

Phylogenetic analysis of porcine astrovirus in domestic pigs and wild boars in South Korea

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Abstract Porcine astrovirus (PAstV) belongs to genetically divergent lineages within the genus Mamastrovirus. In this study, 25/129 (19.4 %) domestic pig and 1/146 (0.7 %) wild boar fecal samples tested in South Korea were positive for PAstV. Positive samples were mainly from pigs under 6 weeks old. Bayesian inference (BI) tree analysis for RNA-dependent RNA polymerase (RdRp) and capsid (ORF2) gene sequences, including Mamastrovirus and Avastrovirus, revealed a relatively geographically divergent lineage. The PAstVs of Hungary and America belong to lineage PAstV 4; those of Japan belong to PAstV 1; and those of Canada belong to PAstV 1, 2, 3, and 5, but not to 4. This study revealed that the PAstVs of Korea belong predominantly to lineage PAstV 4 and secondarily to PAstV 2. It was also observed that PAstV infections are widespread in South Korea regardless of the disease state in domestic pigs and in wild boars as well.

Keywords Pig · Astrovirus · Phylogeny

Astroviruses are single-stranded positive-sense RNA viruses of approximately 7 kb in length, with spherical, non-enveloped virions of about 30 nm in diameter. These viruses generally exhibit a distinctive five- or six-pointed star-shape appearance when viewed by electron microscopy (EM) [1]. As a cause of gastroenteritis in young children, astrovirus infections are currently second only to rotavirus infections in importance, but in animals

their association with enteric diseases is not well documented, with the exception of turkey and mink astrovirus infections [2].

Family Astroviridae is separated into two genera. Viruses of the genus Mamastrovirus infect mammals, and those of Avastrovirus infect avian [3]. Avastroviruses include duck astrovirus 1 (DAstV-1), turkey astrovirus 1 and 2 (TAstV-1 and TAstV-2), and avian nephritis virus (ANV) [2]. Mamastroviruses appear to have a broad host range, including human [1], sheep [4], cow [5], pig [6], dog [7], cat [8], red deer [9], mouse [10], mink [11], bat [12], cheetah [13], brown rat [14], roe deer [15], sea lion and dolphin [16], and rabbit [17].

Porcine astrovirus (PAstV) was first detected by EM in the feces of a diarrheic piglet [6] and was later isolated in culture [18]. Molecular characterization of the capsid (ORF2) gene from this isolate followed some years later [19]. Since then, research groups have successfully used PCR approaches to investigate the presence and diversity of PAstV [20–22]. PAstV has been detected in several countries, including South Africa [23], the Czech Republic [20], Hungary [22], Canada [21], and Colombia [24]. In South Korea, there have been studies done on astrovirus but were only limited to its detection in human infection. There has been no attempt yet to know the extent of astrovirus infection in the pig population of the country. It was, therefore, the aim of this study to investigate the genetic groups of Korean PAstV in domestic pigs and wild boars and to identify the incidence of co-infection with other porcine enteric viruses as well.

A total of 129 fecal samples of domestic pigs (60 piglets under 3 weeks old, 45 weaned pigs, 14 growing-finishing pigs, and 10 sows over 1 year old) was collected from six piggery farms with good breeding facilities in four provinces of South Korea from January to June 2011. Out of

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these collected samples 90 were from diarrheic and 39 were from non-diarrheic pigs. A total of 146 fecal samples of wild boars over 1 year old was collected from the wildlife areas in five provinces of South Korea during the hunting season from December 2010 to January 2011. Out of these collected samples 34 were from diarrheic and 112 were from non-diarrheic boars.

Viral RNA was extracted from the feces using TRIzol LS^b according to the manufacturer's instructions. PAsV was detected in fecal specimens by RT-PCR, as previously described [22], with primers specific for the RdRp and ORF2 regions of PAsV (PAsV-F, 5'-TGACATTTT GTGGATTACAGTT-3' and PAsV-R: 5'-CACCCAGG GCTGACCA-3'). The RT-PCR process resulted in the amplification of a 799-nt-long fragment at an annealing temperature of 45 °C. Products of the expected size were cloned with the pGEM-T Vector System IITM (Promega, Cat. No. A3610, USA). The cloned gene was sequenced with T7 and SP6 sequencing primers on an ABI Prism[®] 3730xi DNA Sequencer (Applied Biosystems, Foster City, CA, USA) at the Macrogen Institute (Macrogen, Seoul, Korea). The sequences of all the positive samples for PAsV were submitted to GenBank under accession numbers JQ696831–JQ696856. The astroviruses used in this study are listed in Table 1 along with their GenBank accession numbers.

