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BRIEF REPORT

Isolation and genetic characterization of novel bovine parechoviruses from Japanese black cattle

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Abstract

Novel bovine parechoviruses (Bo ParVs) were isolated from the feces of Japanese black cattle. Phylogenetic analysis revealed that the novel Bo ParVs formed an independent cluster, exhibiting 72.2–75.6% nucleotide sequence identity to previous Bo ParVs, suggesting that they represent a new genotype. Bo ParVs, including the novel Bo ParVs, shared sequence similarity with each other in the 3' untranslated region (3'UTR) and exhibited low sequence similarity (<38.9% identity) to other parechoviruses. However, a secondary structure prediction of the 3'UTR revealed that the Bo ParVs shared conserved motifs in domain 2 with parechovirus B and E, suggesting some evolutionary constrains in this region.

Parechoviruses are small, icosahedral, non-enveloped viruses belonging to the genus *Parechovirus* within the family *Picornaviridae* [1]. They possess a non-segmented positive-sense RNA genome with 7339–7608 nucleotides (nt), including a single long open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs) and a poly-A tail [1]. The ORF encodes a polyprotein, which is divided into the P1, P2, and P3 regions encoding the capsid structural proteins VP4–VP1 and the non-structural proteins 2A–2C and 3A–3D, respectively. Their 5'UTRs include a type II or type IV internal ribosomal entry site (IRES) upstream of the translational start site [2]. Bovine parechovirus (Bo ParV) is a candidate for a new species in the genus *Parechovirus* whose genome sequence was first discovered in a public database of metagenomic libraries [3]. Although Bo ParV sequence reads have been detected in various tissues, including lymphatic, central nervous system, and digestive tissues, based on public RNA-seq data from cows, the only currently available nearly complete genome sequence of Bo ParV (Bo ParV/2018/4/KOR) was assembled from sequence reads from the lower digestive tract of a cow sampled in 2018 in South Korea [3]. In 2021, the first Bo ParV, Den1/2021/JPN, was isolated in Japan from diarrheic feces of a lactating cow infected with group C rotavirus, using MA104 cells [4]. Subsequently, three additional Bo_ParVs were isolated from diarrheal calves in Japan in 2022, and 6.3% of bovine fecal samples were found to be positive for Bo ParV using qRT-PCR [5]. Therefore, Bo ParV is considered to have an affinity for the intestinal tract. Furthermore, only five nearly complete genome sequences of Bo ParV are currently available from South Korea and Japan. In the present study, novel Bo ParVs were isolated from the feces of Japanese black cattle with or without diarrhea and genetically characterized.

To identify bovine enteric viruses in Japanese black cattle, fecal samples were collected directly from the rectums of four diarrheic calves (eight-day-old to three-month-old) and 40 healthy cattle (one-day-old to adult) kept on a farm located in the Chubu region of the main island of Japan in 2021. Fecal samples were diluted at a 1:9 (w/v) ratio using Eagle's minimal essential medium (EMEM) (Nissui, Tokyo, Japan) and then centrifuged at 12,000 × *g* for 10 minutes. The resulting supernatants were activated by adding equal volumes of 20 µg/mL trypsin (cat. no. 0303; Sigma-Aldrich, MO, USA) and incubated for 1 hour at 37 °C. The activated

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samples were then inoculated onto the African green monkey kidney cell line Marc-145. Confluent monolayers of Marc-145 cells in 48-well plates were washed three times with EMEM and subsequently inoculated with 0.1 mL supernatant from the activated samples. After incubation at 37 °C for 1 hour for adsorption, the cells were washed three times using EMEM. Subsequently, the cells were incubated with EMEM containing 1.0 µg of trypsin per mL for 7 days at 37 °C in a 5% CO_2 atmosphere. If a cytopathic effect (CPE) was not observed after 7 days, the cells and supernatant were subjected to three cycles of freezing and thawing before harvesting. Further passages were conducted following the same procedure. CPE was observed in seven out of 44 samples two or three days after inoculation in the second passage.

