Studies on the genetic recombinations in the enteric RNA viruses detected from cattle and pigs in Japan

（日本の牛及び豚から検出された腸内 RNA ウイルスにおける遺伝子組換えに関する研究）

Mika Ito-Yamamoto

TABLE OF CONTENTS

Abbreviations

ToV: torovirus

BToV: bovine torovirus

- **BToV Ishi:** BToV Ishikawa/2010
- **BToV Kago:** BToV Kagoshima/2014
- **BToV Tochi:** BToV Tochigi/2013
- **BToV Tokyo:** BToV Tokyo/2014

PToV: porcine torovirus

- **PToV Tottori:** PToV Tottori/2015

EToV: equine torovirus

AstV: astrovirus

MAstV: mamastrovirus

PoAstV: porcine astrovirus

- **43/USA:** PoAstV2/USA/43/USA/2010
- **KNU14-07:** PoAstV2/KOR/KNU14-07/2014
- **51/USA:** PoAstV2/USA/51/USA/2010
- **US-IA122:** PoAstV2/USA/US-IA122/2011
- **US-MO123:** PoAstV3/USA/US-MO123/2011
- **US-IL 135:** PoAstV4/USA/US-IL135/2011
- **35/USA:** PoAstV4/USA/35/2010
- **AstV-LL-2:** PoAstV5/CHN/LL-2/2006

NGS: next generation sequencing

S:	spike glycoprotein
M:	membrane glycoprotein
HE:	hemagglutinin esterase
ORF:	open reading frame
N:	nucleocapsid phosphoprotein
UTR:	untranslated region
HRT-18-Aichi cell:	human rectal adenocarcinoma-18-Aichi cell
$TCID_{50}$:	50% median tissue culture infectious dose
RACE:	rapid amplification of cDNA end method
RDP:	recombination detection program
nt:	nucleotide
aa:	amino acid
L1, L2:	lineage 1, lineage 2
CDS:	coding sequence

Notes

Contents of the present thesis were published in the following articles.

1. Ito M, Tsuchiaka S, Naoi Y, Otomaru K, Sato M, Masuda T, Haga K, Oka T, Yamasato H, Omatsu T, Sugimura S, Aoki H, Furuya T, Katayama Y, Oba M, Shirai J, Katayama K, Mizutani T, and Nagai M. Whole genome analysis of Japanese bovine toroviruses reveals natural recombination between porcine and bovine toroviruses. Infect Genet Evol 38, 90-95, 2016.

2. Ito M, Kuroda M, Masuda T, Akagami M, Haga K, Tsuchiaka S, Kishimoto M, Naoi Y, Sano K, Omatsu T, Katayama Y, Oba M, Aoki H, Ichimaru T, Mukono I, Ouchi Y, Yamasato H, Shirai J, Katayama K, Mizutani T, and Nagai M. Whole genome analysis of porcine astroviruses detected in Japanese pigs reveals genetic diversity and possible intra-genotypic recombination. Infect Genet Evol 50, 38-48, 2017.

Preface

Livestock hygiene service centers perform diagnosis of various samples brought directly from the farm. Viral diseases are diagnosed by, examining isolated virus strains; further diagnosis can be performed using diagnostic tools such as antibody tests. In recent years, genetic testing has become increasingly common and is now included in pathological appraisal guidelines (notification of Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, and Forestry and Fisheries). However, many enteric viruses are not successfully isolated using established methods; hence, diagnosis is often dependent on genetic testing.

RNA polymerase and reverse transcriptase do not perform proofreading, thus allowing RNA viruses to mutate at high rates during RNA synthesis and form a group of RNA viruses called quasispecies. Isolated and detected viruses are merely representative of quasispecies, and hosts are often co-infected with multiple strains of viruses [1]. RNA viruses are also prone to genetic recombination at homologous or comparable sites on two parental RNAs [1, 2]. Recombination helps to eliminate errors in RNA synthesis and leads to virus evolution due to genetic changes [1]. Therefore, it is difficult to design primers corresponding to the genetic diversity caused by gene mutation or genetic recombination, especially in RNA viruses [3].

Recently, next generation sequencing (NGS) technology that does not require

specific primers for PCR amplification or concentration has been developed, which makes it possible to analyze the entire genomic sequence of an isolated virus [4]. NGS can also be performed directly from field samples and is hence an effective method for analyzing RNA viruses that are difficult or impossible to propagate in cultured cells in laboratories [3]. In this study, an isolated virus, and the feces of cattle and pigs collected for the diagnosis of infectious diseases from different areas of Japan were metagenomically analyzed by NGS to elucidate the genetic diversity and interspecies transmission of enteric RNA viruses. As a result, previously unreported genetic recombination of Torovirus (ToV) and Astroviruses (AstVs) were discovered.

This study consists of two Chapter I and II describing the genetic recombination of ToV and AstV, respectively. ToVs, belonging to the subfamily *Torovirinae*, are members of the family *Coronaviridae*, order *Nidovirales* [5]. ToVs have a single-stranded positive-sense RNA genome of nearly 28 kb, containing two large open reading frames (ORFs), ORF1a and ORF1b, encoding the RNA polymerase which is important for replication and transcription of the genome, and four ORFs encoding structural proteins; spike glycoprotein (S), membrane glycoprotein (M), hemagglutinin esterase (HE), and nucleocapsid phosphoprotein (N) [6-9]. ToVs were identified in various species of animals, including humans, and are thought to cause diarrhea and respiratory diseases [10-15]. Berne virus (EToV Berne), the first isolated ToV, was isolated from a horse with diarrhea in 1972 in Switzerland [16]. In 1979, a bovine ToV (BToV), named Breda virus, was detected in calves with diarrhea in

United States (US). BToV Breda causes enteric disease in gnotobiotic reared calves [15]. BToVs are found throughout the world including North America [17, 18], Central America [19], Europe [20-22], Asia [23-25] and South Africa [26]. BToVs have been detected in 2.9-36.4% of fecal samples obtained from cattle with diarrhea [18, 25, 27-30]. Porcine ToV (PToV) is also prevalent in pigs all over the world. However, their pathogenicity in swine is still unclear [6, 31, 32]. 6.4% of fecal samples from Korean piglets were tested positive for PToVs [32], 81% of serum samples from adult sows in the Netherlands had EToV-neutralizing antibody titers of ≧10 [12].

In the first study of the genetic diversity among ToVs, Smits et al. [8] reported genetic recombination between BToV and PToV in the HE region, and that these recombinant BToV mutants appeared in the mid-1990's. In addition, it has been shown that the mutation rate of the HE gene is higher compared to the coding regions of the N and S genes [9, 23, 24]. To gain more information on genetic diversity, relationship, and evolution of ToVs, whole genome analysis of Japanese BToVs and PToV was performed. The data obtained in this study showed natural interspecies recombination events among Japanese BToVs, which originated from genetic recombination of BToV Breda1 and PToV strains.

AstVs are the members of the *Astroviridae* family, which is divided into two genera: *Mamastrovirus* (MAstVs) and *Avastrovirus*. AstVs have non-enveloped positive-sense, single-stranded RNA of about 6.4-7.3 kb that contains three overlapping ORFs, namely ORF1a, ORF1b, and ORF2 [33]. A ribosomal frame shift

mediated by ribosomal slippage and a hairpin structure results in the translation of ORF1ab [34]. ORF1a and ORF1b encode nonstructural proteins, a serine protease, and an RNA-dependent RNA polymerase, respectively [35]. ORF2, encoding the viral capsid protein, is translated from a subgenomic RNA [36]. The International Committee on Taxonomy of Viruses (ICTV) classified AstV into six MAstV species based on their host species in the Ninth ICTV Report [37]. However, because of the many recently discovered MAstVs, the *Astroviridae* Study Group updated the taxonomy based not only on host range but also on genetic differences (mean aa genetic distances between and within genotypes range between 0.368-0.781, and 0-0.318, respectively) in the complete ORF2 sequence. To date, following these standardized criteria, 33 and 7 distinct species of MAstV (*Mamastrovirus* 1-33) and *Avastrovirus*, respectively, have been proposed [38].

AstVs have been found in the feces of the large range of animals around the world and are known to cause gastroenteritis in humans [39]. However, their association with intestinal diseases in mammals other than humans has not been sufficiently proven, and the clinical significance of these infections is not well understood. In recent years, non-enteric AstV infections in mammals, such as encephalitis in humans [40-46], minks [47], and cattle [48-51], as well as the isolation of AstVs from the brains of piglets suffering from congenital tremors [52], and from nasal swabs of piglets with acute respiratory disease [53] have been reported. These observations suggest that the clinical importance and impact of AstVs are increasing. Porcine AstV (PoAstV) was

first identified in the 1980s by electron microscopy of pig feces [54, 55]. For PoAstVs, there is one report that an experimental oral infection of 4-day-old pigs resulted in mild diarrhea [56]; however, PoAstVs are commonly found in the feces of apparently healthy pigs [39]. The prevalence of PoAstV is high as 0-83% and 80% based on serological and virological surveillance, respectively [56, 57].

Like other RNA viruses, AstVs have been reported many genetic diversities. From genetic and evolutionary studies, AstVs are thought to possess not only the capacity to infect a wide variety hosts but also to cross species barriers and become adapted to new hosts [39, 58]. In addition, several sites including the ORF1b /ORF2 junctions have been reported as putative recombination breakpoints [59-62]. To contribute to the whole genome sequence data available for PoAstVs, a metagenomics approach was used to sequence and analyze nearly complete genomes of PoAstVs from Japanese pigs. A broad diversity of genotypes (PoAstV2, PoAstV3, PoAstV4, PoAstV5) was detected from diseased and healthy pigs and phylogenetic analysis and recombination analysis revealed multiple possible recombination events between PoAstVs.

The results obtained in the present studies should contributes to the elucidation of genetic diversity and interspecies transmission of the enteric RNA viruses, BToV and PoAstV.

Chapter I

Whole genome analysis of Japanese bovine toroviruses reveals natural recombination between porcine and bovine toroviruses

Introduction

In ToVs, the gene region that determines the host has not been studied, but in coronaviruses, the variations in host range and tissue tropism is mainly due to variations in S protein [63], BoTV S gene and coronavirus S gene are similar in molecular properties [29]. Since S protein is thought to be involved in viral infectivity and is recognized by neutralizing antibody, serotype classification by S gene sequence has been carried out [11, 23, 28, 29].

In 2004, BToV was successfully isolated in cultured cells for the first time [64], but the isolation of BToV has only been reported in Japan [23, 24, 65]. To date, PToV has not yet been successfully isolated. So far, only three whole genome sequences of ToV, namely BToV Breda1 (AY 427798) [7], PToV NPL/2014 (KM 403390) which was identified in the US in 2014 [31], and PToV SH1 (JQ 860350) which was identified in China in 2010 [6], are available on the database. In the present study, to gain more information on genetic diversity, relationship, and evolution of ToVs, whole genome analysis of Japanese BToVs and PToV was performed.

Materials and Methods

Viruses

Four BToVs and one PToV were studied. BToV Ishikawa/2010 (BToV Ishi) was isolated using HRT-18-Aichi cells [24, 64] from a fecal sample of 28-month-old Holstein cattle with diarrhea in the Ishikawa Prefecture in 2010 [65]. BToV Kagoshima/2014 (BToV Kago), BToV Tochigi/2013 (BToV Tochi) and BToV Tokyo/2014 (BToV Tokyo) were detected in the course of metagenomics of diarrhea fecal samples obtained from 16-day-old Japanese black cattle, 12-day-old dairy cow (unknown type), 16-day-old Holstein cattle in the Kagoshima, Tochigi and Tokyo Prefectures in 2014, 2013 and 2014, respectively. These were preselected torovirus-positive fecal samples by diagnostic RT-PCR. PToV Tottori/2015 (PToV Tottori) was identified in the course of metagenomics in fecal samples collected from 28 healthy two-month-old pigs in 2015 in Tottori Prefecture.

Whole genome sequencing

Since viruses were not isolated from samples using HRT-18-Aichi cells by repeating passage thrice, fecal suspensions (20% v/v in sterile phosphate-buffered saline, pH7.4) of BToV Kago, BToV Tochi, BToV Tokyo and PToV Tottori were used for RNA extraction. Viral RNA was extracted from 0.25 mL supernatant of BToV Ishi

culture ($10^{5.3}$ $TCID_{50}$/mL) or 0.25 mL fecal suspensions by using TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, USA), followed by the treatment of the RNA with DNase I (TaKaRa Bio, Shiga, Japan). cDNA library was constructed using NEBNext® Ultra RNA Library Prep Kit for Illumina version 2.0 (New England Biolabs, Ipswich, MA, USA) as described by Nagai et al. [66]. The libraries obtained were loaded onto a MiSeq cartridge (MiSeq Reagent Kit V2 (300 cycles); Illumina, San Diego, CA, USA) and sequenced using a MiSeq bench-top sequencer (Illumina) with 151 paired-end reads. To obtain complete genome sequence of BToV Ishi and BToV Kago, rapid amplification of cDNA end method (RACE) (5'-Full RACE Core Set and 3'-Full RACE Core Set; TaKaRa Bio, Shiga, Japan) was employed.