To investigate the relationship between Astroviruses and other economically important viral diseases that cause diarrhea in piglets in Asia, screening tests were conducted to detect Porcine Epidemic Diarrhea Virus (PEDV), Transmissible Gastroenteritis Virus (TGEV), and Porcine Group A Rotavirus (GAR), as previously described [25]. The primer pairs used in this study were P1 (TTCTGA GTCA CGAACAGCCA, 1466–1485) and P2 (CATATG CAGCCTGCTCTGAA, 2097–2116) for the S gene of PEDV, T1 (GTGGTTTTGGTYRTAAATGC, 16–35) and T2 (CACTAACCAACGTGGARCTA, 855–874) for the S gene of TGEV, and rot3 (AAAGATGCTAGGGACAAA ATTG, 57–78) and rot5 (TTCAGATTGT GGAGCTA TTCCA, 344–365) for the segment 6 region of group A Rotavirus. The sizes of the expected products of multiplex RT-PCR were 859 bp for TGEV, 651 bp for PEDV, and 309 bp for rotavirus, which could be differentiated by agarose gel electrophoresis.

Out of the 129 domestic pig fecal samples tested, 25 were positive for PAsV. Prevalence of PAsV in weaned pigs (35.6 %, 16/45) was higher than that in suckling piglets (15.0 %, 9/60) and in growing-finishing pigs (9.1 %, 1/14) (Table 2). Only one wild boar which is coming from the province of Gyunggi tested positive for PAsV (0.7 %, 1/146). The low prevalence of PAsV in wild boars might have been due to the fact that usually older animals (over 1 year old) have lesser susceptibility to

infection and generally wild pigs are more resistant to many diseases than the domesticated ones.

The percentage of samples that were PAsV-positive differed among the six pig farms: Chungchong A, 28.6 % (10/35); Chungchong B, 22.9 % (8/35); Kangwon, 20.0 % (4/20); Gyunggi A, 7.1 % (1/14); Gyunggi B, 20.0 % (3/15), and Gyungsang, 0 % (0/10). The low or no incidence of PAsV in Gyunggi A (growing-finishing pigs) and Gyungsang (sows) can likewise be attributed to the lesser susceptibility of adult pigs to infection. The proportion of non-diarrheic and diarrheic pig fecal samples was 14.0 % (18/129) and 6.2 % (8/129), respectively. These results suggest that PAsV is widespread in South Korea regardless of the disease status (with or without clinical manifestations) of pigs. Although Astroviruses are highly prevalent in young pigs and are mostly present in diarrheic pigs, PAsV is a common finding as well in the fecal samples of apparently healthy pigs [21, 22]. The clinical significance of PAsV infection remains to be clarified.

The clinical symptoms of diarrhea are frequently reported to be associated with Rotavirus, Coronavirus, and Calicivirus-like infections in piglets [6, 18, 20, 21, 26]. Although PEDV and TGEV infections were not identified in any of the fecal samples, porcine GAR infection was identified in 6.2 % (17/275) of the samples collected from suckling pigs under 3 weeks old (Table 2). Furthermore, coinfection with PAsV and GAR was observed in two cases (one in diarrheic and one in non-diarrheic pig fecal samples). However, it is not cleared yet if GAR is directly associated with astrovirus infection in pigs.

All Astrovirus sequences were aligned initially with the ClustalX 1.8 program [27]. The nucleotide sequences were translated and the nucleotide and amino acid sequence identities among the astrovirus strains were calculated with BIOEDIT 7.053 [28]. Bayesian trees were generated with MrBayes 3.1.2 [29, 30] using best-fit models which were selected with ProtTest 1.4 [31] for amino acid alignment. Markov Chain Monte Carlo (MCMC) analyses were run with 1,000,000 generations for each amino acid sequence.