Viral RNA was extracted from the cell culture supernatants using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA). Subsequently, cDNA libraries for deep sequencing were constructed using an NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacture's guidelines. After quantification of the cDNA using a Qubit 4.0 fluorometer (Invitrogen, Carlsbad, CA, USA), next-generation sequencing (NGS) was performed using an Illumina MiSeq benchtop sequencer (Illumina, San Diego, CA, USA). Sequence data analyzed using MiSeq Reporter (Illumina) to generate FASTQ-formatted sequence data files. The 151-nt paired-end reads were trimmed using the command "Trim Sequences" in the NGS core tools with default parameters in CLC 7.5.5 Genomics Workbench (CLC bio, Aarhus, Denmark) and then assembled *de novo* into contigs in CLC 7.5.5 with default parameters. The quality of the viral contigs was assessed by mapping the original reads back to the contigs, and a sufficient depth of mapped reads was observed (Supplementary Fig. S1). Nearly complete genome sequences of Bo ParV were obtained from six of the seven samples (3, 8oya, 8ko, 10, 18, and 101). Two of these samples (3 and 101) were obtained from diarrheic calves, while the remaining four were from two healthy calves and two healthy adult cows (Supplementary Table S1). To obtain the complete sequence of the 3'UTR, the 3' end of the viral genome was amplified by RT-PCR using a gene-specific forward primer and the reverse primer TX30SXN [6] (Supplementary Fig. S2) and sequenced directly by the Sanger method. The whole-genome sequences from samples 3 and 101, 8oya and 8ko, and 10oya and 18 were found to be identical. These sequences—Bo ParV/Mayo3/2022/JPN (Mayo3), Bo ParV/Mayo8oya/2022/JPN (Mayo8oya), and Bo ParV/Mayo18/2022/JPN (Mayo18)—have been deposited in the DNA Data Bank of Japan under the accession numbers LC790729 to LC790731.

Bo ParV sequences were aligned using ClustalW [7], and pairwise sequence identity values were calculated using CLC Genomics Workbench 7.5.5 (CLC bio). Phylogenetic analysis based on nucleotide sequences was performed using the maximum-likelihood (ML) method with the best-fit model (K2+G for 5'UTR, T92 + G + I for VP1, GTR+G for 2C+3CD, and 3'UTR for HKY) in MEGA7 [8]. The trees were evaluated by bootstrap analysis with 1000 replicates [9]. In the phylogenetic trees, except for the 3'UTR, Mayo3, Mayo8oya, and Mayo18 branched distantly from other Bo ParVs and formed an independent cluster with 100% bootstrap support (Fig. 1A, B, and C). Pairwise complete nt and aa sequence identity was calculated using CLC Genomics Workbench. In the VP1 sequence comparison, Mayo3, Mayo8oya, and Mayo18 exhibited 72.2–75.6% nt and 85.7–87.0% aa sequence identity to the other Bo ParV strains, which shared 85.1–99.1% nt and 95.7–100% aa sequence identity with each other. The VP1 sequences of parechovirus A and parechovirus B strains were 62.1–73.5% and 65.3–74.0% identical at the nucleotide level and 63.3–80.6% and 69.7–86.1% identical at the aa level (Table 1A). No exact genotype classification criteria have been established for parechoviruses; however, previous reports have referred to the criteria used for enteroviruses: 75% nt sequence identity and 88% aa sequence identity [10–13]. The nt sequence identity of the VP1 region between Bo ParVs in this study and previous Bo ParVs is borderline, but the aa sequence identity is below 88%, suggesting that these viruses might represent a novel genotype of Bo ParV. The 2C+3CD regions of Mayo3, Mayo8oya, and Mayo18, and other Bo_ParVs showed higher similarity than the VP1 region (85.6–86.1% nt sequence identity and 97.3–98.6% aa sequence identity) (Supplementary Table S2). Similarity plot analysis performed using SimPlot software v. 3.5.1 [14] showed that the new Bo ParV isolates have a non-structural protein region from 3A to 3D that is most similar to those of the previously reported Bo ParV (Supplementary Fig. S3). The 2C+3CD sequence identity values obtained when excluding Mayo3, Mayo8oya, and Mayo18 and parechovirus A and parechovirus B strains were 89.6–99.0%, 76.0–80.8%, and 72.2–78.7% at the nt level and 98.2–98.8%, 91.9–98.0%, and 83.0–91.9% at the aa level (Supplementary Table S2). These results indicate that the non-structural genes, especially the 3A to 3D regions, are more conserved among Bo ParVs than they are among other parechoviruses. The Bo ParVs from this study and previous Bo_ParVs shared only 69.2–71.5% sequence identity in the 5'UTR while showing high sequence similarity (96.2–100% identity) in the 3'UTR. However, the Bo ParVs exhibited great sequence divergence from other parechoviruses (19.1–38.9%) in the 3'UTR (Table 1B).