Genome analysis

The sequence data were collected using the MiSeq Reporter program (Illumina) to generate reads in FASTQ format. Collected reads were trimmed and assembled into contigs by *de novo* assembly using CLC Genomics Workbench 6.5.1 (CLC bio, Aarhus, Denmark). The sequences were aligned using ClustalW [67] in MEGA5.22 [68]. Pairwise sequence identity calculations were performed using CLC Genomics Workbench 6.5.1. The phylogenetic tree was constructed by the maximum likelihood method statistically supported by bootstrapping with 1,000 replicates [69] by using MEGA5.22. Recombination analysis was performed using SimPlot software v. 3.5.1 [70] and the Recombination Detection Program (RDP) v. 4.58 [71, 72].

Results

Deep sequencing and determination of whole genome sequences

Deep sequencing was performed using the Illumina MiSeq sequencing system. The total ToV sequence read counts (percentage of ToV sequence reads: ToV sequence reads/total reads) of BToV Ishi, BToV Kago, BToV Tochi, BToV Tokyo and PToV Tottori samples were 198,526 (13.2%), 31,685 (2.4%), 492 (0.04%), 1,527 (0.4%) and 1,805 (0.05%), respectively. An approximately 28- kb contig was obtained from BToV Ishi and BToV Kago samples with sufficient average sequence read depth of 1,007 (maximum depth: 2,384) and 162 (maximum depth: 320), respectively; however, large contigs were not obtained from BToV Tochi, BToV Tokyo and PToV Tottori samples. Since nearly complete sequences of contig were obtained, complete genome length of BToV Ishi and BToV Kago were determined using the 5' and 3' RACE method. The complete genome lengthes of BToV Ishi and BToV Kago excluding poly (A) were 28,309 nucleotides (nt) and 28,301 nt, respectively, which were similar to the length of published sequences of ToVs; BToV Breda1 (28,475 nt), PToV NPL/2014 (28,305 nt) and PToV SH1 (28,301 nt). The nucleotide sequence identities among complete genome sequences of BToV Ishi and BToV Breda1, PToV NPL/2014, PToV SH1, and BToV Kago were 82.3%, 86.3%, 85.3% and 97.6%, respectively. The sequences of BToV Ishi and BToV Kago were deposited in the DNA Data Bank of Japan

DDBJ/EMBL/GenBank database under the accession numbers LC088094 and LC088095, respectively.

Pairwise nucleotide sequence identity comparison

Pairwise alignment for comparing nucleotide sequences of BToV Ishi and BToV Kago to the other BToV and PToVs was performed using the whole genomic region of the 5' untranslated region (UTR), ORF1a, ORF1b, S, M, HE, N, and the 3' UTR (Table 1). BToV Ishi shared high sequence identities (96.8-99.4%) with BToV Kago for all genomic regions analyzed. Japanese BToVs showed high identities with PToVs in 5' UTR, ORF1a, ORF1b, N and 3' UTR (86.9-95.1%) and showed low identities with PToVs in S, M, and HE (70.2-80.3%). On the other hand, Japanese BToVs showed high identities with BToV Breda1 in S, M, and HE (87.9-95.7%) and showed low identities with BToV Breda1 in 5' UTR, ORF1a, ORF1b, N and 3' UTR (68.9-83.0%). These results suggest the occurrence of recombination events between BToV Breda1 and PToVs.

Recombination analysis

To investigate the recombination events, the complete genomes of BToV Ishi, BToV Kago, PToV NPL/2014, PToV SH1, and BToV Breda1 were aligned using ClustalW program in MEGA5.22 and standard similarity plot analysis was performed using SimPlot software v. 3.5.1 with BToV Ishi and BToV Kago sequences as separate

Table 1

Pairwise nucleotide sequence comparison of BToV Ishikawa/2010 and BToV Kagoshima/2014 to the other BToV and PToVs.

	Pairwise nucleotide identity (%)								
	BToV Ishikawa/2010					BToV Kagoshima/2014			
	PToV NPL/2014	PToV SH1	BToV Breda1	EToV Berne	BToV Kago	PToV NPL/2014	PToV SH1	BToV Breda1	EToV Berne
5'UTR	91.7	92.0	75.4	63.3	99.4	91.7	92.3	75.6	63.4
ORF1a	88.7	86.9	77.0	67.3	97.3	88.5	86.9	76.9	67.2
ORF1b	92.7	91.9	83.0	81.1	98.2	92.6	91.8	82.8	81.2
S	73.4	73.2	95.7	75.7	96.9	73.6	73.4	95.3	75.6
M	79.6	80.3	94.3	84.3	98.6	78.8	79.5	94.0	84.5
HE	70.8	71.7	88.3	- [a]	96.8	70.2	71.2	87.9	- [a]
N	92.9	90.2	69.1	67.1	98.2	93.1	90.2	69.1	67.3
3'UTR	95.1	90.8	68.9	66.9	98.8	93.9	90.8	68.9	66.9

[a] The complete HE sequence of EToV Berne strain is not available

queries (Fig. 1A, B). Both SimPlot graph indicated that the sequences of Japanese BToV had high similarity with those of PToVs except in the 3' end of ORF1b, S, M, and most of the HE coding regions, which had high similarity with that of BToV Breda1. The bootscanning analysis using RDP v. 4.58 was performed to identify the presumed recombinant breakpoints. Schema of ToV genome structure is shown in Fig. 2A. Beginning and end breakpoint positions were mapped near the 3' of ORF1b and HE regions, respectively (Fig. 2B). At the starting putative recombination breakpoint, 25-nt sequences of Japanese BToVs corresponding to nt 20,138-20,162 of BToV Ishi showed low identities (72-76%) with that of BToV Breda1, whereas 25 nt sequences of Japanese BToVs corresponding to nt 20,161-20,185 showed high identities (100%) with that of BToV Breda1. At the end putative recombination breakpoint, 25 nt sequences of Japanese BToV corresponding to nt 27,404-27,428 of BToV Ishi showed low identities (84-88%) with those of PToVs; however, the 25-nt Japanese BToV sequences corresponding to nt 27,418 to 27,442 showed high identities (96-100%) with those of PToVs (Fig. 2C).

Phylogenetic analysis

For further confirmation of the recombination event, phylogenetic trees of nine genomic regions were constructed using nucleotide sequences of BToVs and PToV determined in this study together with ToV and PToV genome sequences available in GenBank. Although the genome sequences of BToV Tochi, BToV Tokyo, and PToV

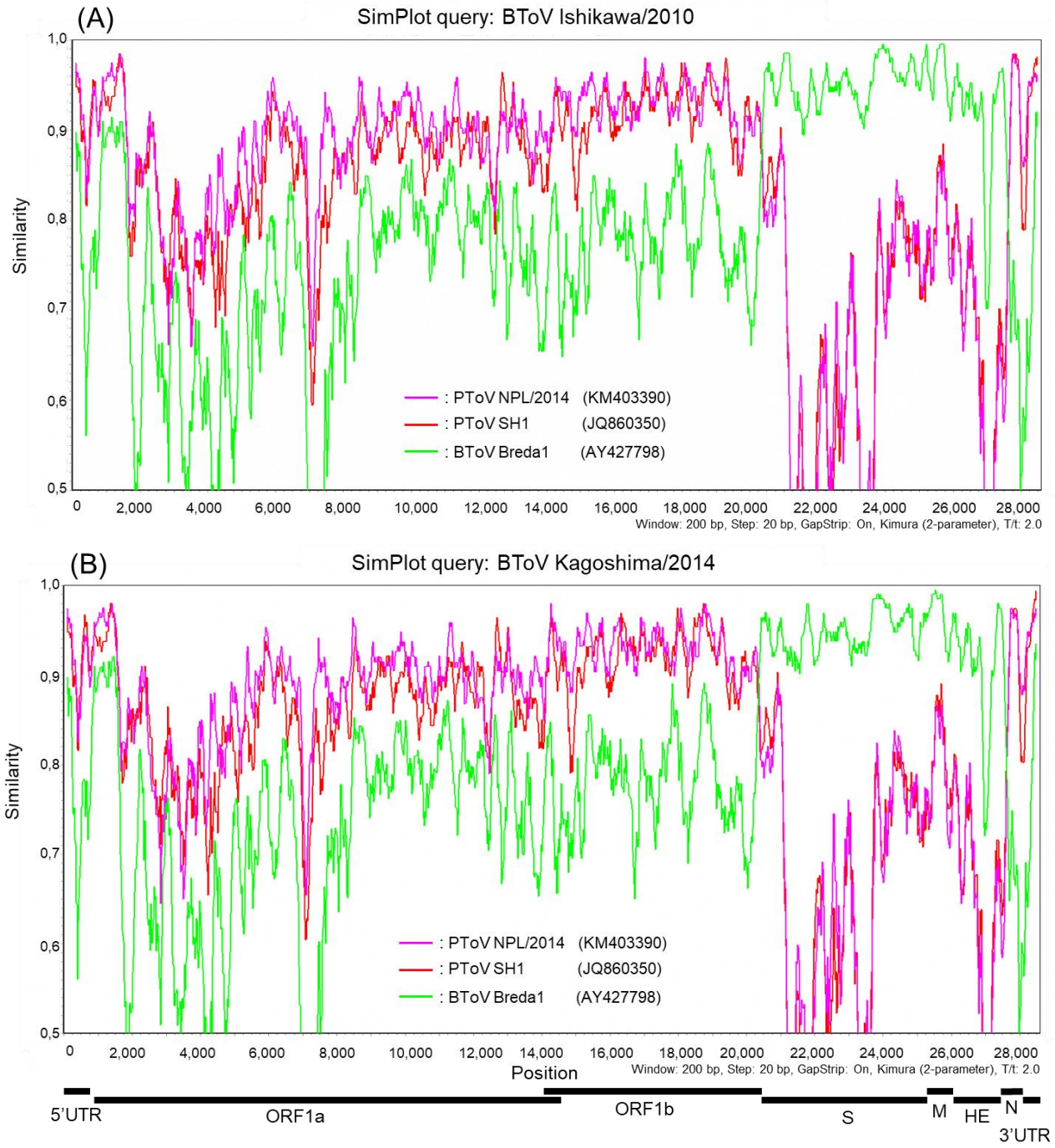

Fig. 1. Similarity plot analysis of the entire genome length of PToV NPL/2014 (pink line), PToV SH1 (red line), BToV Breda1 (green line), and BToV Ishikawa/2010 (A), and BToV Kagoshima/2014 (B) as query sequences, using a sliding window of 200 nt and a moving step size of 20 nt.

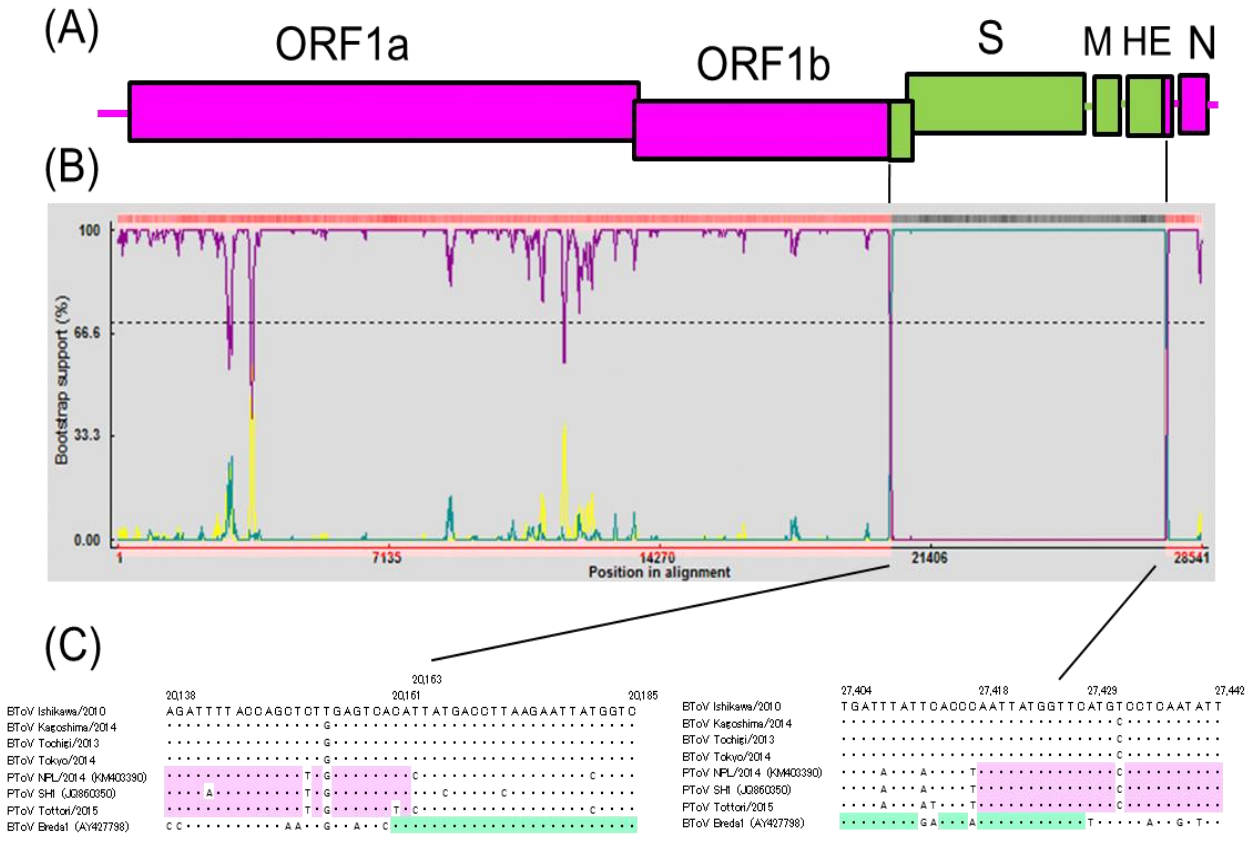

Fig. 2. Detection of recombination breakpoint in Japanese BToV genomes. (A) Genome organization of ToV. Presumed recombinant breakpoints were mapped to the 3' end of ORF1b and of HE coding regions. Pink boxes indicate sequence regions originating from PToV. Green boxes indicate sequence regions originating from BToV Breda1 strain. (B) Bootscan analysis of BToV Ishikawa/2010 vs PToV NPL/2014 (purple line), BToV Ishikawa/2010 vs BToV Breda1 (green line) and PToV NPL/2014 vs BToV Breda1 (yellow line). Cut-off of the bootstrapping test (>70%) is indicated by the break-point. (C) Sequence of recombination junctions of Japanese BToVs. Dots indicate sequences similar to that of BToV Ishikawa/2010. Identities between Japanese BToVs and PToV (NPL/2014 and SH1) are highlighted in pink; identities between Japanese BToV and BToV Breda1 are highlighted in green.