Bayesian posterior probabilities by MrBayes 3.1.2 were estimated on the basis of a 70 % majority rule consensus of the trees. For each analysis, a chicken astrovirus (NC_003790) was specified as the outgroup and a graphic output was produced with TreeView 1.6.1 [32]. The best models of the RdRp and ORF2 amino acid sequences were obtained using ProtTest 1.4, which showed WAG + I + G and WAG + G + F, respectively, according to the results of the Akaike information criterion (AIC).

BI trees for the RdRp (Fig. 1) and ORF2 (Fig. 2) amino acid sequences revealed the presence of five unrelated PAsV lineages. The first lineage (PAsV 1) contained the PoAsV12-3 strain (HM756258) from Canadian pig and two porcine strains (Y15938 and AB037272) derived from Japanese pigs,

Table 1 Astrovirus isolates used in the present study

Virus strains or name	Place and year	Species	Accession nos.
Human astrovirus 1	South Korea, 2006	Human	JN887820
Human astrovirus 2		Human	*L06802
Human astrovirus 3		Human	AF292074
Human astrovirus 4	China, 2007	Human	DQ344027
Human astrovirus 5	Brazil	Human	DQ028633
Human astrovirus 6		Human	AF292077
Human astrovirus 7		Human	AF248738
Human astrovirus 8		Human	AF260508
HMO astrovirus A	Nigeria, 2007	Human	NC_013443
HMO astrovirus B	Nizeria, 2007	Human	GQ415661
HMO astrovirus C	Thailand, 2001	Human	GQ415662
Astrovirus VA1	USA, 2008	Human	FJ973620
Astrovirus MLB1	Australia, 1999	Human	FJ222451
Feline astrovirus		Cat	*AF056197
Dog astrovirus	Italy, 2005	Dog	FM213330
Mink astrovirus		Mink	AY179509
Rabbit astrovirus		Rabbit	JN052023
Rat astrovirus	Hong Kong, 2007	Rat	HM450382
Ovine astrovirus		Sheep	NC_002469
CcAstV-1	Denmark, 2010	Deer	HM447045
CcAstV-2	Denmark, 2010	Deer	HM447046
Dolphin AstV1	USA, 2007	Dolphin	FJ890355
Cali. sea lion AstV 1	USA, 2006	Sea Lion	FJ890351
Cali sea lion AstV 2	USA, 2008	Sea Lion	FJ890352
PAstV-2/2007/HUN	Hungary, 2007	Pig	GU562296
PoAstV12-3	Canada, 2006	Pig	HM756258
PoAstV12-4	Canada, 2006	Pig	HM756259
PoAstV14-4	Canada, 2006	Pig	HM756260
PoAstV16-2	Canada, 2006	Pig	HM756261
Porcine astrovirus	Japan	Pig	*Y15938
Porcine astrovirus	Japan, 1983	Pig	*AB037272
Porcine astrovirus CC12		Pig	JN088537
PAstV/1104/MN	USA, 2010	Pig	JF272548
PAstV/1115/MS	USA, 2010	Pig	JF272559
PAstV/1102/IL	USA, 2010	Pig	JF272546
PAstV/1116/PA	USA, 2010	Pig	JF272560
PAstV/1117/MN	USA, 2010	Pig	JF272561
PAstV/1118/NC	USA, 2010	Pig	JF272562
PAstV/1122/MN	USA, 2010	Pig	JF272566
PAstV/1123/MN	USA, 2010	Pig	*JF272567
PAstV/1124/IL		Pig	JF272568
PAstV/1128/VA	USA, 2010	Pig	JF272572
PAstV/1125/NE	USA, 2010	Pig	JF272569
PAstV/1134/KS	USA, 2010	Pig	JF272578
PAstV/1137/NE	USA, 2010	Pig	JF272581

