from January to September 2016. Bats were captured by net for sample collection, and released immediately after sampling. Oral swabs and feces were transported in viral transport medium (Copan Diagnostics, Murrieta, CA, USA) after being collected from individual bats, and were stored at -70 °C until use. Bat carcasses were autopsied for the collection of internal organs including the brain, lung, intestine, heart, and liver; all collected tissues were immediately ground in phosphate-buffered saline (PBS) containing 1% antibiotic-antimycotic solution (Corning Inc., Corning, NY, USA). The supernatant of the ground tissues was also stored at -70 °C until use. RNA was extracted within 1 week of the date the samples were collected.

PCR, cloning, and RNA sequencing

Total RNA was extracted from oral swab medium, feces medium, and ground tissue supernatant at a final volume of 200 µl using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) and eluted in 70 µl RNase-free water. Random cDNA was synthesized using the SuperScript III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primer pairs for the molecular detection of astrovirus in Korean bats were described previously by Chu *et al.* [18]. The primers for hemi-nested RT-PCR (for first PCR, AS1-F1: GARTTYYGATTGGRCKCGKTAYGA; AS2-F1: GARTTYGATTGGRCKAGGTAYGA; AS-R: GGYTTKACCCACATNCCRAA; for second PCR, AS3-F1: GCKTAYGATGGKACKATHCC; AS4-F2: AGGTAYGATGGKACKATHCC; AS-R) target a partial region of the *RNA-dependent RNA-polymerase* (*RdRp*) gene in astrovirus, which has a length of 422 bp. Amplification was performed using the Platinum Green Hot Start PCR master mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols; briefly, both PCRs were performed at a final volume of 50 µl containing 10 µl forward and reverse primers, 2.5 µl cDNA, and PCR master mix (2×). Sequences were amplified under the following conditions: initial incubation at 94 °C for 1 min, followed by 35 cycles of denaturation 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 68 °C 30 s, with a final extension at 68 °C for 5 min in a Mastercycler (Eppendorf, Hamburg, Germany). The amplified 422-bp products were purified using the QIAquick PCR purification kit (Qiagen) and cloned into pTOP V2-TA plasmids (Enzynomics, Daejeon, Korea) for DNA sequencing. Three clones from the PCR products were selected and sequenced using BigDye 1.1 terminator cycle sequencing reagents on an ABI PRISM 3130 Automated Capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). For the amplification of a partial region of ORF2 (capsid protein), primers were designed against a conserved region following alignment of available astroviral sequences. The following primer sets were used: for the first PCR, cap523F: TYACNCCTATGGTTGG; cap1290R: TRTTRTTYTGAGCATC; and for the second PCR, cap523F; cap1193R: CAAACCACCANCCACC. Amplification was performed under the following conditions: initial incubation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C for 40 s, and extension at 72 °C for 40 s, with a final extension at 72 °C for 5 min in a Mastercycler. The final 670-bp amplicons were purified and sequenced as described above.

Phylogenetic analysis

Phylogenetic analysis was carried out based on the partial 422-bp *RdRp* sequence of bat astrovirus. Available sequences were retrieved from GenBank. Multiple alignments of astroviral sequences were generated by the Clustal W algorithm in BioEdit v. 7.0.9.0 [19]. Phylogenetic trees were constructed using the neighbor-joining (NJ) method in MEGA 7 [20]. The topologies of the NJ trees were evaluated by the maximum likelihood method and bootstrap values were determined by 1,000 iterations using MEGA 7.

Results

Identification of bat astrovirus

Bat astroviruses were identified from thirty-four samples collected between January and September 2016 (Fig. 1). They were collected from the Sunchang, Yeongcheon, and Danyang regions of Korea. The highest detection rate for astroviral genes was found in Sunchang at 61.5% (8/13 bats), followed by 53.8% in Yeongcheon (7/13 bats). In two areas of Danyang (DY1 and DY4), the detection rate of astrovirus genes was found to be 7.4% (7/94 bats) and 48% (12/25 bats), respectively (Table 1). In addition, the detection of astroviral genes by PCR was confirmed in samples collected in January, March, April, and July (Fig. 1). In particular, the highest detection rate (50%, 20/40 bats) was found in the samples collected during April, followed by January (25%, 2/8 bats). The detection rates in March and July were 10.5%