The secondary structures of the viral RNAs were predicted using RNAfold in the ViennaRNA package (version 2.4.18) [15]. The 3'UTR of the Bo_ParVs is 75 nucleotides long, excluding the poly-A tract. Secondary structure

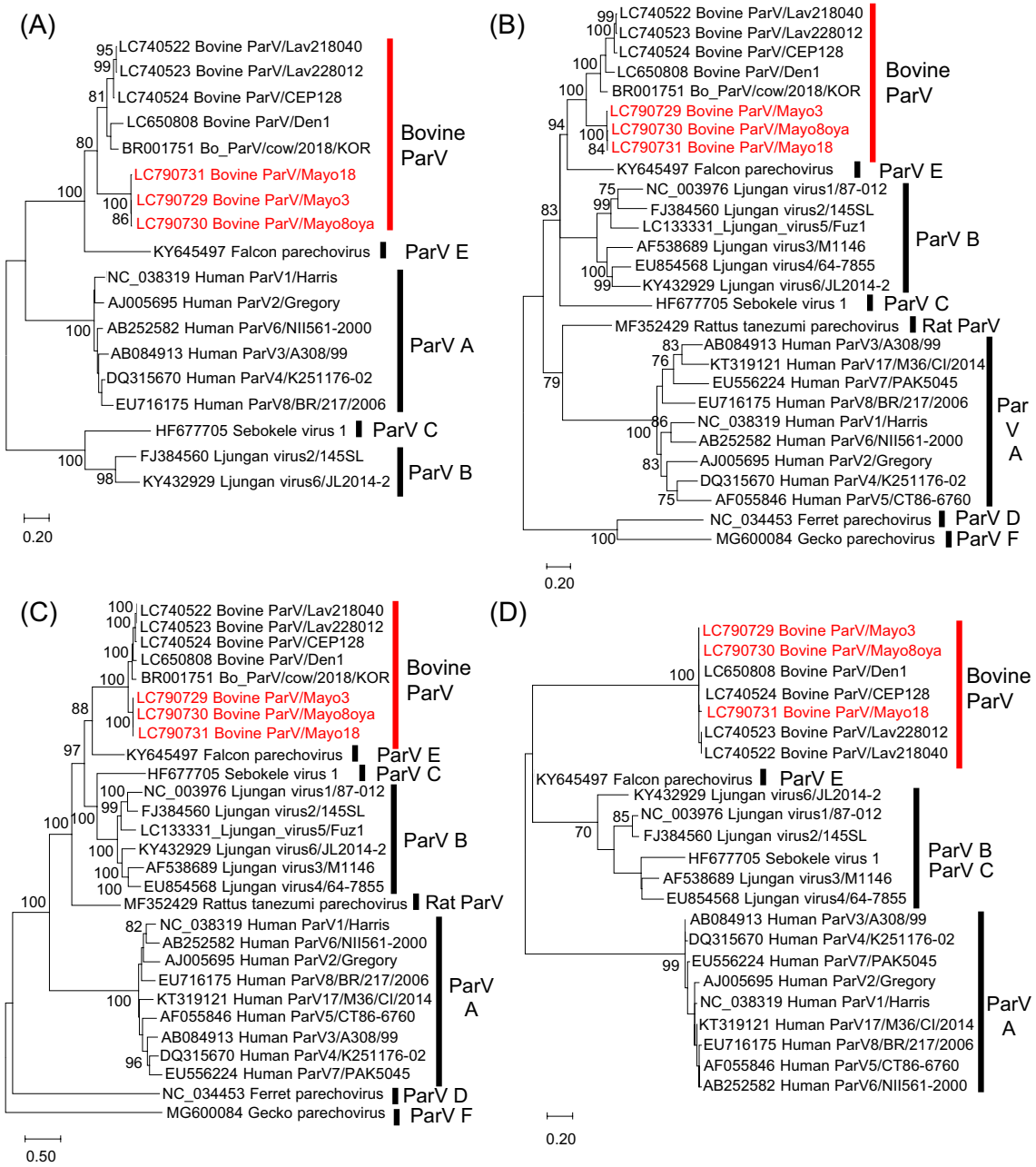

Fig. 1 Phylogenetic analysis based on nucleotide sequences of the 5′UTR (**A**), VP1 region (**B**), 2C+3CD region (**C**), and 3′UTR (**D**) of the Bo_ParVs identified in this study (red text) and of other parechoviruses, obtained from the DDBJ/EMBL/GenBank database. Phylogenetic trees were constructed using the maximum-likelihood method in MEGA7 with best-fit models (K2+G for the 5′UTR, T92+G+I for VP1, GTR+G for 2B+3CD, and HKY for the 3′UTR). Bootstrap values above 70 (1000 replicates) are shown. The bars represent the corrected genetic distances