Table 1 continued

Virus strains or name	Place and year	Species	Accession nos.
PAstV/1138/MEX	USA, 2010	Pig	JF272582
PAstV/1139/NC	USA, 2010	Pig	JF272583
PAstV/1140/OK	USA, 2010	Pig	JF272584
PAstV/1142/MN	USA, 2010	Pig	JF272586
PAstV/1154/IL	USA, 2010	Pig	JF272598
PAstV/1155/IL	USA, 2010	Pig	JF272599
PAstK4	Korea/Ch, 2011	Pig	JQ696831
PAstK5	Korea/Ch, 2011	Pig	JQ696832
PAstK12	Korea/Ch, 2011	Pig	JQ696833
PAstK21	Korea/Ch, 2011	Pig	JQ696834
PAstK22	Korea/Ch, 2011	Pig	JQ696835
PAstK29	Korea/Ch, 2011	Pig	JQ696836
PAstK31	Korea/Gy, 2011	Wild boar	JQ696837
PAstK32	Korea/Ch, 2011	Pig	JQ696838
PAstK37	Korea/Ch, 2011	Pig	JQ696839
PAstK54	Korea/Ch, 2011	Pig	JQ696840
PAstK60	Korea/Ka, 2011	Pig	JQ696841
PAstK63	Korea/Ch, 2011	Pig	JQ696842
PAstK65	Korea/Gy, 2011	Pig	JQ696843
PAstK73	Korea/Ch, 2011	Pig	JQ696844
PAstK76	Korea/Gy, 2011	Pig	JQ696845
PAstK78	Korea/Ka, 2011	Pig	JQ696846
PAstK103	Korea/Ka, 2011	Pig	JQ696847
PAstK110	Korea/Ka, 2011	Pig	JQ696848
PAstK114	Korea/Ch, 2011	Pig	JQ696849
PAstK118	Korea/Ka, 2011	Pig	JQ696850
PAstK119	Korea/Ch, 2011	Pig	JQ696851
PAstK120	Korea/Ka, 2011	Pig	JQ696852
PAstK123	Korea/Ch, 2011	Pig	JQ696853
PAstK124	Korea/Ch, 2011	Pig	JQ696854
PAstK126	Korea/Ch, 2011	Pig	JQ696855
PAstK127	Korea/Ch, 2011	Pig	JQ696856
WBAstV-1	Hungary, 2011	Wild boar	JQ340310
AFCD11	Hong Kong, 2005	Bat	EU847145
AFCD57	Hong Kong, 2005	Bat	EU847144
LD38	China, 2007	Bat	FJ571065
LD77	China, 2007	Bat	FJ571066
LD71	China, 2007	Bat	FJ571067
LS11	China, 2007	Bat	FJ571068
Chicken astrovirus1		Chicken	NC_003790

Most of the isolates were used for both RdRp and ORF2 analysis. Few others (with asterisk (*) on the corresponding accession number) were used only for ORF2 analysis. The new sequences are marked in bold fonts

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Table 2 Porcine astrovirus infection status of diarrheic and healthy pigs and its association with other viruses

Animal species	Age	Province	No. of diarrheic and healthy pigs	Reverse transcript (RT)-PCR				
				PAstV	PGAR	PEDV	TGEV	
Domestic pigs	Suckling pigs (≤ 3 weeks; $n = 60$)	Gyunggi and Chungchung	Diarrheic ($n = 33$)	4/33 (12.1 %)	10/33 (30.3 %)	0	0	
			Healthy ($n = 27$)	5/27 (18.5 %)	7/27	0	0	
	Weaned pigs (≤ 6 weeks; $n = 45$)	Kangwon and Chungchung	Diarrheic ($n = 39$)	13/39 (33.3 %)	0	0	0	
			Healthy ($n = 6$)	3/6 (50 %)	0	0	0	
	Growth-finishing pigs (≤ 22 weeks; $n = 14$)	Gyunggi	Diarrheic ($n = 11$)	1/11 (9.1 %)	0	0	0	
			Healthy ($n = 3$)	0	0	0	0	
	Sows (≥ 1 year, $n = 10$)	Gyungsang	Diarrheic ($n = 7$)	0	0	0	0	
			Healthy ($n = 3$)	0	0	0	0	
	Wild boar	Wild boar (≥ 1 year, $n = 146$)	Kangwon	Diarrheic ($n = 4$)	0	0	0	0
				Healthy ($n = 17$)	0	0	0	0
Gyunggi			Diarrheic ($n = 14$)	1/48 (2.1 %)	0	0	0	
			Healthy ($n = 34$)	0	0	0	0	
Gyungsang			Diarrheic ($n = 4$)	0	0	0	0	
			Healthy ($n = 21$)	0	0	0	0	
Jena			Diarrheic ($n = 3$)	0	0	0	0	
			Healthy ($n = 15$)	0	0	0	0	
Chungchung			Diarrheic ($n = 9$)	0	0	0	0	
			Healthy ($n = 25$)	0	0	0	0	
Total	$n = 275$			27/275 (9.8 %)	17/275 (6.2 %)			