Tottori had gaps owing to insufficient sequence read counts, sequence regions with no gap and corresponding to BToV Ishi were used: 5' UTR (161 nt, 499-659), ORF1a (1,800 nt, 3,396-5,195), ORF1b (915 nt, 18,425-19,339), S (594 nt, 20,889-21,482 and 3,863 nt, 21,693-25,548), M (304 nt, 25,752-26,055), HE (1,015 nt, 26,427-27,477), N (485 nt, 27,629-28,113) and 3'UTR (172 nt, 28,114-28,285). However, the sequences of regions S (20,889-21,482) of PToV Tottori, S (21,693-25,548) of BToV Tokyo, and N (27,629-28,113) of PToV Tottori were not obtained. The phylogenetic trees of S and N coding regions were constructed without these strains. The four Japanese BToVs branched separately from BToV Breda1, and clustered with PToV strains in the 5' UTR, ORF1a, ORF1b, N and 3' UTR phylogenetic trees (Fig. 3A-C, H and I). In addition, Japanese BToVs in this study together with other BToV strains from Japan and other countries selected from GenBank, clustered with BToV Breda1 but not with PToVs in the S, M, and HE phylogenetic trees (Fig. 3D-G). Ito et al. [11, 23, 28] classified Japanese BToVs using 5' portion of S coding region into three genotypes, Clusters 1-3. In the phylogenetic tree, the 5' portion of S coding region of BToVs in this study showed that BToV Ishi and BToV Tochi clustered with Cluster-1 and Cluster-2 strains, respectively, while BToV Kago and BToV Tokyo clustered with Cluster-3 strain, though enough bootstrap support was not obtained (Fig. 3D). BToV Tochi separately clustered with BToV Ishi, BToV Kago, and BToV Tokyo in the S (21,693-25,548), M, and HE phylogenetic trees (Fig. 3E-G).

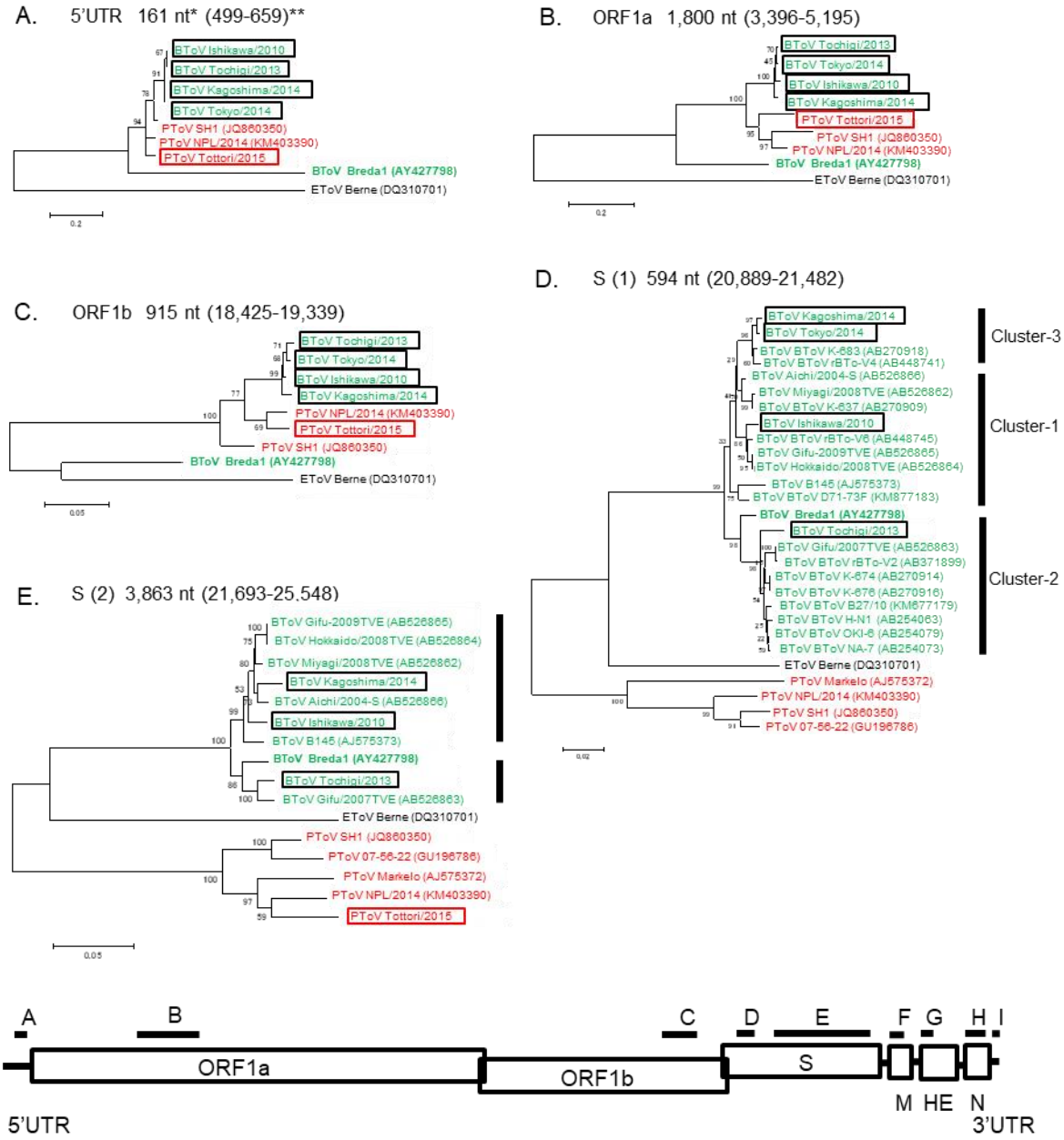

Fig. 3. Phylogenetic trees based on sequences of 5'UTR (A), ORF1a (B), ORF1b (C), S (D, E), M (F), HE (G), N (H), and 3'UTR (I). Phylogenetic trees were constructed using the maximum likelihood method in MEGA5.22 with bootstrap values (1,000 replicates). Scale bar indicates nucleotide substitutions per site. The BToV strains are represented by green (BToV Breda1 is indicated by boldface), whereas the PToV strains are represented by red. The BToV strains analyzed in this study are shown by black open square. *: Length of nucleotide sequences using analysis. **: Nucleotide position of BToV Ishikawa/2010.

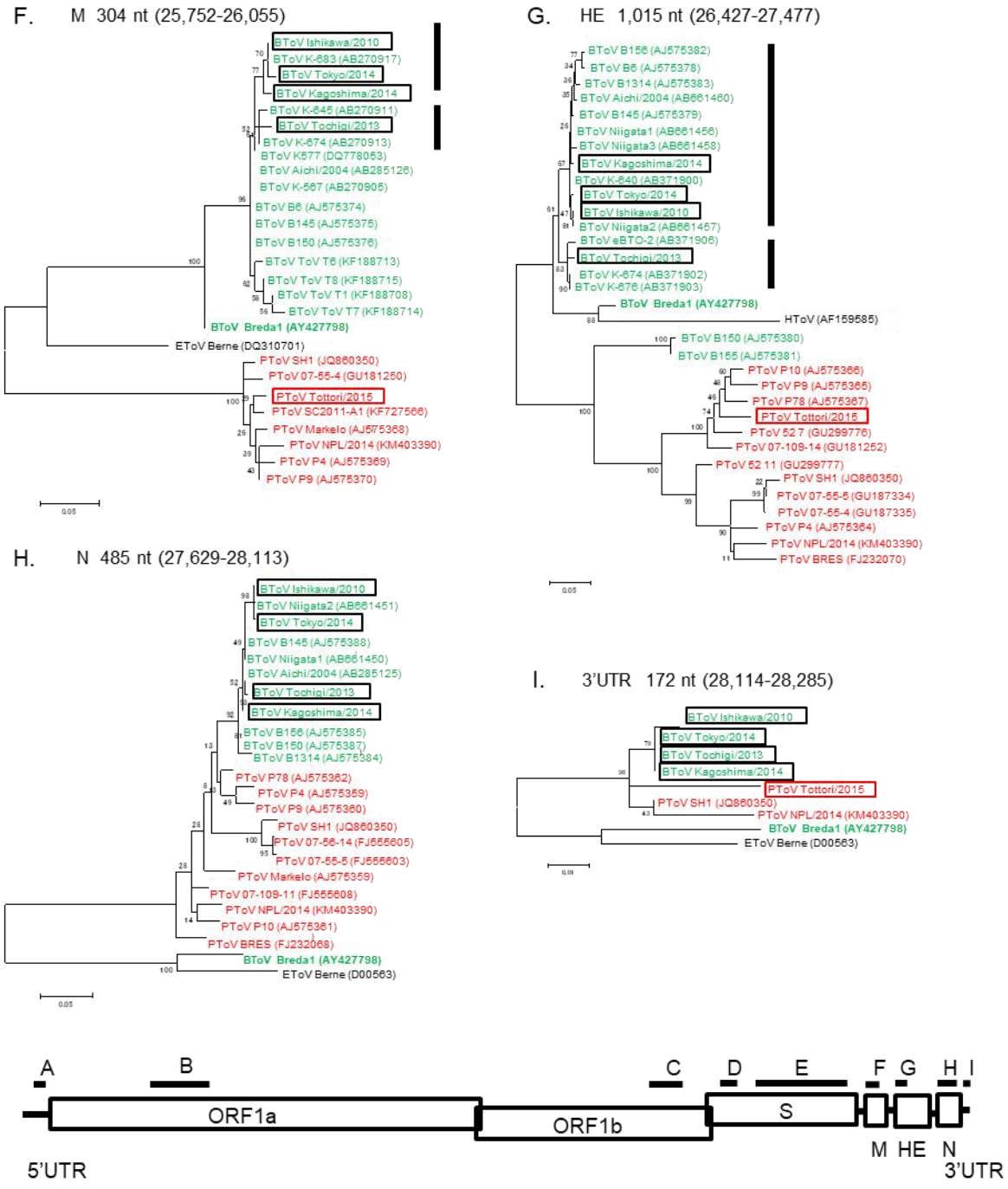

Fig. 3. (*continued*)

Discussion

In the present study, whole genome sequencing of Japanese BToVs and PToV was performed by deep sequencing using supernatants of virus cultures and fecal suspensions. A sufficient number of sequence reads and contigs of nearly 28kb was obtained from 0.25 mL of $10^{5.3}$ $TCID_{50}$/mL BToV Ishi cell culture supernatant. Only one sample among three 0.25mL fecal suspension samples gave a sufficient number of sequence reads and large contigs, though the number of sequence reads from this sample was lower than that from the viral culture supernatant. Unfortunately, BToV was not isolated from fecal sample of BToV Kago by using HRT-18-Aichi cells, which are useful for BToV isolation [24, 64, 65], possibly because the samples were preserved at -20°C at the veterinary clinic. Although numerous sequence reads originating from bacterial species and hosts might interfere and reduce the number of sequence reads from fecal suspensions, the whole genome sequence of BToV was obtained from this sample by using the deep sequencing strategy.

The results of sequence comparison through pairwise alignment, similarity plot, bootscanning, and phylogenetic tree analyses of Japanese BToVs with BToVs and PToVs sequences from database were indicative of interspecies recombination between BToV Breda1 and PToV at the 3' end of ORF1b and the end of HE coding region. Smits et al. [8] provided evidence for the recombination of ToV sequences

between BToV Breda1 and PToV in the HE coding region. Italian and Dutch BToVs identified before the year 2000 were reported to possess a single recombination breakpoint located approximately 1,100 nucleotides from the ATG initiation codon of the HE coding region. The putative recombinant breakpoint in the HE coding region of Japanese BToVs was quite identical to that of European BToVs. In the phylogenetic tree analyses, Japanese BToVs were found to be closely related to European BToVs in S, M, HE, and N coding regions. These finding suggest that Japanese BToVs and European BToVs have a common ancestor. However, owing to the lack of sequence information of 5' UTR, ORF1a and ORF1b of European BToVs, Japanese BToVs were not further compared with European BToVs. The HE coding region of ToV shares sequence homology to that of coronavirus and Influenza C virus, however it is suggested that HE region of these viruses were introduced through independent heterologous recombination events [73]. Homologous recombination of HE coding region was also reported in PToVs [8, 9], suggesting that the HE coding region may be a hot spot for recombination events.