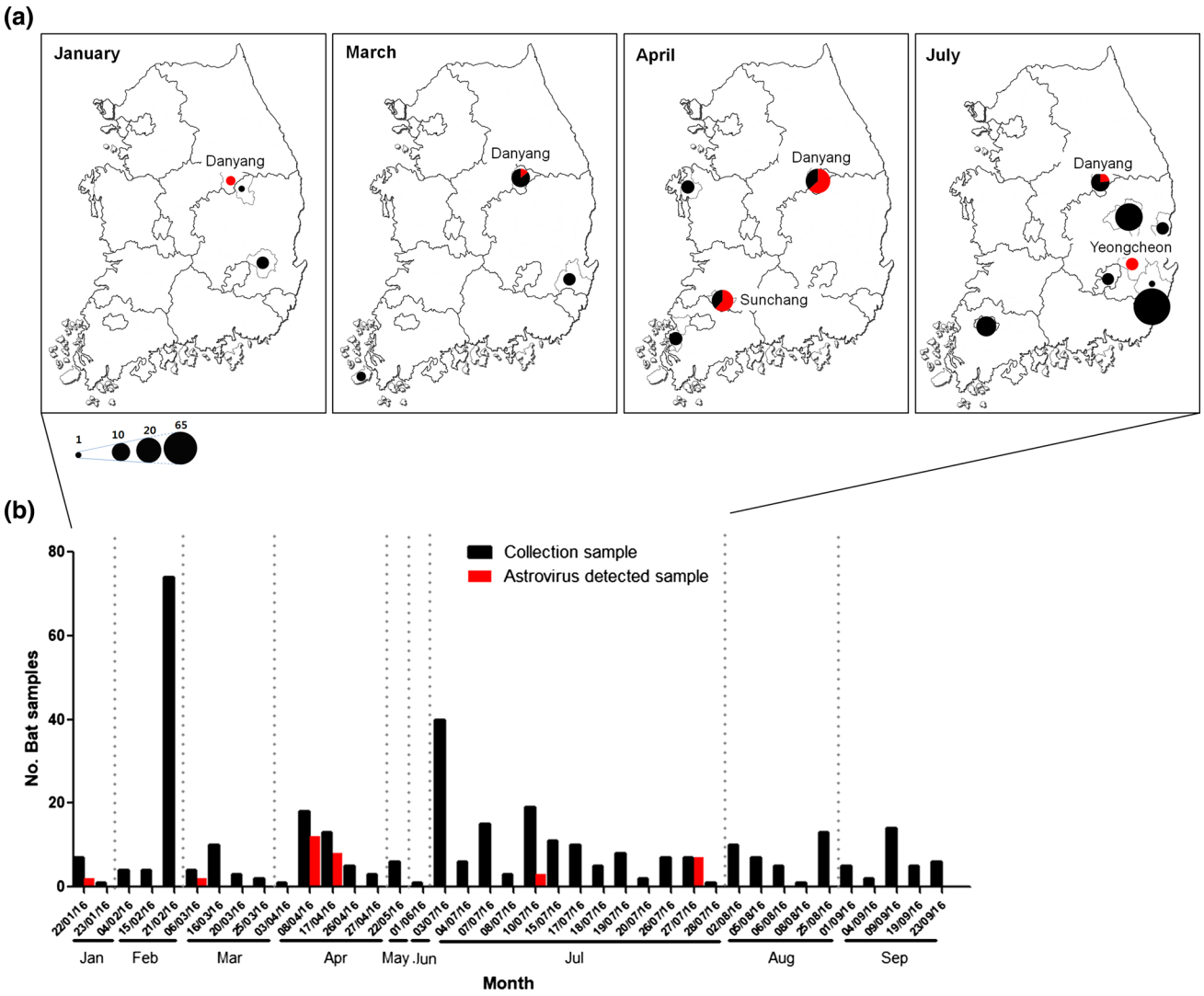

Fig. 1 Spatiotemporal distribution of Korean bat astrovirus. (a) Geographical distribution of astrovirus-positive bats. The size of the circles indicates the number of collected bat samples. (b) Seasonal distribution of bat astroviruses from January to September 2016. The vertical dashed lines separate each month

(2/19 bats) and 7.2% (10/138 bats), respectively (Fig. 1b). Astrovirus was detected consistently in Danyang during January (2/2 bats, 100%), March (2/14 bats, 14.3%), April (12/19 bats, 63.2%), and July (3/13 bats, 23.1%). Additionally, the eight astroviruses detected in Sunchang were only found during April, and the seven astroviruses detected in Yeongcheon were only found during July (Fig. 1a).