prediction analysis revealed that Bo ParVs possess two potential stem-loops that are structurally similar to those found in the 3'UTR of Ljungan virus 1-4 (LV-1/bank voles/87-012/SWE, LV-2/bank voles/145SL/SWE, LV-3/vole/M1146/USA, and LV-4/64-7855/USA), belonging to the species *Parechovirus beljungani,* and Falcon/2014/HUN, belonging to the species *Parechovirus efalco* [16, 17]. Although the 3'UTR of Bo ParV strains was the shortest among parechoviruses, the predicted stem-loop structures in domain II, corresponding to the 3' one-third of the 3'UTR, were conserved among these viruses (Fig. 2). The 3'UTR is important for maintaining viral genomic RNA stability and

Table 1 Pairwise nucleotide (lower left) and amino acid (upper right) sequence identities (%) of theVP1 among parechoviruses (A)

(A) VP1	amino / nucleotide	Novel Bo ParV			Bo ParV					ParV A									ParV B						ParV C	ParV D	ParV E	ParV F
		Mayo3	MayoSoya	Mayo18	Den1	2018/KOR	LaV218040	Lav228012	CEP128	EV22	Gregory	A308/99	K251176	CT86-6760	NII561	PAK5045	BR/217	M36/CI	87-012	145SL	M1146	64-7855	Fuzl	JL2014-2	Sebokele	Ferret	Falcon	Gecko
Novel	Bo ParV/Mayo3/2021/JPN		99.7	100	85.7	87.0	86.1	86.1	85.7	31.2	34.9	33.9	32.1	33.8	34.6	29.9	33.2	32.2	49.7	47.8	50.0	48.8	48.8	51.2	46.9	30.6	58.6	31.8
Bo	Bo ParV/Mayo8oya/2021/JPN	99.0		99.7	85.7	87.0	86.1	86.1	85.7	31.2	34.9	33.9	32.1	33.8	34.6	29.9	33.2	32.2	49.7	47.8	50.0	48.8	48.8	51.2	46.9	30.6	58.3	31.8
ParV	Bo ParV/Mayo18/2021/JPN	99.3	99.5		85.7	87.0	86.1	86.1	85.7	31.2	34.9	33.9	32.1	33.8	34.6	29.9	33.2	32.2	49.7	47.8	50.0	48.8	48.8	51.2	46.9	30.6	58.6	31.8
	LC650808_Bo ParV/Den1/2021/JPN	72.4	72.3	72.2		96.7	98.3	98.3	98.0	32.6	35.2	34.6	32.5	33.8	34.6	30.9	33.6	32.6	51.2	48.2	49.1	47.8	49.4	49.4	47.2	30.6	57.4	32.1
	BR001751 Bo ParV/cow/2018/KOR	75.4	75.4	75.2	85.7		96.0	96.0	95.7	32.6	35.6	34.2	32.8	34.1	34.2	31.2	33.6	32.6	51.2	48.5	49.7	47.5	49.7	50.3	48.8	30.9	59.3	32.1
Bo ParV	LC740522_Bo ParV/LaV218040/2022/JPN	75.6	75.5	75.5	85.4	84.5		100	99.7	32.9	35.2	33.9	32.1	33.4	33.9	31.2	33.2	32.6	51.9	48.8	49.7	47.8	49.7	49.1	46.9	30.9	57.7	31.8
	LC740523_Bo ParV/Lav228012/2022/JPN	75.3	75.2	75.2	85.1	84.2	99.1		99.7	32.9	35.2	33.9	32.1	33.4	33.9	31.2	33.2	32.6	51.9	48.8	49.7	47.8	49.7	49.1	46.9	30.9	57.7	31.8
	LC740524_Bo ParV/CEP128/2022/JPN	74.9	74.8	74.5	85.1	84.9	96.0	96.0		33.2	35.2	33.9	32.1	33.4	33.9	31.2	33.2	32.6	51.9	48.8	49.7	47.8	49.7	49.1	46.9	30.9	57.4	31.5
	NC_038319_Human_parechovirus_1_EV22	38.7	38.5	38.7	37.7	37.8	38.9	38.5	38.1		78.6	70.2	77.5	74.9	80.6	68.5	73.9	69.1	30.5	30.5	32.8	32.2	30.2	30.0	28.6	26.9	30.1	24.9
	AJ005695_Human_parechovirus_2_Gregory	39.2	39.2	39.2	37.9	38.6	37.7	37.8	37.6	71.5		70.5	75.7	72.8	72.6	68.4	73.4	67.7	34.0	31.8	33.8	34.1	32.4	31.2	30.2	27.9	32.0	25.9