This is the first report to present the identification of a natural recombination event between BToV Breda1 and PToV at ORF1b. Approximately 76% of the genomes of BToV Ishi and BToV Kago are similar to that of PToV. Natural recombination events are common in coronaviruses, resulting in evolution and emergence of new variants that are pathogenic to poultry, cats, pigs, and humans [74-84]. Co-infection of a certain cells in bovine or swine with BToV Breda1 type virus and PToV is a pre-

requisite for the generation of new recombinant viruses by double-template switch. The generated new recombinant virus, which has tropism to bovine cells, might adapt to bovine hosts and spread among cattle.

Although the four Japanese BToVs evaluated in this study were collected from distinct regions, the sequences of genomic regions apart from the S, M, and HE coding regions were closely related to each other and showed high identity (97.3-99.4%). Based on the sequences of the S, M, and HE coding regions, these BToVs were divided into at least two groups (Fig. 3D-G). The four Japanese BToVs showed genetic diversity, they all possessed a recombination genome. When it was thought that the Breda1 strain was the original BToV, the recombination of BToVs occurred before it was introduced to Japan, or it was occured before spreading all over the country. The Japanese and foreign BToV strains retrieved from the database clustered closely together in the phylogenetic tree, suggesting that all BToVs investigated in this study, except BToV Breda1, underwent recombination at the same genomic regions. Strains genetically closely related to Japanese BToVs are distributed worldwide.

Hurst et al. [85] found the interaction between the coronavirus nucleocapsid protein and the replicase-transcriptase complex, from that viral mutants did not proliferate when only the N gene region of the coronavirus mouse hepatitis virus (MHV) was replaced with its counterpart from the closely related bovine coronavirus (BCoV). ToVs may be similar as well, which may be the reason why Japanese BToV holds both N and ORF1ab region of PToV. Moreover, the continuation of the

combination of PToVs N protein and replicase-transcriptase complex is presumed to be more advantageous for the proliferation.

In conclusion, a natural recombination event was identified between BToV Breda1 and PToV of Japanese BToVs. The four Japanese BToVs possessed two recombination breakpoints mapped to the 3' end of ORF1b coding region and to the 3' end of HE coding region and nearly 76% of the genome is similar to the PToV genome. Homologous recombination events of the virus genome are crucial for their evolution, and are significant factors for changes in host range and virulence of viruses. The data obtained in this study may provide important evidence to evaluate the epidemiological basis of ToV in cattle and swine population and to understand the mechanisms underlying the evolution of BToV.

Summary

BToV belongs to the subfamily *Toroviridae* within the family *Coronaviridae*, and are pathogens, causing enteric diseases in cattle. In Japan, BToVs are distributed throughout the country and cause gastrointestinal infection of calves and cows. In this study, complete genome sequences of two Japanese BToVs and partial genome sequences of two Japanese BToVs and one PToV from distant regions in Japan were determined and genetic analysis were performed. Pairwise nucleotide comparison and phylogenetic analyses revealed that Japanese BToVs shared high identity with one another and showed high similarities with BToV Breda1 strain in S, M and HE coding regions. Japanese BToVs showed high similarities with PToVs in ORF1a, ORF1b and N coding regions and the 5' and 3' untranslated regions, suggesting a natural recombination event. Recombination analyses mapped the putative recombinant breakpoints to the 3' ends of the ORF1b and HE regions. These findings suggest that the interspecies recombinant nature of Japanese BToVs resulted in a closer relationship between BToV Breda1 and PToVs.

Chapter II

Whole genome analysis of porcine astroviruses detected in Japanese pigs reveals genetic diversity and possible intra-genotypic recombination

Introduction

The N-terminal half of AstV ORF2 encodes the particle assembly domain that is conserved among AstVs, whereas the C-terminal half forms the hypervariable receptor-interaction domain [86]. Because this hypervariable domain is believed to form the capsid spike and to contain neutralizing epitopes [87], ORF2 is used for the presumption of AstV serotypes by PCR with subsequent sequencing [88]. High sequence diversity was found in ORF2; ORF1b is least divergent [89]. This is a naturally expected event, as the region coding for the viral capsid protein is subject to selective pressure.

PoAstVs were first isolated using porcine cell lines in 1990 [56], but reports of successful isolation are limited. However, genetic diagnosis of AstVs and PoAstVs have been reported in several countries around the world [39]. A study in Quebec in Canada showed that pigs may have several AstVs most likely derived from different ancestry [57]. Presently, five genotypes of PoAstV (PoAstV1-PoAstV5) are recognized [90]. In Japan, although PoAstVs were detected in the 1980s, only two sequences of PoAstV1 have been reported [91, 92]. Furthermore, only a few whole genome sequences of PoAstV are available in the DDBJ/EMBL/GenBank database. In this study, to contribute to the whole genome sequence data available for PoAstVs, a metagenomics approach was used to sequence and analyze nearly complete genomes

of PoAstVs from Japanese pigs.

Materials and Methods

Viruses

A total of 145 fecal samples were collected from 38 pig farms in the mainland of Japan in 2014-2015, and were taken from 2 to 120-day-old pigs that were clinically healthy (73 samples) or had diarrhea (72 samples). Samples from single pigs (124 samples) or 2-3 pigs (pooled, 21 samples) were diluted 1:9 (w/v) in sterile phosphate-buffered saline and stored in a -80℃ freezer until use.

Whole genome sequencing

The method was as described in Chapter I. However, RACE was not employed.

Genome analysis

The method was as described in Chapter I. However, the updated program was used. That is, CLC Genomics Workbench 7.5.5, MEGA6.06, Recombination Detection Program (RDP) v. 4.80. The contigs that exhibited sequence similarities with AstVs and that were > 5,930 nt in length with the sufficient read coverage were analyzed.

Results

Complete or nearly complete sequencing of the PoAstV coding sequence

Contigs were generated from trimmed sequence reads and generated contigs were evaluated by map reads to reference command in CLC Genomics Workbench with strictest parameter setting (mismatch cost, 2; insertion cost, 3; deletion cost, 3; length function, 1.0; and similarity function, 1.0), and 5′ and 3′ sequences with insufficient read depth (<3) were omitted. Only contigs that showed sequence similarities with AstVs and that were longer than 5,930 nt were used in this study. Thirty-six AstV contigs were identified from 9 to 100-day-old pigs with or without diarrhea. Sample data and strain names corresponding to contigs are summarized in Tables 2 and 3. Of interest, six and two samples (excluding pooled samples) contained two strains (Bu4-2-1 and Bu4-2-2, Bu4-6-1 and Bu4-6-2, Bu-5-10-1 and Bu5-10-2, Iba-464-4-1 and Iba464-4-2, Ishi-Ya7-1 and Ishi-Ya7-2, and MoI2-3-1 and MoI2-3-2) and three strains (HgTa2-1-1, HgTa2-1-2, and HgTa2-1-3, and MoI2-1-1, MoI2-1-2, and MoI2-1-3), respectively, each pigs were thought to be co-infected with multiple strains (Tables 2 and 3). The nt sequences of these 36 strains were deposited in DDBJ/EMBL/GenBank under accession numbers LC201585-LC201620 (Table 3).

Phylogenetic analyses and pairwise nucleotide and amino acid comparison

Table 2

Summary of samples used in this study and a number of astrovirus contigs identified in each sample.

Sample name	Sample status	Ages of host (days)	Health status of host	Region	Number of Astrovirus contigs	Co-infection with other viruses
Bu2-5	Single	9	Healthy	Gunma	1	Rotavirus A, Kobuvirus
Bu4-2	Single	14	Healthy	Niigata	2	Kobuvirus, Enterovirus
Bu4-4	Single	16	Healthy	Niigata	1	Rotavirus A, Kobuvirus, Enterovirus, Picobirnavirus
Bu4-6	Single	20	Healthy	Niigata	2	Rotavirus A, Kobuvirus, Enterovirus, Picobirnavirus
Bu5-10	Single	10	Healthy	Tochigi	2	Rotavirus A, Enterovirus, Sapovirus, Kobuvirus
Bu7-9	Single	18	Mild diarrhea	Gifu	1	Kobuvirus
Bu8-4	Single	25	Diarrhea	Chiba	1	Rotavirus A, Enterovirus, Picobirnavirus
Buta17	Single	14	Diarrhea	Tottori	1	Kobuvirus
HgOg2-1	Single	60	Healthy	Tottori	1	Enterovirus, Sapelovirus
HgOg2-4	Single	60	Healthy	Tottori	1	Enterovirus, Picornavirus, Sapovirus
HgTa2-1	Single	60	Healthy	Tottori	3	Rotavirus C, Enterovirus, Sapelovirus, Sapovirus
HgTa2-2	Single	60	Healthy	Tottori	1	Picobirnavirus, Sapovirus, Enterovirus
HgTa2-3	Single	60	Healthy	Tottori	1	Rotavirus A, Picobirnavirus, Sapelovirus, Enterovirus
HgYa2-3	Single	60	Healthy	Tottori	1	Enterovirus, Sapolevorus, Picobirnavirus
HkKa2-1	Single	60	Healthy	Tottori	1	Picobirnavirus, Posavirus, Sapovirus
Iba-464-4	Single	30	Diarrhea with PED	Ibaraki	2	Enterovirus, Rotavirus H
Ishi-Im1	Pooled	54	Healthy	Ishikawa	2	Posavirus, Techovirus, Picobirnavirus, Sapovirus
Ishi-Im3	Pooled	30	Healthy	Ishikawa	1	Sapelovirus, Posavirus, Picobirnavirus, Sapovirus
Ishi-Ya4	Single	100	Diarrhea	Ishikawa	1	Enterovirus
Ishi-Ya6	Single	94	Diarrhea	Ishikawa	1	Picobirnavirus
Ishi-Ya7	Single	80	Diarrhea	Ishikawa	2	Sapelovirus, Sapovirus
Ishi-Ya8	Single	80	Diarrhea	Ishikawa	1	Posavirus, Picobirnavirus
MoI2-1	Single	60	Healthy	Tottori	3	Enterovirus, Picornavirus, Sapovirus
MoI2-2	Single	60	Healthy	Tottori	1	Rotavirus C, Enterovirus, Sapelovirus, Teschovirus
MoI2-3	Single	60	Healthy	Tottori	2	Rotavirus C, Enterovirus

Table 3

Summary of genomic information of porcine astroviruses obtained from deep sequencing in this study.