Bat astroviruses were discovered in the following four bat species of the thirteen analyzed: *Miniopterus fuliginosus, Myotis macrodactylus, M. petax,* and *Rhinolophus ferrumequinum*. The highest positive rate was identified in *M. fuliginosus* at 25.3% (25/99 bats), followed by *M. petax* at 25% (4/16 bats). The presence of astroviral RNA sequences in *M. macrodactylus* and *R. ferrumequinum* were confirmed at 10% (1/10 bats) and 9.3% (4/43 bats), respectively. The detection rate of bat astrovirus was the greatest in tissue samples (21/57, 36.8%), followed by feces (13/244, 5.3%). No bat astroviruses were detected in oral swabs.

Genetic diversity and phylogenetic analysis of bat astroviruses

A total of ninety-three astroviral sequences were fully identified from multiple clones isolated from thirty-four bats (accession No. MG840952-MG841043 and MH254918), and newly found astroviruses from Korean bats were tentatively denominated in the order of BtAstV-sample type and number-clone number. The amino acid identity within each clone from the same bat ranged from 50.8–100%. The lowest amino acid identity within each clone from individual

Table 1 Detection rate of bat astrovirus at various collection sites in Korea

State/Country	City/Province	Site	Habitat	Positive samples/ Total (%)
Gangwon	Yeongwol	YW1	Forest	0/18
Gyeongnam	Changwon	CW1	Abandoned mine	0/7
Gyeongbuk	Gyeongju	GJ1	Abandoned mine	0/8
		GJ2	Forest	0/1
		GJ3	Around the private house	0/15
	Andong	AD1	Forest	0/35
		AD2	Forest	0/2
	Yeongdeok	YD1	Abandoned mine	0/3
		YD2	Forest	0/5
	Yeongju	YJ1	Abandoned mine	0/7
		YJ2	Forest, stream	0/5
	Yeongcheon	YC1	Abandoned mine	7/13 (53.8%)
	Cheongsong	CS1	Forest, valley	0/1
Gwangju	Gwangsan	Gwj1	Forest	0/11
Daegu	Dalseong	DS1	Cave	0/5
Ulsan	Ulju	US1	Abandoned mine	0/6
		US2	Around the private house	0/52
		US3	Forest	0/8
Jeonnam	Muan	MAU	Cave	0/5
	Jindo	JD1	Abandoned mine	0/2
Jeonbuk	Sunchang	SC1	Abandoned mine	8/13 (61.5%)
Chungnam	Seosan	SSU	Abandoned mine	0/3
Chungbuk	Danyang	DY1	Cave	7/94 (7.4%)
		DY2	Cave	0/2
		DY3	Cave	0/1
		DY4	Cave	12/25 (48%)
		DY6	Forest, valley	0/6
		DY7	Forest	0/10

bats was in BT34. The amino acid identity between BtAstV-BT34-1 and BtAstV-BT34-2 was 50.8%, and the amino acid identity between BtAstV-BT34-1 and BtAstV-BT34-3 was 55.8%. However, the amino acid identity between BtAstV-BT34-2 and BtAstV-BT34-3 was 75.8% (violet squares in Fig. 2). In addition, the amino acid identity of BtAstV-BT42-1 was 57.5% when compared to both BtAstV-BT42-2 and BtAstV-BT42-3 (orange triangles in Fig. 2).

The amino acid identity among the Korean bat astrovirus sequences ranged from 46.6–100%, and the amino acid identity within single bat species captured from the same habitat also had a wide range of 53.4–100%. The amino acid identity among bat astrovirus sequences from group B in Fig. 2 was 100%, and the amino acid identity of bat astrovirus sequences within group E in Fig. 2 was ≥ 99.1% (Fig. 2). However, the pairwise amino acid similarity among Korean bat astroviruses from group F (BtAstV-BF73-1, BtAstV-BF73-3, and BtAstV-BT34-1) and D (BtAstV-BT54-1, BtAstV-BF67-2, and BtAstV-BF67-3) in Fig. 2 were the lowest, at 46.6–48.3%. Additionally, the pairwise amino acid similarity among the astroviruses from group F and B in Fig. 2 were confirmed to be 47.5–51.6%. In group C in Fig. 2, the bat astrovirus detected from *R. ferrumequinum* (BtAstV-BF67-1) and the bat astrovirus detected from *M. fuliginosus* (BtAstV-BT53-1) were closely clustered, with an amino acid identity of ≥ 99.1%. Furthermore, in group A in Fig. 2, bat astroviruses identified from *M. macrodactylus* (BtAstV-BT40-1, BtAstV-BT40-2, and BtAstV-BT40-3) were clustered with the astroviruses confirmed from *M. fuliginosus* (BtAtV-BT53-2 and BtAstV-BF78-2); the average amino acid identity among the bat astroviruses in group A in Fig. 2 was ≥ 94.1%.