	AB084913_Human_parechovirus_3_A308/99	38.3	38.4	38.7	37.9	36.9	37.8	37.8	37.2	66.8	66.8		68.2	64.8	72.6	77.3	71.1	80.0	32.4	30.5	31.9	33.8	30.5	31.2	30.2	26.0	32.0	27.4
	DQ315670_Human_parechovirus_4_K251176-02	36.7	36.8	36.8	37.2	37.3	37.8	37.4	37.5	70.2	71.1	67.9		80.0	71.9	66.5	74.9	67.1	29.8	28.5	31.1	31.1	29.5	29.3	29.8	27.2	30.0	24.6
ParV A	AF055846_Human_parechovirus_5_CT86-6760	37.1	36.6	36.8	38.7	37.4	38.4	38.2	37.9	69.9	69.2	63.0	72.6		70.6	64.8	71.5	65.0	30.1	29.2	30.8	31.8	30.4	29.3	29.5	26.9	31.0	25.2
	AB252582_Human_parechovirus_6_NII561-2000	38.9	38.9	39.1	39.6	39.9	39.8	39.6	39.5	71.9	65.8	67.5	67.7	65.7		63.3	71.3	67.9	30.8	30.2	32.2	33.1	29.9	30.9	29.9	26.9	31.4	26.5
	EU556224_Human_parechovirus_7_PAK5045	38.1	38.0	38.0	39.0	38.5	40.5	40.1	39.5	64.7	65.7	69.1	66.8	63.3	62.3		71.6	76.5	30.2	28.9	30.9	31.2	28.6	28.1	28.0	24.7	29.5	24.9
	EU716175_Human_parechovirus_8_BR/217/2006	38.4	38.3	38.5	37.8	37.8	38.5	38.2	37.7	69.1	67.4	68.4	68.5	66.5	65.1	68.3		68.2	31.8	30.5	32.2	34.1	30.2	30.6	28.6	26.3	28.6	24.6
	KT319121_Human_parechovirus_17_M36/CI/2014	35.9	36.0	36.0	36.7	37.1	37.0	36.9	36.8	65.0	65.0	73.5	67.5	62.1	65.4	70.9	67.4		30.2	28.9	31.6	32.5	29.9	30.0	28.9	25.3	29.8	26.8
	NC_003976_Ljungan_virus_1_87-012	52.6	52.3	52.3	52.0	52.9	54.1	53.8	53.9	35.6	34.4	35.3	33.7	34.8	34.9	32.3	34.3	33.4		79.2	71.6	69.7	86.1	69.7	45.6	31.5	53.5	34.9
	FJ384560_Ljungan_virus_2_145SL	51.8	51.7	51.5	52.1	51.7	53.0	52.9	52.9	34.8	33.3	33.5	32.5	33.5	35.0	32.7	33.1	34.2	71.9		71.3	72.2	82.0	70.0	45.6	30.6	52.0	33.0
ParV B	AF538689_Ljungan_virus_3_M1146	54.0	53.8	53.9	52.2	50.7	52.6	52.3	52.6	33.2	34.4	32.4	33.7	32.4	34.4	33.3	35.6	33.5	67.1	66.1		80.7	71.9	79.1	44.1	30.9	53.2	34.2
	EU854568_Ljungan_virus_4_64-7855	53.7	53.6	53.8	52.2	51.8	52.6	52.7	52.3	35.6	34.0	33.9	33.8	34.1	36.2	33.3	35.2	34.9	66.2	67.0	72.1		71.6	83.2	45.3	30.6	51.7	32.1
	LC133331_Ljungan_virus_5_Fuzl	52.5	52.4	52.4	54.8	51.2	53.0	52.8	53.0	34.4	34.7	33.8	34.2	34.2	35.5	32.6	33.6	33.1	72.6	70.6	66.1	67.4		71.0	46.9	30.3	53.2	34.6
	KY432929_Ljungan_virus_6_JL2014-2	52.7	52.7	52.8	50.8	51.5	51.8	51.5	51.1	34.9	32.9	34.8	33.1	32.8	36.2	33.2	34.8	34.7	65.3	66.6	70.0	74.0	68.6		45.6	30.3	51.7	33.0
ParV C	HF677705_Sebokele_virus_1	50.1	50.2	50.3	50.3	50.7	50.2	50.2	50.5	34.7	34.3	35.3	34.5	33.9	36.3	32.6	33.9	33.7	52.2	49.9	50.1	49.1	51.3	50.8		31.7	51.7	33.8
ParV D	NC_034453_Ferret_parechovirus	40.3	40.6	40.4	39.4	39.3	38.6	38.4	38.9	31.5	31.9	31.1	32.8	32.2	33.8	32.0	31.8	31.7	40.1	40.7	41.2	40.6	41.2	40.6	41.8		33.5	39.3
ParV E	KY645497_Falcon_parechovirus	57.2	57.1	57.1	58.6	57.6	57.0	57.2	57.5	34.4	35.2	37.1	35.0	33.3	35.6	35.3	35.1	34.4	54.8	52.7	54.8	52.4	54.7	52.2	49.7	40.6		33.8
ParV F	MG600084_Gecko parechovirus	38.5	38.4	38.4	39.4	39.6	38.8	38.3	38.7	32.2	30.2	32.2	32.0	32.5	33.0	31.5	31.9	31.0	42.8	41.9	42.4	42.1	42.0	41.3	42.6	52.1	37.6	