Strains	Abbreviated name of strains	Porcine astrovirus genotype	Reads and sequences obtained from deep sequencing				DDBJ Accession No.	Length of nucleotide sequence		
			Total reads	Astrovirus reads	Rate of Astrovirus reads (%)	Sequence Length (excluding poly-A)		ORF1a	ORF1ab	ORF2
PoAstV2/JPN/Bu5-10-1/2014	Bu5-10-1	2	780,414	4,301	0.6	6,299	LC201585	2,475	3,938	2,253
PoAstV2/JPN/HgOg2-1/2015	HgOg2-1	2	181,984	948	0.5	6,333	LC201586	2,475	3,938	2,298
PoAstV2/JPN/HgOg2-4/2015	HgOg2-4	2	399,976	4,725	1.2	6,368	LC201587	2,475	3,938	2,328
PoAstV2/JPN/HgYa2-3/2015	HgYa2-3	2	722,010	4,899	0.7	6,347	LC201588	2,475	3,938	2,304
PoAstV2/JPN/Ishi-Ya4/2015	Ishi-Ya4	2	371,918	3,617	1.0	6,347	LC201589	2,475	3,938	2,316
PoAstV2/JPN/Ishi-Ya6/2015	Ishi-Ya6	2	1,250,140	1,278	0.10	6,330	LC201590	2,475	3,938	2,298
PoAstV2/JPN/Ishi-Ya7-2/2015	Ishi-Ya7-2	2	464,514	1,498	0.3	5,930	LC201591	2,478	3,941	1,962 (incomplete)
PoAstV2/JPN/Ishi-Ya8/2015	Ishi-Ya8	2	413,176	2,004	0.5	6,372	LC201592	2,475	3,938	2,331
PoAstV2/JPN/Ishi-Im3/2015	Ishi-Im3	2	3,041,700	31,887	1.0	6,366	LC201593	2,475	3,938	2,319
PoAstV2/JPN/Iba-464-4-1/2015	Iba-464-4-1	2	2,016,670	70,197	3.5	6,351	LC201594	2,475	3,938	2,307
PoAstV3/JPN/Bu2-5/2014	Bu2-5	3	660,138	25,061	3.8	6,397	LC201595	2,535	4,061	2,454
PoAstV3/JPN/Bu4-2-1/2014	Bu4-2-1	3	629,140	46,019	7.3	6,404	LC201596	2,535	4,061	2,457
PoAstV3/JPN/Bu4-4/2014	Bu4-4	3	957,684	11,286	1.2	6,389	LC201597	2,535	4,061	2,457
PoAstV3/JPN/Bu7-9/2014	Bu7-9	3	379,772	2,383	0.6	6,374	LC201598	2,535	4,061	2,463
PoAstV3/JPN/Bu8-4/2014	Bu8-4	3	563,365	2,142	0.4	6,351	LC201599	2,535	4,061	2,454
PoAstV4/JPN/Bu4-2-2/2014	Bu4-2-2	4	629,140	3,501	0.6	6,646	LC201600	2,643	4,088	2,490
PoAstV4/JPN/Bu4-6-1/2014	Bu4-6-1	4	446,798	4,449	1.0	6,656	LC201601	2,643	4,088	2,490
PoAstV4/JPN/Bu4-6-2/2014	Bu4-6-2	4	446,798	2,297	0.5	6,513	LC201602	2,634	4,079	2,437 (incomplete)
PoAstV4/JPN/Bu5-10-2/2014	Bu5-10-2	4	780,414	1,268	0.2	6,476	LC201603	2,631	4,076	2,397 (incomplete)
PoAstV4/JPN/Buta17/2014	Buta17	4	1,225,306	623	0.05	6,494	LC201604	2,634 (incomplete)	4,079 (incomplete)	2,423 (incomplete)
PoAstV4/JPN/HkKa2-1/2015	HkKa2-1	4	1,128,024	2,591	0.2	6,674	LC201605	2,631	4,076	2,526
PoAstV4/JPN/HgTa2-1-1/2015	HgTa2-1-1	4	685,940	13,453	2.0	6,482	LC201606	2,631	4,079	2,401 (incomplete)
PoAstV4/JPN/HgTa2-1-2/2015	HgTa2-1-2	4	685,940	3,111	0.5	6,316	LC201607	2,597 (incomplete)	4,042 (incomplete)	2,282 (incomplete)
PoAstV4/JPN/HgTa2-3/2015	HgTa2-3	4	1,320,004	954	0.07	6,625	LC201608	2,643	4,088	2,472
PoAstV4/JPN/MoI2-1-1/2015	MoI2-1-1	4	2,804,452	87,262	3.1	6,669	LC201609	2,643	4,088	2,511
PoAstV4/JPN/MoI2-1-2/2015	MoI2-1-2	4	2,804,452	25,726	0.9	6,623	LC201610	2,631	4,076	2,478
PoAstV4/JPN/MoI2-2/2015	MoI2-2	4	1,495,394	57,546	3.8	6,628	LC201611	2,631	4,076	2,478
PoAstV4/JPN/MoI2-3-2/2015	MoI2-3-2	4	277,718	19,109	6.9	6,620	LC201612	2,631	4,076	2,478
PoAstV4/JPN/Ishi-Ya7-1/2015	Ishi-7-1	4	464,514	18,402	4.0	6,723	LC201613	2,631	4,079	2,625
PoAstV4/JPN/Iba-464-4-2/2015	Iba-464-4-2	4	2,016,670	3,936	0.2	6,014	LC201614	2,622 (incomplete)	4,067 (incomplete)	1,955 (incomplete)
PoAstV5/JPN/HgTa2-1-3/2015	HgTa2-1-3	5	685,940	2,350	0.3	6,439	LC201615	2,586	4,058	2,208
PoAstV5/JPN/HgTa2-2/2015	HgTa2-2	5	862,726	4,600	0.5	6,432	LC201616	2,586	4,058	2,208
PoAstV5/JPN/MoI2-1-3/2015	MoI2-1-3	5	2,804,452	14,959	0.5	6,430	LC201617	2,586	4,058	2,208
PoAstV5/JPN/MoI2-3-1/2015	MoI2-3-1	5	277,718	118,918	42.8	6,439	LC201618	2,586	4,058	2,208
PoAstV5/JPN/Ishi-Im1-1/2015	Ishi-Im1-1	5	1,809,416	1,120	0.06	6,378	LC201619	2,586	4,058	2,199
PoAstV5/JPN/Ishi-Im1-2/2015	Ishi-Im1-2	5	1,809,416	2,218	0.12	6,352	LC201620	2,586	4,058	2,208

Firstly, because complete ORF2 sequences were needed for AstV demarcation [38], a phylogenetic analysis using ORF2 aa sequences (including incomplete coding sequences of seven strains) was performed. Ten, 5, 15, and 6 Japanese PoAstVs were clustered with the reference strains of PoAstV2, PoAstV3, PoAstV4, and PoAstV5, respectively, sharing 26.5-41.1% nt and 15.5-28.4% aa identities in ORF2 with other genotypes (Fig. 4). Sequence identities in ORF1a and ORF1b between each genotype were 29.7-46.1% nt and 16.6-33.3% aa, and 48.5-60.8% nt and 42.1-59.5% aa, respectively (Tables 4-7). PoAstV3 and PoAstV5 strains formed single clusters corresponding to *Mamastrovirus 22* and *Mamastrovirus 24*, respectively, proposed by Guix et al. [38], while clusters with PoAstV2 and PoAstV4 were subdivided into two and four lineages. One PoAstV3 strain, Bu7-9, and one PoAstV5 strain, Ishi-Im1-1, showed considerable sequence differences from other PoAstV3 and PoAstV5 strains, respectively, in ORF2 (Fig. 4, Tables 5, 7). The PoAstV5 cluster included ovine and bovine AstV strains. Two PoAstV2 lineages, tentatively named PoAstV2 lineages 1 (PoAstV2 L1) and 2 (PoAstV2 L2), corresponded to *Mamastrovirus 31* and *32*, respectively. The identities in ORF 2 between the lineages of PoAstV2 L1 strain and PoAstV2 L2 strain was 52.2-64.5% nt and 47.8-65.4% aa, whereas the identities within each lineage were > 58.0% nt and > 54.3% aa in the L1 lineage, and > 61.7% nt and > 58.6% aa in the L2 lineage (Table 8). Strains in the PoAstV2 cluster were closely related to bovine AstV strains, Ishikawa9728/2013, Ishikawa24-6/2013, and Kagoshima2-3-2/2015, reported by Nagai et al. [66], and the PoAstV2 L2 cluster

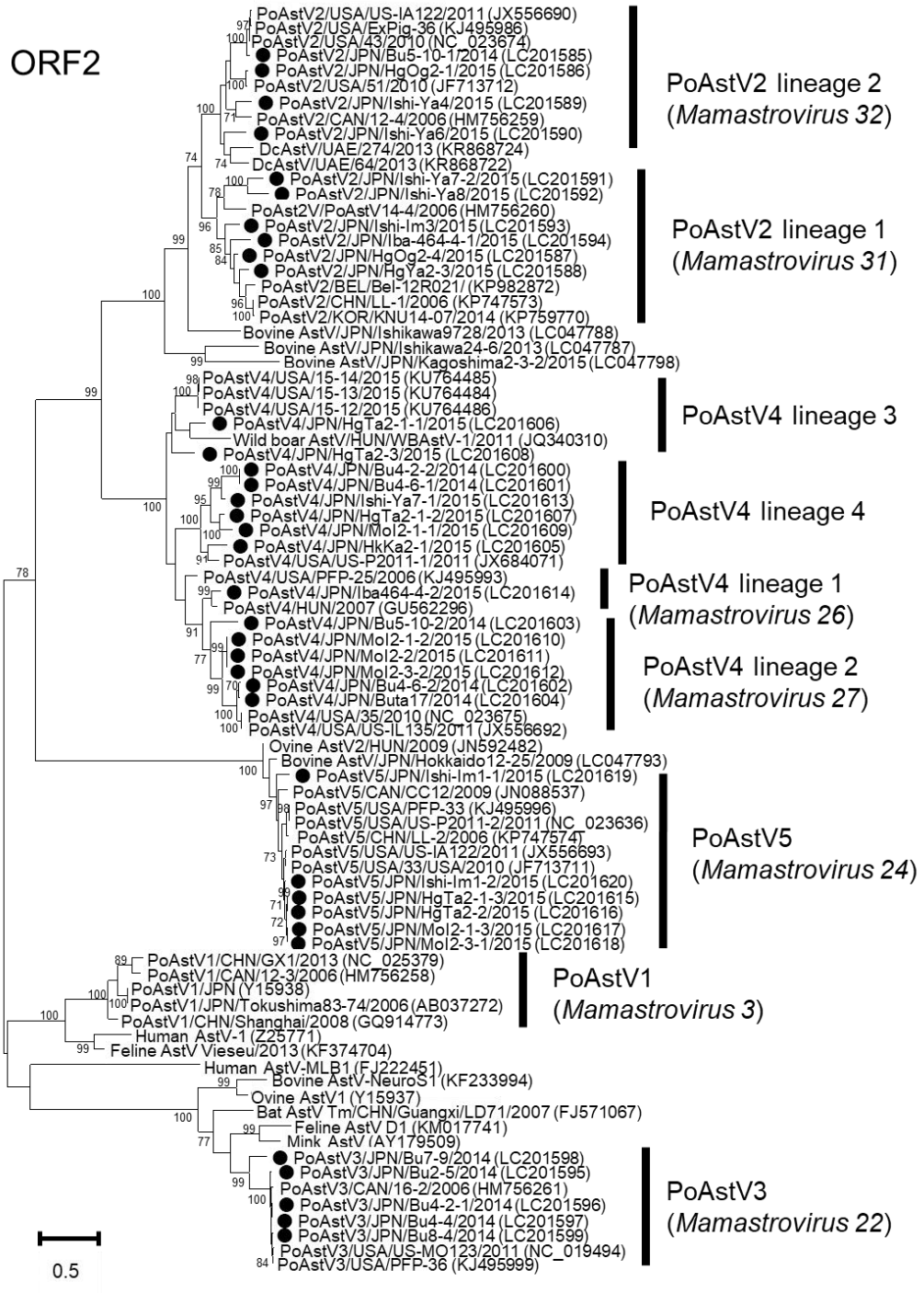

Fig. 4. Results of a phylogenetic analysis based on deduced amino acid sequences of AstV ORF2. The trees were constructed using the maximum likelihood method (rtREV + G + F model) in MEGA6.06 with 1,000 bootstrap values. The scale bar indicates amino acid substitutions per site. ● indicates PoAstV strains identified in this study. Percent bootstrap support is indicated by the value at each node, with values < 70 omitted.

Table 4

Pairwise nucleotide (lower left) and amino acid (upper right (grey shades)) sequence identities (%) between three complete CDS (ORF1a, ORF1b, and ORF2) of Japanese PoAstV2s and other PoAstVs, and dromedary and bovine astroviruses.