The detection of partial ORF2 (capsid protein) sequences from several astroviral RdRp positive samples—BT6 and BT27 of *R. ferrumquinum*, BT51 of *M. petax*, and BF69 and BF80 of *M. fuliginosusi*—was performed. Two partial ORF2 (632 bp) sequences (BT6 and BT27) were obtained and the phylogenetic distance was calculated. The ORF2

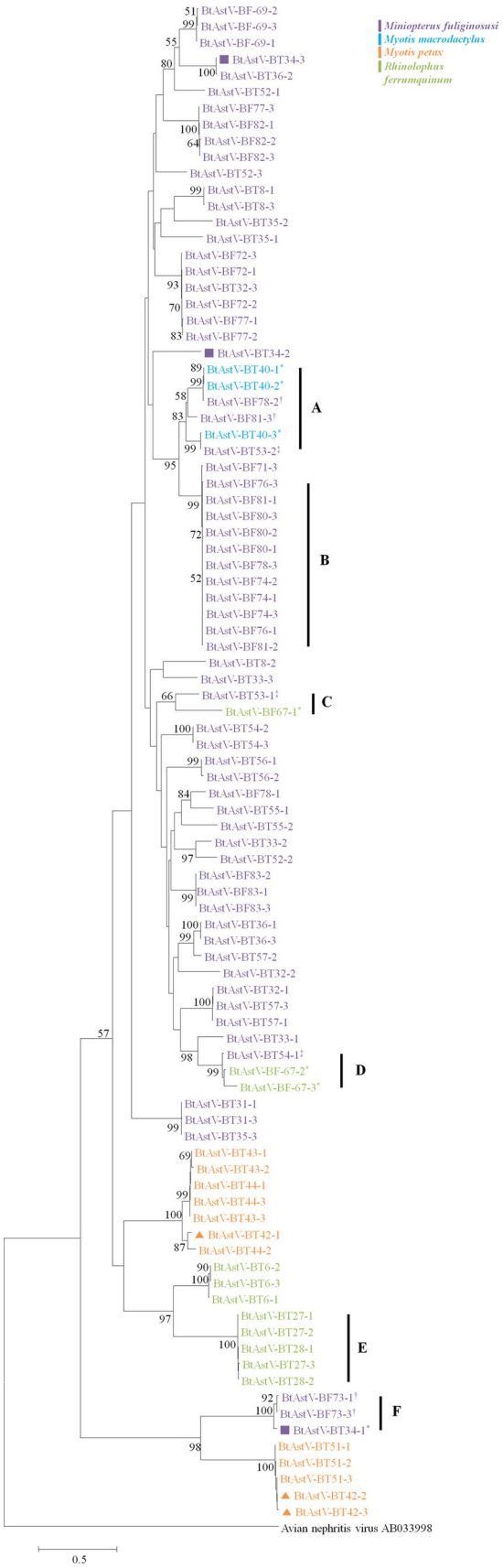

Fig. 2 Phylogenetic analysis of the partial *RdRp* sequence from astroviruses in Korean bats. The phylogenetic tree, based on the partial *RdRp* nucleotide sequences (422 bp), was reconstructed by neighbor-joining (NJ) using MEGA 7. The numbers at each node indicate bootstrap values as a percentage of 1,000 iterations; scale bars indicate the number of nucleotide substitutions per site. *Miniopterus fuliginosus*, *Myotis macrodactylus*, *M. petax*, and *Rhinolophus ferrumquinum* are shown in violet, blue, orange, and green, respectively. *, Sunchang; †, Danyang; ‡, Yeongcheon. BT, bat tissue; BF, bat feces; BO, bat oral sample (colour figure online)

amino acid identity between BtAstV-BT6 and BtAstV-BT27 was found to be 86.8%. However, BtAstV-BT6 and BtAstV-BT27 showed an ORF2 amino acid identity ranging from 73.3–74.8% and 74.3–74.8%, respectively, when compared to bat astrovirus LC03 (FJ571047) and LS11 (FJ571068), which were originally detected in China (Fig. 3).