The total number of diarrheic and healthy domestic pigs with PAstV was 18/90 (20.0 %) and 8/39 (20.5 %), respectively

PAstV porcine astrovirus, PGAR porcine group A rotavirus, PEDV porcine epidemic diarrhea virus, TGEV transmissible gastroenteritis virus

and it adjoined the lineage of the human and feline astrovirus strains. The second lineage (PAstV 2) contained three Korean strains (JQ696839, JQ69845, and JQ69852), two Canadian strains (HM756259 and HM756260), and deer strains (HM447045 and HM447046). The third lineage (PAstV 3) contained the PoAstV16-2 strain (HM756261) from Canadian pig, and it adjoined the lineage containing human astrovirus. The fourth lineage (PAstV 4) contained only strains of domestic pigs and of a wild boar, including 1 Hungarian (GU562296), 22 Korean, and 19 American strains (Figs. 1, 2). The fifth lineage (PAstV 5) contained the PoAstV CC12 strain (JN088537) derived from Canadian pig.

Interestingly, rat astrovirus (HM450382) and porcine PoAstV CC12 (JN088537) have formed two different lineages on the Bayesian trees for the RdRp and ORF2 amino acid sequences (Figs. 1, 2). Astrovirus strains contained in Group 1 (G1), 2 (G2), and 4 (G4) on the two Bayesian trees showed similar topologies. However, Astrovirus strains in Group 3 (G3) on the Bayesian tree for the ORF2 amino acid sequence were divided into G3 and Group 5 (G5) on the Bayesian tree for the RdRp amino acid sequence (Figs. 1, 2). A strain isolated from a Hungarian wild boar in 2011 [33] belonged to PAstV 4 or Group 4 (G4) that also contained PAstK31 derived from Korean wild boar.

A previous study suggested that the number of PAstV lineages extends to a total of five, all of which most likely

represent distinct species of different origins [34]. However, with the available AstVs research data from countries around the world, future studies could unveil diverse genetic lineages. In this study, the porcine astrovirus strains appeared to be phylogenetically related to not only prototypical human astroviruses (as was already known) but also the recently discovered novel human strains. This finding suggests the existence of multiple cross-species transmission events between the hosts and the other animal species.

Several recent studies have shown that bats form multiple independent lineages [35, 36]. Bat astrovirus strains in this study also showed independent lineages and specifically, the LD71 (FJ571067) strain had a close relationship with astrovirus strains of human, sheep, mink, and sea lion (Figs. 1, 2).

A previous study suggested that porcine AstVs have played an active role in pigs in the evolution and ecology of the Astroviridae [21]. Recent studies have shown evidence of multiple recombination events between distinct PAstV strains and between PAstV and human astrovirus (HAstV) in the variable region of ORF2 [24], as well as interspecies recombination between porcine and deer astroviruses [37].

A study of the molecular epidemiology and genetic diversity of human astrovirus in South Korea from 2002 to 2007 revealed genotype 1 to be the most prevalent, accounting for 72.19 % of strains, followed by genotypes 8