	Po2-Bu5-10-1			Po2-HgOg2-1			Po2-HgOg2-4			Po2-HgYa2-3			Po2-Ishi-Ya4			Po2-Ishi-Ya6			Po2-Ishi-Ya8			Po2-Ishi-Im3			Po2-Iba-464-4-1			Po2-US-IA122			Po2-KNU14-07			Po2-USA			Po2-43		
	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2
PoAstV2/JPN/Bu5-10-1/2014				85.7	97.2	71.7	83.5	87.1	58.0	77.3	86.9	57.8	80.6	95.0	63.0	81.5	86.3	63.0	82.4	87.3	48.3	77.1	86.9	56.5	77.5	95.0	58.5	90.3	96.0	88.5	90.6	87.1	58.9	85.2	96.0	88.3	85.9	95.8	89.1
PoAstV2/JPN/HgOg2-1/2015	79.2	91.9	69.6				81.2	87.5	56.6	81.5	87.5	58.8	83.2	94.3	66.5	89.5	86.9	62.8	81.8	87.3	48.8	79.5	86.7	57.6	80.8	94.6	59.1	86.9	95.6	71.4	80.9	87.7	58.8	96.0	96.6	70.9	97.2	95.4	72.5
PoAstV2/JPN/HgOg2-4/2015	77.9	79.4	60.7	75.3	78.7	60.8				79.8	95.0	80.8	76.6	87.7	53.0	83.6	95.0	53.7	89.0	95.0	56.2	79.5	95.4	79.8	77.2	88.1	74.1	82.9	87.7	57.4	82.7	95.4	73.7	81.2	87.1	57.0	81.7	88.5	57.5
PoAstV2/JPN/HgYa2-3/2015	72.0	79.6	60.3	75.2	78.5	59.9	73.6	88.6	78.0				76.4	86.3	55.3	83.4	94.4	55.3	79.2	92.9	56.9	90.9	92.5	66.6	76.2	86.3	71.2	76.6	87.3	57.6	78.1	96.6	75.7	81.9	86.3	56.9	82.1	87.3	57.3
PoAstV2/JPN/Ishi-Ya4/2015	75.0	87.7	64.6	75.6	87.2	67.0	71.2	80.1	57.8	70.0	79.9	60.4				80.4	85.7	69.1	80.4	86.7	49.1	75.4	86.7	53.2	77.4	98.0	56.2	80.5	94.8	64.9	75.6	86.9	55.1	82.8	94.8	64.1	82.8	94.8	64.8
PoAstV2/JPN/Ishi-Ya6/2015	75.2	79.4	63.0	79.6	79.0	63.3	76.7	87.5	57.6	75.9	86.8	58.3	73.8	78.6	69.4				81.8	92.3	49.6	81.8	93.3	53.1	79.7	86.1	52.7	82.4	86.5	61.4	80.7	94.1	54.9	89.2	85.9	61.2	89.7	87.1	61.7
PoAstV2/JPN/Ishi-Ya8/2015	75.9	79.9	55.4	75.7	80.2	55.1	81.6	88.1	59.5	72.5	86.8	59.5	73.5	79.1	55.3	75.7	85.7	54.7				78.2	95.8	60.8	79.7	87.3	54.3	82.9	87.1	47.6	81.9	93.9	55.8	81.8	86.9	47.6	82.2	87.3	48.6
PoAstV2/JPN/Ishi-Im3/2015	72.2	80.0	59.0	74.6	79.0	59.0	73.9	87.8	75.9	82.1	86.4	66.6	70.9	79.3	58.0	75.0	86.6	56.9	72.7	88.6	62.5				75.7	87.1	69.3	76.4	86.3	56.3	78.8	92.9	67.1	80.5	86.1	56.2	80.6	87.1	56.5
PoAstV2/JPN/Iba-464-4-1/2015	70.6	88.4	60.5	72.2	86.5	62.4	70.5	78.6	70.6	70.1	79.0	70.2	69.9	90.6	59.4	71.6	77.5	56.8	70.8	79.0	58.4	70.1	78.2	66.5				78.7	94.4	60.0	77.1	87.1	72.2	80.5	94.8	59.3	80.0	94.8	59.7
PoAstV2/USA/US-IA122/2011 (JX556690)	83.5	88.0	84.7	80.0	88.0	69.4	76.5	80.4	60.0	72.2	80.4	60.4	74.6	88.0	65.8	75.0	79.5	62.5	75.8	80.1	55.0	72.2	79.0	59.9	71.8	88.6	61.8				84.5	87.5	58.9	86.6	96.6	93.7	87.4	97.2	94.9
PoAstV2/KOR/KNU14-07/2014 (KP759770)	82.5	79.2	60.8	74.4	79.2	61.5	76.8	87.5	72.0	72.9	89.0	72.9	71.0	78.2	59.1	73.6	87.1	59.1	74.8	87.6	59.6	72.8	87.1	67.0	70.5	78.8	70.4	79.2	79.5	60.6				80.9	86.3	57.5	81.5	87.7	57.9
PoAstV2/USA/ExpPig-36 (KJ495986)	79.2	88.7	84.3	86.6	87.7	69.2	75.4	79.3	59.6	75.1	79.0	60.2	76.2	88.2	64.7	80.0	78.7	61.6	75.6	78.9	53.9	73.7	78.4	59.1	72.0	88.8	61.1	79.9	90.0	89.0	75.4	78.4	60.0				97.1	96.6	96.0
PoAstV2/USA/43/2010 (NC_023674)	78.8	87.7	85.0	86.7	86.9	69.4	75.0	79.3	60.3	75.2	79.3	60.1	75.4	87.0	64.4	79.4	79.3	61.5	75.0	79.3	54.4	73.3	78.9	59.5	72.2	87.9	60.4	79.2	90.9	89.4	74.6	78.8	60.2	89.2	91.1	90.0			
PoAstV2/USA/51/2010 (JF713712)	78.7	87.4	69.6	86.7	87.0	84.2	74.8	78.8	61.0	75.3	78.8	60.7	75.5	87.0	66.8	79.2	79.3	63.9	75.1	79.3	55.3	73.5	79.1	59.7	72.4	88.1	61.5	79.1	90.5	69.6	74.7	78.6	61.2	89.2	91.3	69.7	99.5	97.4	69.2
PoAstV2/BEL/Bel-12R021/ (KP982872)	78.3	79.4	60.7	74.3	79.4	59.8	76.0	88.1	72.1	72.7	88.3	72.1	71.2	79.1	58.3	74.2	86.9	57.6	74.4	87.8	58.7	72.8	86.6	65.7	70.7	79.0	69.2	80.7	80.2	60.3	81.8	89.3	79.0	73.2	79.5	59.5	73.8	79.3	59.5
PoAstV2/CHN/LL-1/2006 (KP747573)	71.5	78.6	60.8	73.3	78.8	61.2	72.0	87.4	71.7	82.7	88.2	72.8	73.1	78.5	59.1	74.1	88.6	58.6	70.9	86.2	59.6	80.6	87.3	67.7	69.2	78.2	70.6	71.2	78.9	60.7	72.6	89.0	95.2	73.5	79.3	59.7	73.4	78.6	60.0
DcAstV/UAE/64/2013 (KR868722)	71.0	79.4	59.9	73.9	79.1	60.7	71.7	85.5	55.7	79.2	85.4	55.6	70.1	79.3	61.6	74.0	85.1	61.8	72.3	86.0	53.3	77.8	85.0	54.0	68.7	78.5	55.6	71.3	79.4	59.3	73.0	85.4	55.9	73.2	78.6	60.2	73.7	78.8	60.6
DcAstV/UAE/274/2013 (KR868724)	70.8	79.0	61.8	73.5	78.9	64.1	71.7	85.3	55.9	78.8	85.1	56.2	70.0	78.4	64.2	73.9	85.1	62.0	71.6	85.7	54.6	77.4	84.5	55.8	68.4	77.8	56.1	70.9	78.8	62.1	72.2	85.5	56.7	73.1	78.6	62.5	73.2	78.4	62.8
BoAstV/JPN/Ishikawa24-6/2013 (LC047787)	67.2	76.9	48.9	67.6	75.3	50.6	67.6	76.7	46.5	68.2	75.7	47.5	66.8	75.4	48.1	68.2	76.0	48.2	67.3	76.1	47.3	67.2	76.7	46.0	68.0	76.1	46.6	67.1	76.1	48.8	67.0	76.2	46.6	67.6	76.7	49.4	67.1	76.1	49.1
BoAstV/JPN/Ishikawa9728/2013 (LC047788)	63.5	75.2	47.7	63.4	74.4	46.6	63.9	74.1	47.2	65.2	73.2	47.9	62.8	73.6	46.2	63.9	73.4	45.6	63.6	73.8	46.9	64.7	73.7	46.8	65.2	73.7	47.5	63.7	74.4	47.1	64.5	73.3	47.8	64.3	74.1	48.2	63.7	75.5	47.9
BoAstV/JPN/Kagoshima2-3-2/2015 (LC047798)	49.2	65.2	43.1	49.9	64.4	42.5	49.7	65.5	41.6	48.0	65.2	41.7	49.0	63.1	42.2	49.5	65.7	42.3	48.7	65.8	40.7	48.6	64.3	41.5	48.6	63.9	41.4	49.7	65.4	43.7	49.7	65.6	41.8	50.0	64.3	44.0	50.0	63.7	42.8
PoAstV1/CHN/GX1/2013 (NC_025379)	31.2	57.9	31.4	31.7	58.7	31.0	30.8	58.3	32.5	31.5	58.5	32.0	31.8	57.9	31.5	31.0	58.7	31.1	30.7	58.5	32.3	32.2	58.7	31.0	31.3	58.0	32.1	31.0	58.0	30.9	30.6	58.2	33.1	31.4	57.9	30.8	31.2	58.6	31.4
PoAstV3/USA/US-MO123/2011 (NC_019494)	34.1	52.8	27.7	33.3	53.4	28.7	33.6	53.2	27.1	33.5	52.9	27.6	32.8	52.4	27.6	33.6	53.9	29.0	33.4	53.9	27.3	33.1	53.3	28.4	33.4	52.0	27.8	33.6	53.0	28.1	32.6	54.0	26.8	33.7	52.7	27.7	33.4	52.8	27.5
PoAstV4/USA/35/2010 (NC_023675)	42.1	59.4	37.0	42.7	59.4	37.3	42.4	60.4	37.6	41.8	60.8	38.0	42.4	59.7	37.9	42.7	59.7	38.3	43.1	60.4	36.9	41.9	59.9	36.9	41.1	58.7	36.4	41.8	60.4	37.2	41.9	60.6	37.2	43.0	60.4	36.5	42.5	60.6	36.5
PoAstV5/USA/US-IA122/2011 (NC_023636)	32.1	52.1	27.9	33.2	52.2	28.5	32.2	52.0	29.5	33.0	52.4	29.2	32.8	52.7	28.6	32.8	52.0	28.4	32.2	51.8	29.4	32.6	51.7	29.5	33.6	50.9	29.9	32.4	52.2	28.5	32.6	51.8	28.7	33.1	51.9	28.7	32.8	53.3	28.7