Discussion

In the present study, we identified the infection of four Korean bat species—*M. fuliginosus*, *M. macrodactylus*, *M. petax*, and *R. ferrumequinum*—with various astroviruses. These bats are distributed across a wide geographical area that includes Korea, Japan, and Russia [21–24]. The discovery of astroviruses from these bat species has been reported in China and Hungary, but not Korea [25–27].

Bat astroviruses are known to have an exceedingly high genetic diversity. In a previous study, the amino acid similarity of the *RdRp* gene within bat astroviruses in Asia and Europe was verified to be ~49.2% [26, 28]. In this study, the amino acid identity of the partial *RdRp* gene in Korean bat astroviruses ranged from 46.6–100%, and the amino acid identity of astroviruses within a single bat species captured from the same habitat also ranged from 53.4–100%. Moreover, the amino acid identity of multiple astroviral strains from within an individual bat was 50.8–100%. This amino acid identity range within multiple clones of bat astroviruses indicated the existence of astrovirus haplotypes in Korean bats; the very low amino acid identity may indicate the possibility of infection by two or more astrovirus species in individual bats, or hyperpolymorphisms within identical astrovirus strains; however, more systematic studies and more genomic sequences are needed to verify this. Additionally, astroviruses are known to widely spread among Chiroptera and show no host restriction [15, 18, 25, 26]. Our result showed that astrovirus sequences in multiple clones from one *M. fuliginosus* bat were closely clustered with bat astroviruses from different host species: *M. macrodactylus* and *R. ferrumequinum*. In group D, the clustering between *M. fuliginosus* (BtAstV-BT54-1) and *R. ferrumequinum* (BtAstV-BF67-2) showed an amino acid similarity of ≥ 99%; this was supported by high posterior probabilities (pp = 0.99) from