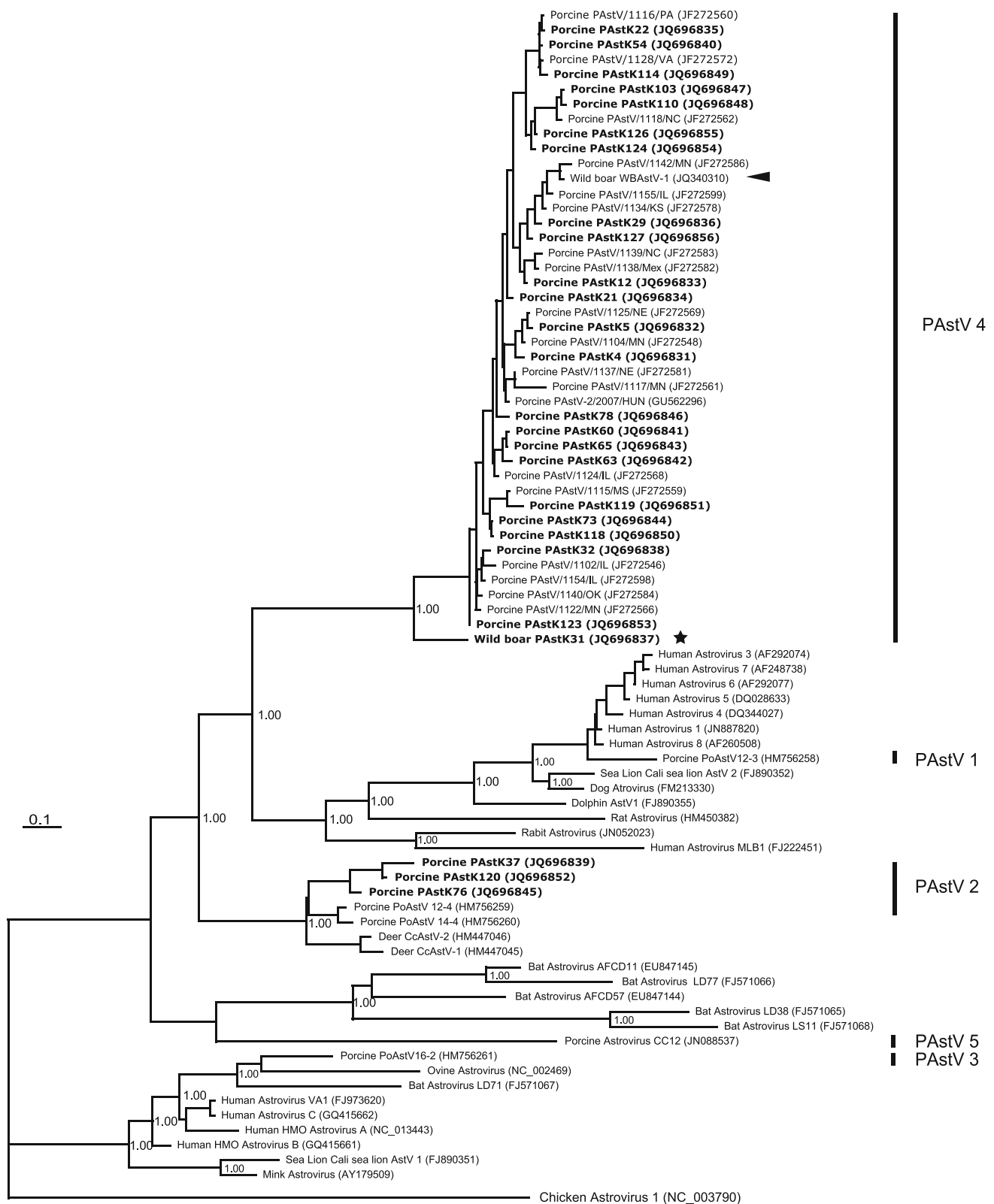


Fig. 1 Phylogenetic relationship of porcine astrovirus and prototypical astrovirus species based on the partial RdRp (ORF1b) amino acid sequence analysis. The best model of the RdRp amino acid sequence by ProTest 1.4 was WAG + I + G (deltaAIC: 0.00, AIC: 7839.43, AICw: 0.53, -lnL: -3760.72). The tree was constructed using the BI method

to show the phylogenetic relationship between the global Mamastrovirus strains and the Avastrovirus strain as the outgroup. Korean PASTVs are shown in **bold** prints, and strains isolates from Korean and Hungarian wild boar are marked with a *star* and an *arrow*, respectively. The *scale bar* indicates the number of nucleotide substitutions per site