	Po2-51			Po2-Bel-12R021			Po2-LL-1			D-64			D-274			Bo-Ishikawa24-6			Bo-Ishikawa9728			Bo-Kagoshima2-3-2			Po1-GX1			Po3-MO123			Po4-35/USA			Po5-US-IA122		
	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2
PoAstV2/JPN/Bu5-10-1/2014	85.9	96.2	72.5	84.0	86.7	58.0	76.1	87.7	58.8	76.4	87.1	60.5	76.2	86.7	59.7	70.7	83.1	37.9	66.2	83.7	43.2	39.6	67.1	33.1	22.2	54.4	19.3	23.0	48.3	17.1	31.2	58.9	25.7	18.2	46.7	19.7			
PoAstV2/JPN/HgOg2-1/2015	97.3	95.8	90.7	80.2	87.3	56.7	79.9	88.3	58.8	79.8	87.1	63.8	79.5	86.7	61.0	72.0	83.5	38.8	67.5	83.9	41.3	41.1	67.3	31.2	22.5	55.0	19.7	22.7	48.7	17.2	32.2	58.9	26.1	18.6	46.3	20.1			
PoAstV2/JPN/HgOg2-4/2015	81.7	88.3	56.5	81.7	95.6	72.9	77.8	94.6	73.3	78.6	92.9	50.4	78.3	92.3	52.8	70.3	83.1	35.3	67.6	81.6	42.2	41.9	67.9	29.8	21.9	56.3	20.7	21.9	49.1	17.1	31.3	58.5	25.8	18.8	47.1	20.3			
PoAstV2/JPN/HgYa2-3/2015	82.1	87.3	57.8	78.4	94.6	75.3	91.5	95.2	75.7	87.2	93.1	51.8	86.8	93.1	53.4	71.2	83.1	36.9	68.6	80.8	43.7	40.1	67.9	31.2	21.9	55.3	21.2	21.9	49.2	16.7	31.6	59.5	25.7	19.2	47.3	21.0			
PoAstV2/JPN/Ishi-Ya4/2015	82.9	95.4	64.7	76.4	86.5	53.1	78.1	86.5	55.2	75.4	85.3	63.6	74.8	84.5	63.6	70.8	82.3	37.6	66.6	83.5	42.4	40.4	66.3	31.9	21.1	55.3	20.7	22.7	47.5	16.1	32.0	58.1	25.8	19.2	46.5	19.8			
PoAstV2/JPN/Ishi-Ya6/2015	89.7	86.9	62.6	80.9	94.4	54.6	81.9	95.8	54.9	80.2	92.3	62.0	80.2	91.7	63.3	72.6	82.3	38.9	67.2	81.6	42.3	41.6	68.4	33.3	22.5	55.0	20.3	23.6	48.1	17.0	33.1	58.9	26.0	18.6	46.5	20.6			
PoAstV2/JPN/Ishi-Ya8/2015	82.2	87.5	48.9	81.0	92.5	54.3	77.0	92.3	55.6	79.5	92.5	47.8	79.4	92.3	47.5	71.1	82.9	36.1	67.0	81.4	42.9	41.5	67.1	29.1	21.5	56.1	19.7	22.5	48.9	16.1	32.6	58.7	24.3	18.9	46.5	19.3			
PoAstV2/JPN/Ishi-Im3/2015	80.6	87.1	57.8	78.1	92.1	65.0	88.9	93.3	67.7	85.8	91.9	51.3	85.5	91.5	52.7	70.5	82.9	36.0	68.1	81.2	41.7	40.2	66.9	29.7	22.4	55.9	19.3	22.2	48.5	17.0	31.0	58.7	25.9	19.4	46.9	19.8			
PoAstV2/JPN/Iba-464-4-1/2015	80.0	95.4	58.6	77.6	86.7	69.9	74.6	87.3	72.5	75.7	85.9	52.5	75.7	85.1	52.4	73.0	82.7	35.4	68.1	83.5	42.6	39.9	66.7	30.1	21.3	55.0	20.7	22.7	47.9	17.6	31.4	57.9	26.1	19.8	46.5	21.1			
PoAstV2/USA/US-IA122/2011 (JX556690)	87.4	97.0	71.5	87.9	87.3	56.5	75.2	87.9	58.5	76.5	86.7	60.6	76.2	86.3	59.5	70.9	83.3	37.6	66.7	83.5	43.3	40.7	66.9	32.4	22.4	54.3	19.5	23.1	48.1	17.5	32.8	59.1	25.0	18.0	46.5	19.7			
PoAstV2/KOR/KNU14-07/2014 (KP759770)	81.5	86.9	58.3	88.5	95.0	84.2	77.1	95.2	97.6	78.4	94.4	52.5	78.1	94.3	53.3	71.2	82.3	36.5	68.1	80.0	42.8	41.1	67.9	31.6	21.1	55.5	20.9	22.9	49.6	17.0	30.8	59.5	25.9	19.1	46.7	20.9			
PoAstV2/USA/ExpPig-36 (KJ495986)	97.2	97.2	70.8	80.5	86.5	56.8	81.1	87.3	57.4	80.0	85.9	60.0	79.8	85.5	59.3	72.2	83.3	37.9	67.6	83.1	43.8	41.0	67.1	32.5	22.4	55.0	19.4	23.1	49.1	17.4	32.1	58.9	25.6	19.1	46.1	19.8			
PoAstV2/USA/43/2010 (NC_023674)	99.9	99.0	71.5	80.6	87.5	56.4	80.4	88.1	57.8	80.2	87.3	61.2	80.0	86.9	60.1	72.6	83.3	37.6	67.6	82.9	43.4	41.6	66.9	32.4	22.4	55.2	19.4	23.0	48.1	17.5	32.6	59.3	25.3	19.1	46.9	20.1			
PoAstV2/USA/51/2010 (JF713712)				80.6	87.3	56.9	80.5	87.9	58.3	80.2	87.1	62.8	80.0	86.7	59.5	72.8	83.1	38.3	67.6	83.7	41.6	41.6	67.3	30.9	22.4	55.2	19.8	23.0	48.1	16.6	32.6	59.1	25.8	19.1	46.7	20.3			
PoAstV2/BEL/Bel-12R021/ (KP982872)	73.9	79.7	60.0				77.0	94.3	84.9	77.3	92.5	51.5	76.9	91.7	52.7	71.1	81.9	36.6	67.2	79.8	42.2	40.9	69.0	31.0	21.7	56.1	21.1	23.2	48.9	16.6	31.4	58.9	25.7	19.2	46.7	20.9			
PoAstV2/CHN/LL-1/2006 (KP747573)	73.3	78.6	61.2	71.4	88.6	79.5				84.4	93.7	52.5	83.9	93.1	53.1	71.6	82.9	36.5	67.0	81.0	42.8	39.7	68.2	31.6	21.9	55.2	20.7	22.2	49.2	16.9	32.0	59.1	26.1	19.4	47.1	21.0			
DcAstV/UAE/64/2013 (KR868722)	73.6	78.9	61.2	72.7	85.5	55.4	76.7	85.5	55.6				99.0	98.6	64.1	72.8	82.9	38.6	69.0	80.8	41.6	39.7	67.7	31.8	21.3	55.3	19.7	22.8	48.7	16.3	30.6	59.1	25.4	20.1	46.7	19.8			
DcAstV/UAE/274/2013 (KR868724)	73.1	78.5	63.6	72.3	85.3	55.2	76.3	85.3	56.9	97.9	97.4	65.2				72.2	82.7	37.6	68.6	81.0	41.0	39.5	67.5	32.7	21.1	55.2	20.4	22.5	48.7	16.5	30.4	58.7	24.6	19.9	46.5	20.8			
BoAstV/JPN/Ishikawa24-6/2013 (LC047787)	67.0	75.7	50.7	67.4	75.8	46.2	68.0	76.1	46.6	67.2	74.9	45.8	67.0	75.1	47.5				73.3	86.9	36.1	39.7	67.8	41.5	20.4	55.6	17.4	23.4	47.5	16.8	32.2	58.7	21.2	17.8	47.2	19.3			
BoAstV/JPN/Ishikawa9728/2013 (LC047788)	63.6	75.5	47.0	64.5	72.5	47.3	64.2	73.6	47.2	64.3	74.3	45.6	63.9	74.3	44.7	67.7	77.5	46.1				40.4	68.2	32.3	19.8	56.6	19.8	22.4	47.9	15.6	31.2	58.1	25.7	19.3	47.2	19.8			
BoAstV/JPN/Kagoshima2-3-2/2015 (LC047798)	50.1	64.2	42.8	48.9	66.7	41.5	48.8	64.7	41.9	47.9	64.9	41.6	47.3	64.6	41.8	48.1	67.9	45.0	48.4	64.6	43.3				21.0	54.4	17.5	19.9	45.3	16.7	29.8	58.2	24.4	18.0	46.6	19.9			
PoAstV1/CHN/GX1/2013 (NC_025379)	31.3	58.0	32.4	29.7	58.5	32.6	31.5	58.7	32.8	32.0	58.4	32.3	31.6	58.8	32.4	30.5	56.8	28.8	30.0	58.6	28.6	30.1	55.1	28.6				20.6	48.0	18.0	22.8	55.2	21.3	15.9	46.9	18.1			
PoAstV3/USA/US-MO123/2011 (NC_019494)	33.4	52.9	27.8	34.2	53.8	27.7	32.6	53.2	26.5	34.1	52.5	27.7	33.9	52.5	28.6	33.7	51.8	28.7	34.2	52.0	26.2	29.5	50.3	27.5	36.0	52.2	30.0				21.8	48.3	16.0	21.0	42.0	16.5			
PoAstV4/USA/35/2010 (NC_023675)	42.5	60.8	37.5	41.5	60.7	37.6	41.4	60.2	37.2	41.5	60.0	38.5	41.4	59.9	36.7	41.1	59.8	33.7	40.7	59.8	34.7	37.8	58.7	35.0	32.5	57.8	31.4	32.7	51.4	29.3				18.0	45.7	18.0			
PoAstV5/USA/US-IA122/2011 (NC_023636)	32.7	52.9	29.3	32.5	50.6	28.9	32.6	51.8	28.8	32.8	52.9	28.3	32.7	52.6	28.8	32.5	51.4	29.1	32.4	52.8	28.4	29.3	52.0	28.5	31.3	51.9	29.4	33.8	48.2	30.5	35.3	51.5	27.7						

Table 5

Pairwise nucleotide (lower left) and amino acid (upper right (grey shades) sequence identities (%) between three complete CDS (ORF1a, ORF1b, and ORF2) of Japanese PoAstV3s and other PoAstVs.

	Po3-Bu2-5			Po3-Bu4-2-1			Po3-Bu4-4			Po3-Bu7-9			Po3-Bu8-4			Po3-MO123			Po1-GX1			Po2-43			Po4-35			Po5-US-IA122		
	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2
PoAstV3/JPN/Bu2-5/2014				96.0	97.3	95.4	96.0	97.1	95.5	96.2	96.4	65.9	95.5	97.0	95.4	94.4	97.0	94.1	21.9	48.2	23.2	23.7	49.0	19.6	23.3	49.7	17.8	20.3	42.1	18.4
PoAstV3/JPN/Bu4-2-1/2014	90.1	90.6	92.0				100.0	99.8	99.9	95.7	95.4	65.8	95.0	96.2	95.2	94.6	96.2	94.1	21.8	48.6	23.0	23.4	49.0	19.3	22.7	50.3	17.9	20.3	42.3	18.7
PoAstV3/JPN/Bu4-4/2014	90.1	90.5	92.0	100.0	99.9	100.0				95.7	95.2	65.8	95.0	96.0	95.4	94.6	96.0	94.3	21.8	48.4	23.0	23.4	49.0	19.3	22.7	50.1	17.9	20.3	42.3	18.7
PoAstV3/JPN/Bu7-9/2014	90.1	91.3	65.4	90.1	89.8	64.9	90.1	89.7	64.9				95.7	95.4	65.3	94.8	95.8	65.2	22.0	48.2	23.3	23.3	49.5	19.6	22.5	49.9	17.4	20.3	42.3	18.6
PoAstV3/JPN/Bu8-4/2014	89.6	91.5	91.0	89.1	90.2	90.8	89.1	90.1	90.9	89.2	90.5	65.1				94.3	95.4	95.0	21.5	47.8	22.9	23.1	48.4	19.5	22.7	49.5	17.7	20.3	42.1	18.7
PoAstV3/USA/US-MO123/2011 (NC_019494)	88.0	91.2	88.9	86.9	90.5	88.7	86.9	90.5	88.8	87.9	92.0	64.9	87.1	90.4	88.8				21.5	47.6	22.8	23.8	48.8	19.2	23.0	49.0	17.9	20.3	42.3	18.2
PoAstV1/CHN/GX1/2013 (NC_025379)	42.1	52.3	40.2	42.4	53.3	41.1	42.4	53.3	41.1	43.0	52.8	38.4	42.8	53.0	41.0	42.3	52.3	39.8				20.8	55.9	20.7	20.2	56.1	21.7	16.5	46.8	19.7
PoAstV2/USA/43/2010 (NC_023674)	39.0	53.9	31.3	39.0	53.2	31.7	39.0	53.2	31.7	38.9	54.1	31.3	39.0	53.8	31.0	38.4	53.6	32.0	38.8	58.8	37.2				32.6	59.5	24.5	17.1	46.5	19.3
PoAstV4/USA/35/USA/2010 (NC_023675)	33.0	52.7	34.9	33.2	52.9	35.6	33.2	52.9	35.5	32.8	52.5	34.8	32.6	53.0	35.0	32.1	52.0	34.8	34.5	57.3	37.3	43.9	61.2	39.5				17.4	45.8	19.7
PoAstV5/USA/US-IA122/2011 (NC_023636)	34.0	50.5	34.3	33.7	50.2	34.2	33.7	50.2	34.2	33.9	50.0	34.4	33.0	50.6	34.0	33.6	49.7	33.8	34.2	50.8	32.5	35.7	52.9	31.6	30.3	51.7	32.4			

Table 6

Pairwise nucleotide (lower left) and amino acid (upper right (grey shades)) sequence identities (%) between three complete CDS (ORF1a, ORF1b, and ORF2) of Japanese PoAstV4s and other PoAstVs.

	Po4-Bu4-2-2			Po4-Bu4-6-1			Po4-HkKa2-1			Po4-HgTa2-3			Po4-MoI2-1-1			Po4-MoI2-1-2			Po4-MoI2-2			Po4-MoI2-3-2			Po4-Ishi-Ya7-1		
	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2
PoAstV4/JPN/Bu4-2-2/2014				99.7	100.0	99.5	75.1	95.6	64.5	91.6	96.6	55.7	83.2	96.4	70.1	73.8	97.4	54.2	73.8	97.4	54.2	73.8	97.4	54.2	74.1	95.8	75.2
PoAstV4/JPN/Bu4-6-1/2014	99.8	99.9	99.7				75.3	95.6	64.4	91.6	96.6	55.7	83.3	96.4	70.0	73.9	97.4	54.2	73.9	97.4	54.2	73.9	97.4	54.2	74.2	95.8	75.1
PoAstV4/JPN/HkKa2-1/2015	72.9	90.3	66.6	73.0	90.2	66.6				73.6	96.6	54.0	76.6	95.0	63.6	89.2	96.4	50.5	89.2	96.4	50.5	89.2	96.4	50.5	89.0	96.2	62.2
PoAstV4/JPN/HgTa2-3/2015	86.3	92.8	61.1	86.3	92.7	61.1	72.3	91.7	60.0				85.0	95.2	56.5	73.4	96.8	55.3	73.4	96.8	55.3	73.4	96.8	55.3	74.2	96.8	53.0
PoAstV4/JPN/MoI2-1-1/2015	79.7	91.2	71.4	79.6	91.1	71.2	74.9	89.8	66.5	81.0	90.5	61.4				75.4	96.8	55.0	75.4	96.8	55.0	75.4	96.8	55.0	77.4	95.6	69.4
PoAstV4/JPN/MoI2-1-2/2015	72.6	92.8	59.3	72.7	92.9	59.2	83.4	90.8	58.6	73.2	90.8	59.2	75.0	91.7	61.2				100.0	100.0	100.0	100.0	100.0	100.0	93.9	96.4	49.9
PoAstV4/JPN/MoI2-2/2015	72.6	92.8	59.3	72.7	92.9	59.2	83.4	90.8	58.6	73.2	90.8	59.2	75.0	91.7	61.2	100.0	100.0	100.0				100.0	100.0	100.0	93.9	96.4	49.9
PoAstV4/JPN/MoI2-3-2/2015	72.6	92.8	59.3	72.7	92.9	59.2	83.4	90.8	58.6	73.2	90.8	59.2	75.0	91.7	61.2	100.0	100.0	100.0	100.0	100.0	100.0				93.9	96.4	49.9
PoAstV4/JPN/Ishi-Ya7-1/2015	72.3	91.7	73.4	72.3	91.6	73.4	83.6	91.1	64.7	72.8	91.9	58.8	75.4	91.5	69.0	88.3	91.7	56.8	88.3	91.7	56.8	88.3	91.7	56.8			
PoAstV4/USA/35/2010 (NC_023675)	73.1	93.4	57.8	73.1	93.3	57.7	76.7	91.1	58.6	71.7	92.3	60.9	73.9	91.5	58.3	78.8	91.9	75.4	78.8	91.9	75.4	78.8	91.9	75.4	78.3	92.2	55.6
PoAstV4/USA/US-IL135/2011 (JX556692)	74.3	93.9	58.0	74.3	93.8	57.8	86.5	90.8	58.8	73.4	92.1	60.8	75.6	92.3	58.5	83.3	92.1	75.6	83.3	92.1	75.6	83.3	92.1	75.6	84.7	91.9	56.1
PoAstV4/USA/15-12/2015 (KU764486)	70.9	90.9	60.1	70.9	90.8	60.1	76.4	89.6	61.1	70.3	91.9	66.9	72.7	90.7	59.0	79.1	90.3	58.9	79.1	90.3	58.9	79.1	90.3	58.9	79.4	91.3	56.2
Wild_boar_AstV/HUN/WBAstV-1/2011 (JQ340310)	69.0	91.7	59.7	69.0	91.6	59.6	82.3	89.8	58.3	68.3	91.1	58.8	70.7	92.4	61.1	77.4	90.3	59.3	77.4	90.3	59.3	77.4	90.3	59.3	77.7	90.2	60.8
PoAstV1/CHN/GX1/2013 (NC_025379)	38.3	57.4	35.7	38.4	57.4	35.8	38.7	59.1	37.1	38.8	57.2	37.0	38.6	57.6	35.6	38.5	57.6	36.3	38.5	57.6	36.3	38.5	57.6	36.3	38.8	58.3	34.3
PoAstV2/USA/43/2010 (NC_023674)	43.2	58.7	38.3	43.1	58.7	38.4	44.5	60.2	38.3	43.4	59.7	38.5	45.2	58.7	39.5	45.1	58.9	40.1	45.1	58.9	40.1	45.1	58.9	40.1	46.1	58.6	36.3
PoAstV3/USA/US-MO123/2011 (NC_019494)	35.8	51.8	30.7	35.9	51.7	30.6	35.7	52.7	30.3	36.9	53.1	30.0	37.0	53.1	30.3	36.7	52.9	30.5	36.7	52.9	30.5	36.7	52.9	30.5	36.8	52.9	29.5
PoAstV5/USA/US-IA122/2011 (NC_023636)	35.5	52.9	31.1	35.5	52.9	31.2	36.7	52.6	30.6	35.8	53.0	31.3	36.9	52.3	31.4	36.4	53.3	32.0	36.4	53.3	32.0	36.4	53.3	32.0	35.9	52.5	31.1