(B)	5'UTR / 3'UTR	Novel Bo ParV			Bo ParV				ParV A									ParV B					ParV C	ParV E
		Mayo3	MayoSoya	Mayo18	cow/2018	Den1	LaV218040	Lav228012	CEP128	EV22	Gregory	A308/99	K251176	CT86-6760	NII561	PAK5045	BR/217	M36/CI	87-012	145SL	M1146	64-7855	JL2014-2	Sebokele	Sebokele
Novel	Bo ParV/Mayo3/2021/JPN		99.1	98.8	69.6	71.4	70.5	69.5	71.1	43.0	41.6	42.0	42.7	-	43.5	-	43.2	-	-	37.9	-	-	36.7	36.7	51.0
Bo	Bo ParV/Mayo8oya/2021/JPN	100		98.4	69.4	71.2	70.2	69.2	70.5	43.1	41.7	42.0	42.6	-	43.1	-	43.0	-	-	37.6	-	-	36.5	36.7	51.3
ParV	Bo ParV/Mayo18/2021/JPN	98.7	98.7		69.2	71.5	70.6	69.6	70.7	42.9	41.4	42.0	42.7	-	43.2	-	43.0	-	-	37.9	-	-	36.8	36.2	50.9
	BR650808 Bo ParV/cow/2018/KOR	- *	-	-		81.2	79.6	78.8	79.2	43.7	42.6	43.0	42.9	-	44.2	-	43.2	-	-	36.5	-	-	37.0	34.8	51.0
	LC650808 Bo ParV/Den1/2021/JPN	100	100	98.7			83.3	82.4	82.8	44.1	43.1	43.4	44.4	-	45.1	-	44.7	-	-	37.4	-	-	37.1	35.7	53.0
Bo ParV	LC740522 Bo ParV/LaV218040/2022/JPN	98.7	98.7	97.5	-	98.7		97.2	94.9	43.3	42.6	43.5	44.2	-	45.7	-	43.7	-	-	37.1	-	-	37.7	35.4	53.7
	LC740523 Bo ParV/Lav228012/2022/JPN	97.5	97.5	96.2	-	97.5	98.7		95.2	43.2	42.8	43.5	44.0	-	45.7	-	43.2	-	-	36.4	-	-	37.7	35.4	52.9
	LC740524 Bo ParV/CEP128/2022/JPN	98.7	98.7	97.5	-	98.7	97.5	96.2		43.1	42.6	43.6	44.3	-	45.5	-	43.5	-	-	36.7	-	-	37.8	35.7	53.2
	NC_038319_Human_parechovirus_1_EV22	21.2	21.2	21.2	-	21.2	21.2	20.2	20.2		82.9	82.9	85.0	-	84.9	-	80.8	-	-	40.9	-	-	42.1	39.8	37.6
	AJ005695_Human_parechovirus_2_Gregory	25.0	25.0	25.0	-	25.0	25.0	24.0	26.0	86.3		84.5	83.5	-	84.8	-	80.1	-	-	41.0	-	-	40.3	40.2	38.9
	AB084913_Human_parechovirus_3_A308/99	23.8	23.8	23.8	-	23.8	23.8	22.9	22.9	85.3	88.4		85.7	-	86.4	-	82.1	-	-	42.2	-	-	41.9	39.4	38.1