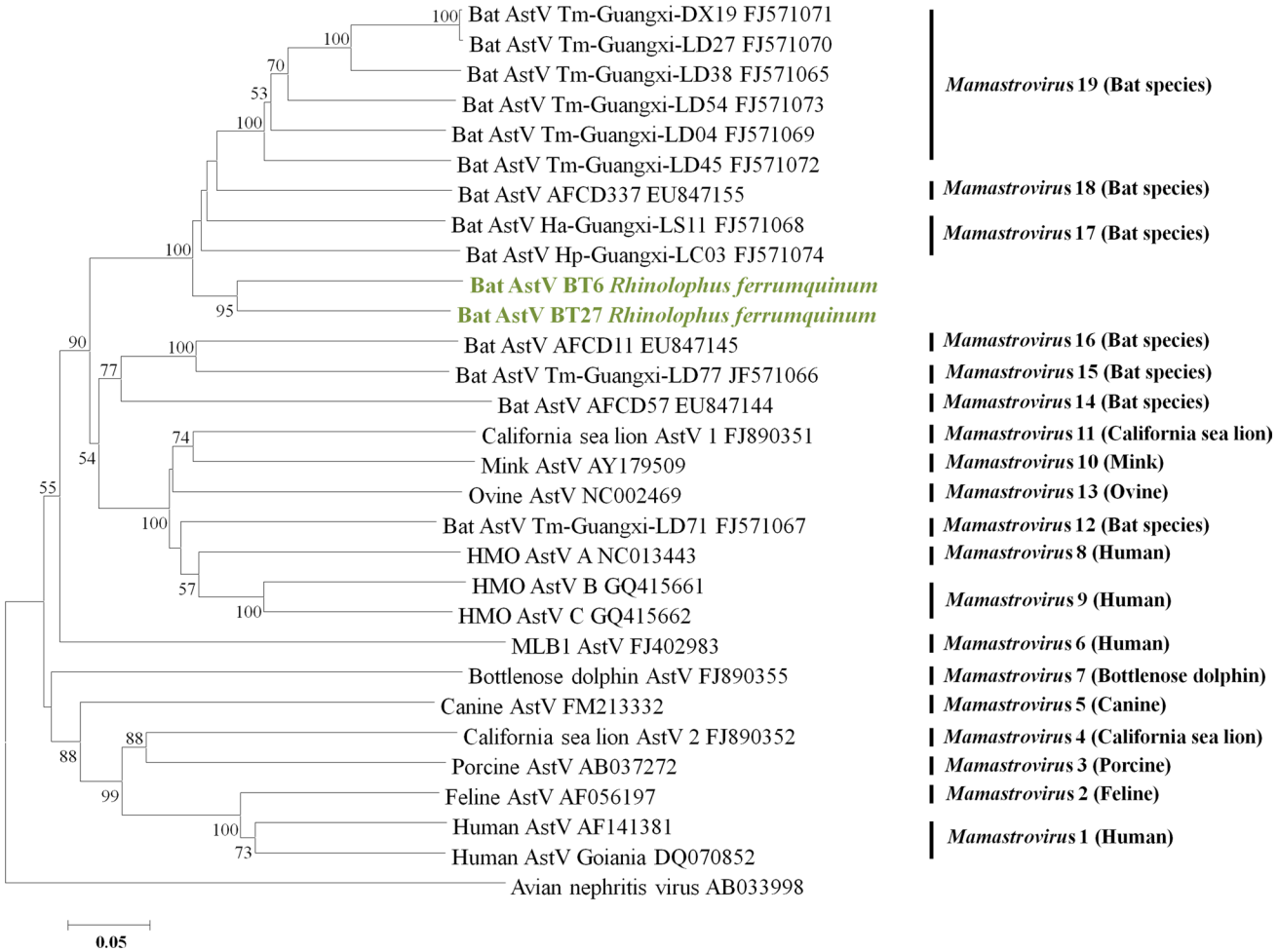

Fig. 3 Phylogenetic analysis of partial ORF2 (capsid protein) sequences from representative Korean bat astroviruses. A phylogenetic tree was generated using the partial ORF2 nucleotide sequences (632 bp) of mamastroviruses by neighbor-joining (NJ) in MEGA 7. The numbers at each node indicate bootstrap values from 1,000 iterations; scale bars indicate the number of nucleotide substitutions per site

phylogenetic topology. These phylogenetic results suggest that genetic variants were generated at high rates within individual bats, and that active transmission events between bat species or families occurred between the *Vespertilionidae* and *Rhinolophidae* families. Furthermore, of the available astrovirus sequences, the partial amino acid sequence of ORF2 from two bat astroviruses was confirmed to have the highest identity (73.3–74.8%) with the bat astrovirus LC03 (FJ571074) and LS11 (FJ571067) sequences from China. It is unclear whether these represent novel astrovirus species according to ICTV guidelines [29], as we could not characterize the entire ORF2 sequence. Therefore, further studies on the characterization of the whole genomes of bat astroviruses is necessary, as Korean bat astroviruses have not been comprehensively analyzed until now.

Various emerging viruses, such as nipah virus, filovirus, coronavirus, and astrovirus in bats, have been known to display seasonal variations in concentration or prevalence [28, 30, 31]. For astrovirus persistence, recent studies have shown that dry roosting at the beginning of the rainy season may increase the rate of transmission [32]. Accordingly, a high concentration and high prevalence of astrovirus was found in the samples collected during spring and autumn, indicating that they were most susceptible in the periods before and after parturition [28]. Our results showed that the prevalence of astrovirus in Korean bats was at its maximum during spring, the period before parturition, and before the rainy season. Knowing the seasonal fluctuations of bat astrovirus infections is required to understand the transmission and dispersal of astroviruses within bat populations.

Korean bat astroviruses were distributed across three regions: Sunchang, Danyang, and Yeongcheon. A recent study showed that no direct transmission occurred across different geographical locations through the analysis of astrovirus haplotypes in individual bats [33]; however, our study identified different phylogenetic topologies.

The indiscriminate phylogenetic clustering of astroviral sequences obtained from different locations implies the direct (or indirect) transmission of bat astroviruses with no geographical restrictions in Korea. Additionally, astrovirus was highly prevalent in feces because astroviruses replicate in the gastrointestinal tract, and transmission has been found to occur through the oral-fecal route [34]. Interestingly, Korean bat astroviruses only showed high detection rates in tissue (including colon) and fecal samples, but not in oral swabs.

In bats, the co-infection and recombination of viruses has been occasionally reported [18, 35]. This suggests that the ecological characteristics of bats may increase the opportunities for recombination in various viruses. The movement of bats due to a decrease in the size of their habitats causes an increase in contact opportunities with domestic animals as well as humans; zoonotic pathogens in bats may thus pose a potential risk of transmission to humans and domestic animals. Therefore, further surveillance studies of bat viruses are required due to the lack of clear knowledge on bat pathogens in South Korea.

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Compliance with ethical standards

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

Ethical approval All applicable international, national, and institutional guidelines for the case and use of animals were followed.

Informed consent Not applicable (Human subjects were not involved in this study).

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