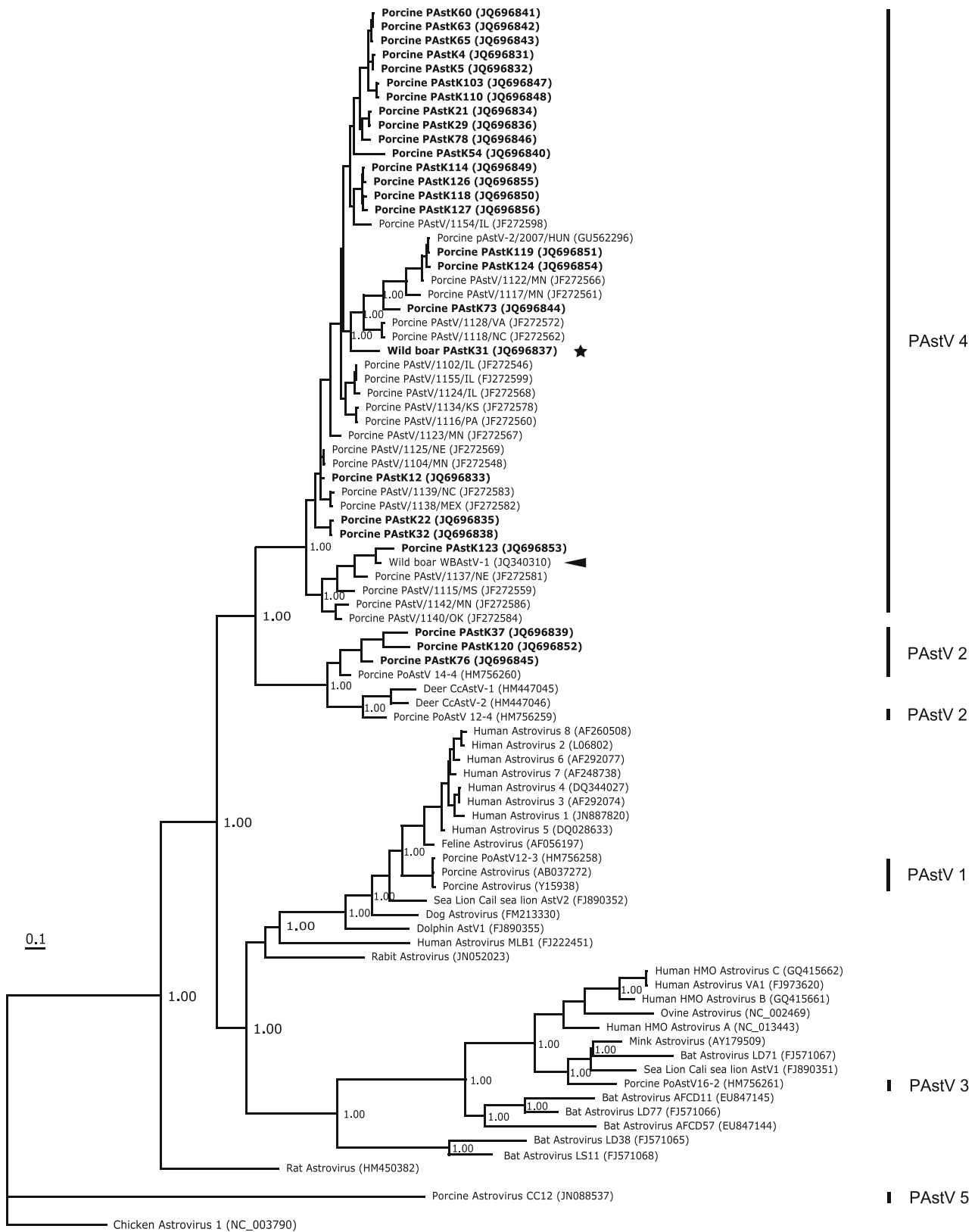


Fig. 2 Phylogenetic relationship of porcine astrovirus and prototypic astrovirus species based on the partial capsid (ORF2) amino acid sequence analysis. The best model of the ORF2 amino acid sequence by ProTest 1.4 was WAG + G + F (deltaAIC: 0.00, AIC: 19367.21,

AICw: 0.66, -lnL: -9496.61). Korean PASTVs are shown in bold prints, and strains isolates from Korean and Hungarian wild boar are marked with a star and an arrow, respectively. The numbers above the nodes represent posterior probabilities

(9.63 %), 6 (6.95 %), 4 (6.42 %), 2 (3.21 %), and 3 (1.6 %) [38]. This finding suggests that little interspecies (between human and pig) transmission has occurred until now in South Korea.

In conclusion, this study extends current knowledge of PAsTV in wild boar and domestic pig. A more extensive study should be done on wild life PAsTVs to further elucidate their potential role in the epidemiological landscape of the astrovirus infection in domestic pig population. To a greater length, continuous surveillance on the prevalence of both PAsTVs will provide a wider understanding of the possible cross-species or human transmissions, in particular.

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