	DQ315670_Human_parechovirus_4_K251176-02	23.8	23.8	23.8	-	23.8	23.8	22.9	22.9	83.2	88.4	96.8		-	88.3	-	87.2	-	-	40.5	-	-	40.3	39.5	39.8
ParV A	AF055846_Human_parechovirus_5_CT86-6760	21.7	21.7	21.7	-	21.7	21.7	20.8	22.6	82.3	86.5	82.3	81.3		-	-	-	-	-	-	-	-	-	-	-
	AB252582_Human_parechovirus_6_NII561-2000	23.6	23.6	23.6	-	23.6	23.6	22.6	24.5	78.4	85.6	80.4	80.4	91.8		-	85.2	-	-	42.4	-	-	41.9	39.7	39.6
	EU556224_Human_parechovirus_7_PAK5045	22.9	22.9	22.9	-	22.9	22.9	21.9	21.9	84.2	88.4	95.8	94.7	82.3	80.4		-	-	-	-	-	-	-	-	-
	EU716175_Human_parechovirus_8_BR/217/2006	24.5	24.5	24.5	-	24.5	24.5	23.6	23.6	85.4	90.6	87.5	86.5	87.5	86.6	87.5		-	-	41.2	-	-	40.5	38.1	38.8
	KT319121_Human_parechovirus_17_M36/CI/2014	24.5	24.5	24.5	-	24.5	24.5	23.6	23.6	85.4	87.5	88.5	87.5	85.4	84.5	88.5	93.8	-	-	-	-	-	-	-	-
	NC_003976_Ljungan_virus_1_87-012	28.9	28.9	28.1	-	28.9	28.1	28.1	28.1	26.7	28.3	28.3	28.3	30.8	30.0	29.2	30.0	29.2		-	-	-	-	-	-
	FJ384560_Ljungan_virus_2_145SL	28.2	28.2	27.4	-	28.2	27.4	27.4	27.4	26.7	27.7	29.2	29.2	30.0	29.2	30.0	29.2	29.2	91.5		-	-	73.5	56.3	36.6
ParV B	AF538689_Ljungan_virus_3_M1146	26.1	26.1	25.2	-	26.1	25.2	25.2	26.1	28.1	29.8	27.8	27.8	29.6	29.6	28.7	29.6	28.7	60.5	61.2		-	-	-	-
	EU854568_Ljungan_virus_4_64-7855	20.0	20.0	19.1	-	20.0	19.1	19.1	20.0	19.3	21.1	21.7	21.7	24.4	23.5	22.6	22.6	21.7	61.0	63.5	73.8		-	-	-
	KY432929_Ljungan_virus_6_JL2014-2	26.8	26.8	25.9	-	26.8	26.8	27.7	25.9	28.1	28.1	27.8	27.8	30.4	30.4	27.8	28.7	29.6	63.6	67.0	75.5	64.8		55.6	36.1
ParV C	HF677705_Sebokele_virus_1	35.2	35.2	34.1	-	35.2	34.1	33.0	35.2	21.9	21.1	22.6	22.6	24.1	23.3	23.5	22.4	21.6	42.2	42.4	46.8	45.4	43.2		36.9
ParV E	KY645497_Falcon_parechovirus	38.9	38.9	38.0	-	38.9	38.0	37.0	38.0	33.3	35.3	35.0	35.9	35.9	35.9	34.2	34.2	35.0	50.4	52.1	48.7	40.9	51.3	39.1	39.1

*: Not done (full-length or comparable sequences could not be obtained)

Pairwise nucleotide sequence identities (%) of the 5'UTR (upper right) and 3'UTR (lower left) among parechoviruses (B). Pink, orange, and yellow show indicate >90%, 90–80%, and 80–70% identities, respectively

for replication of the picornavirus genome [18–20]. Secondary structure analysis of the internal ribosomal entry site (IRES) in the 5'UTR and the putative *cis*-acting RNA element (cre) located in the 3B region, which are necessary for viral RNA replication, showed that these structures in Bo ParVs, including the novel Bo_ParVs, were similar to those of Ljungan virus 1-4 and falcon parechovirus (Supplementary Fig. S4). These results indicate that there are some evolutionary constrains in these regions.

In summary, novel Bo ParVs were isolated from Japanese black cattle with or without diarrhea. The genome sequences of these viruses exhibited differences when compared to previous Bo ParVs, suggesting that they represent a novel genotype of Bo ParV. Secondary structure prediction

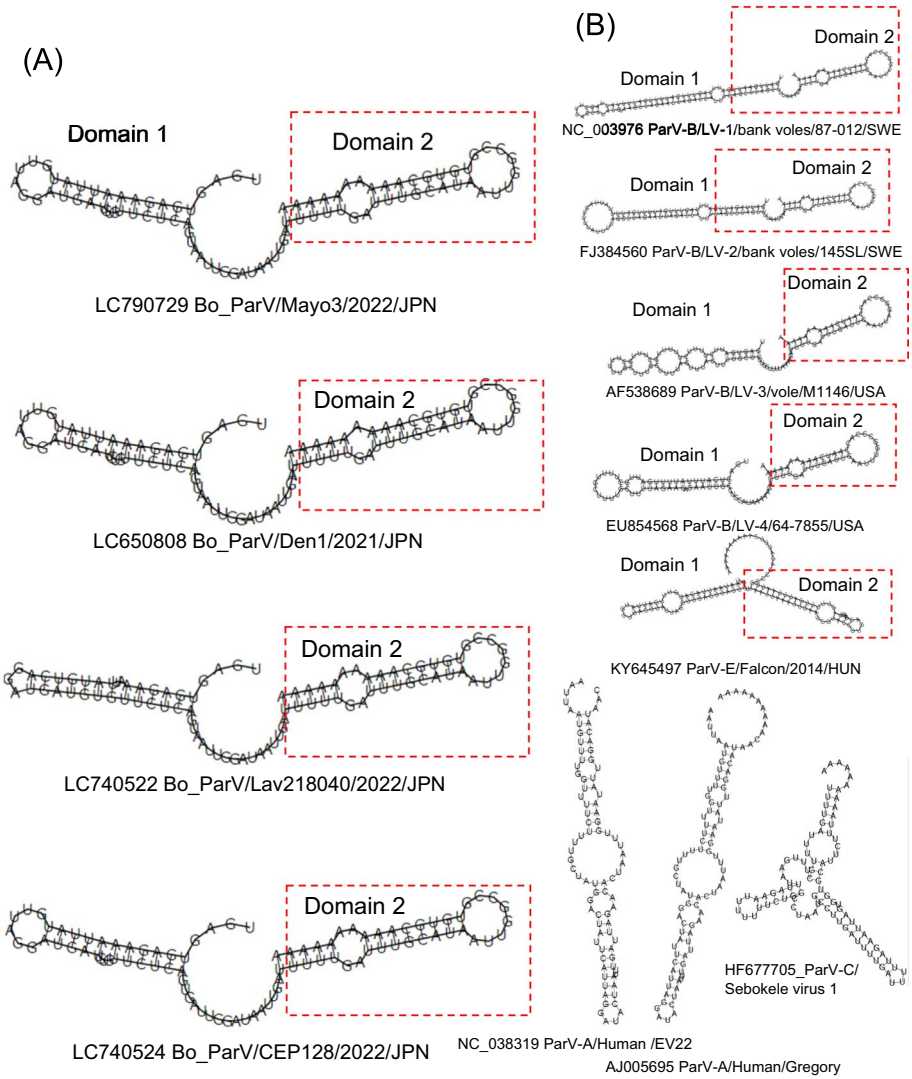

Fig. 2 Secondary structure prediction of the 3′UTR of Bo_ParVs (**A**) and other parechoviruses (**B**) using RNAfold in the ViennaRNA package (version 2.4.18). Analysis of the 3'UTR included 10 nt of the poly(A) tail to simulate the authentic viral RNA molecule

suggested structural conservation in the 5'UTR, cre, and 3'UTR between Bo ParVs and other parechoviruses.

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Data availability The GenBank/EMBL/DDBJ accession numbers for the sequences of the Bovine ParV/Mayo strains determined in this study are LC790729 to LC790731. Other datasets generated or analyzed during the current study are available from the corresponding authors upon reasonable request.

Declarations

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

Ethical approval The fecal samples that were used in this study were collected for pathological testing of cattle; therefore, no specific approval was needed.

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