	Po4-35			Po4-US-IL135			Po4-15-12			WBAst-1			Po1-GX1			Po2-43			Po3-MO123			Po5-US-IA122		
	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2
PoAstV4/JPN/Bu4-2-2/2014	74.4	96.8	52.0	75.7	95.8	52.7	70.2	95.4	52.3	68.0	95.6	53.1	22.8	56.3	24.1	31.8	58.9	26.9	21.3	48.3	16.3	18.4	45.5	19.7
PoAstV4/JPN/Bu4-6-1/2014	74.5	96.8	51.9	75.8	95.8	52.6	70.1	95.4	52.2	67.9	95.6	53.0	22.8	56.3	24.0	31.8	58.9	27.1	21.4	48.3	16.3	18.4	45.5	19.7
PoAstV4/JPN/HkKa2-1/2015	79.6	95.8	49.6	92.4	94.1	50.1	80.1	95.8	53.3	82.5	93.9	51.4	23.9	57.2	22.1	33.1	58.7	25.6	21.4	48.7	15.8	18.4	45.5	20.0
PoAstV4/JPN/HgTa2-3/2015	72.8	96.6	56.3	74.2	95.2	56.4	68.5	96.2	63.2	67.0	94.7	54.6	22.9	56.1	23.6	32.0	58.9	28.4	21.1	48.7	17.3	18.3	44.9	19.7
PoAstV4/JPN/MoI2-1-1/2015	75.7	96.2	53.4	77.0	94.6	53.5	70.5	95.4	53.2	69.0	96.8	53.8	22.9	56.6	22.2	32.7	58.7	27.4	21.6	49.4	16.7	18.4	45.5	19.2
PoAstV4/JPN/MoI2-1-2/2015	81.8	97.4	78.5	90.4	95.6	78.9	81.3	96.0	52.8	77.6	95.3	56.3	24.1	56.6	23.5	33.0	59.1	27.2	21.4	49.4	17.9	18.1	45.9	19.8
PoAstV4/JPN/MoI2-2/2015	81.8	97.4	78.5	90.4	95.6	78.9	81.3	96.0	52.8	77.6	95.3	56.3	24.1	56.6	23.5	33.0	59.1	27.2	21.4	49.4	17.9	18.1	45.9	19.8
PoAstV4/JPN/MoI2-3-2/2015	81.8	97.4	78.5	90.4	95.6	78.9	81.3	96.0	52.8	77.6	95.3	56.3	24.1	56.6	23.5	33.0	59.1	27.2	21.4	49.4	17.9	18.1	45.9	19.8
PoAstV4/JPN/Ishi-Ya7-1/2015	81.7	96.4	48.6	89.8	94.8	48.9	80.9	95.2	48.9	78.1	94.5	50.9	24.6	56.6	22.2	33.3	58.7	25.1	21.8	48.7	15.5	18.8	45.3	18.6
PoAstV4/USA/35/2010 (NC_023675)				81.0	95.4	96.3	81.8	96.6	52.4	76.0	94.9	55.3	23.3	56.3	23.7	32.8	59.5	26.3	22.1	48.9	17.2	18.1	45.3	20.1
PoAstV4/USA/US-IL135/2011 (JX556692)	77.5	92.9	94.9				80.7	94.3	52.1	81.6	93.9	55.7	24.1	55.7	23.1	32.7	57.7	26.3	21.3	48.5	17.2	18.1	44.9	20.0
PoAstV4/USA/15-12/2015 (KU764486)	81.8	91.7	59.0	77.9	91.5	59.3				75.2	94.3	51.5	23.6	56.3	22.8	31.6	58.5	26.3	20.3	49.1	16.9	17.0	45.9	20.8
Wild_boar_AstV/HUN/WBAstV-1/2011 (JQ340310)	76.7	91.2	59.2	80.9	91.8	59.9	76.5	89.6	56.7				21.9	55.8	22.8	31.2	57.6	26.3	20.4	48.4	16.7	17.4	44.6	19.5
PoAstV1/CHN/GX1/2013 (NC_025379)	38.1	57.5	37.5	39.3	57.8	37.2	37.8	58.7	36.5	37.6	56.6	35.6				21.7	56.1	20.2	21.0	47.6	20.6	14.6	46.4	20.5
PoAstV2/USA/43/2010 (NC_023674)	44.6	59.2	39.7	44.7	59.1	39.6	43.7	58.6	39.3	42.3	59.2	37.8	36.2	59.5	37.2				22.6	49.1	16.7	17.2	45.9	19.6
PoAstV3/USA/US-MO123/2011 (NC_019494)	35.5	52.4	30.9	36.7	52.5	31.3	35.3	53.1	29.8	34.8	52.3	29.8	34.1	51.9	32.0	37.1	53.1	31.6				18.9	42.3	18.3
PoAstV5/USA/US-IA122/2011 (NC_023636)	37.1	52.3	31.8	36.7	53.0	32.4	36.5	51.6	30.9	36.2	52.5	31.1	33.7	52.0	32.6	33.8	52.1	31.8	32.6	48.7	32.8			

Table 7

Pairwise nucleotide (lower left) and amino acid (upper right (grey shades)) sequence identities (%) between three complete CDS (ORF1a, ORF1b, and ORF2) of Japanese PoAstV5s and other PoAstVs.

	Po5-HgTa2-1-3			Po5-HgTa2-2			Po5-MoI2-1-3			Po5-MoI2-3-1			Po5-Ishi-Im1-1			Po5-Ishi-Im1-2			Po5-US-IA122		
	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2
PoAstV5/JPN/HgTa2-1-3/2015				100.0	100.0	100.0	99.5	99.6	97.3	99.5	99.6	97.3	97.3	99.0	78.6	98.1	99.6	96.7	96.8	98.8	87.9
PoAstV5/JPN/HgTa2-2/2015	100.0	100.0	100.0				99.5	99.6	97.3	99.5	99.6	97.3	97.3	99.0	78.6	98.1	99.6	96.7	96.8	98.8	87.9
PoAstV5/JPN/MoI2-1-3/2015	96.4	92.9	92.6	96.4	92.9	92.6				100.0	100.0	100.0	97.1	98.8	77.9	98.0	99.4	96.9	96.8	98.8	87.5
PoAstV5/JPN/MoI2-3-1/2015	96.4	92.9	92.6	96.4	92.9	92.6	100.0	100.0	100.0				97.1	98.8	77.9	98.0	99.4	96.9	96.8	98.8	87.5
PoAstV5/JPN/Ishi-Im1-1/2015	89.4	90.7	76.4	89.4	90.7	76.4	89.8	92.6	76.5	89.8	92.6	76.5				96.4	99.0	77.6	96.1	98.4	73.1
PoAstV5/JPN/Ishi-Im1-2/2015	90.0	90.7	91.5	90.0	90.7	91.5	89.6	92.9	93.3	89.6	92.9	93.3	88.9	91.7	76.1				96.0	98.6	87.5
PoAstV5/USA/US-IA122/2011 (JX556693)	86.2	91.4	83.6	86.2	91.4	83.6	86.4	92.5	82.9	86.4	92.5	82.9	86.2	91.9	72.4	86.9	90.9	83.0			
PoAstV5/USA/33/2010 (JF713711)	86.0	91.5	89.0	86.0	91.5	89.0	86.4	93.2	88.4	86.4	93.2	88.4	86.0	91.8	77.0	86.5	91.3	88.3	96.9	97.1	90.9
PoAstV5/CHN/LL-2/2006 (KP747574)	69.4	78.6	82.7	69.4	78.6	82.7	69.3	78.4	82.6	69.3	78.4	82.6	70.1	77.9	70.9	69.6	77.7	82.3	70.0	77.5	77.0
BoAstV/JPN/Hokkaido12-25/2009 (LC047793)	69.1	76.3	68.7	69.1	76.3	68.7	69.3	75.9	68.7	69.3	75.9	68.7	69.2	76.7	66.2	69.0	77.0	69.2	69.1	76.0	64.7
PoAstV1/CHN/GX1/2013 (NC_025379)	40.0	51.4	37.5	40.0	51.4	37.5	39.8	51.6	37.4	39.8	51.6	37.4	39.8	51.7	36.2	39.3	52.0	37.6	39.6	51.6	35.2
PoAstV2/USA/43/2010 (NC_023674)	35.3	52.1	38.7	35.3	52.1	38.7	35.4	51.7	37.8	35.4	51.7	37.8	34.5	51.9	38.2	34.6	51.0	37.9	34.5	52.5	36.6
PoAstV3/USA/US-MO123/2011 (JX556691)	33.6	48.8	34.2	33.6	48.8	34.2	33.2	48.6	34.2	33.2	48.6	34.2	33.2	49.1	34.1	33.3	48.5	34.2	32.7	49.1	35.7
PoAstV4/USA/35/2010 (JF713713)	31.5	51.0	39.2	31.5	51.0	39.2	31.7	51.3	39.2	31.7	51.3	39.2	31.4	50.9	37.6	31.5	51.5	38.9	32.2	51.6	36.6

	Po5-33			Po5-LL-2			Bo-Ho12-25			Po1-GX1			Po2-43			Po3-MO123			Po4-35		
	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2	ORF1a	ORF1b	ORF2
PoAstV5/JPN/HgTa2-1-3/2015	96.8	99.2	93.6	77.6	89.9	84.8	76.3	88.5	72.3	17.1	47.1	21.5	18.5	46.2	24.2	21.7	42.7	18.9	18.3	45.7	21.3
PoAstV5/JPN/HgTa2-2/2015	96.8	99.2	93.6	77.6	89.9	84.8	76.3	88.5	72.3	17.1	47.1	21.5	18.5	46.2	24.2	21.7	42.7	18.9	18.3	45.7	21.3
PoAstV5/JPN/MoI2-1-3/2015	96.6	99.2	93.5	77.6	89.7	84.4	76.3	88.3	72.9	17.0	47.3	21.1	18.4	46.0	23.9	21.7	42.9	18.8	18.2	45.9	21.2
PoAstV5/JPN/MoI2-3-1/2015	96.6	99.2	93.5	77.6	89.7	84.4	76.3	88.3	72.9	17.0	47.3	21.1	18.4	46.0	23.9	21.7	42.9	18.8	18.2	45.9	21.2
PoAstV5/JPN/Ishi-Im1-1/2015	96.0	98.8	78.0	78.0	89.9	70.7	76.5	88.7	68.3	16.6	47.3	21.1	18.6	46.4	23.8	21.8	42.7	19.3	18.1	45.7	21.3
PoAstV5/JPN/Ishi-Im1-2/2015	95.9	99.0	93.3	77.2	89.9	85.1	76.1	88.3	72.9	17.4	46.9	21.6	18.4	46.2	23.9	21.8	42.7	18.9	18.2	45.5	21.3
PoAstV5/USA/US-IA122/2011 (JX556693)	98.6	99.2	92.5	78.2	89.1	78.0	75.7	88.1	68.6	16.6	46.7	20.3	18.4	46.2	22.7	21.4	42.7	18.9	18.4	45.7	20.2
PoAstV5/USA/33/2010 (JF713711)				78.2	89.7	83.3	75.5	88.7	72.5	16.6	46.9	21.5	18.7	46.4	23.9	21.4	42.9	18.8	18.5	45.9	21.3
PoAstV5/CHN/LL-2/2006 (KP747574)	69.9	77.4	81.9				77.1	87.9	75.3	16.6	46.7	20.4	17.5	46.6	22.9	20.9	43.6	18.3	20.1	45.1	20.7
BoAstV/JPN/Hokkaido12-25/2009 (LC047793)	69.0	76.4	68.3	69.3	76.7	68.7				16.2	47.3	21.6	18.4	45.6	22.7	20.6	44.0	17.8	19.3	45.7	20.4
PoAstV1/CHN/GX1/2013 (NC_025379)	39.6	51.5	37.1	37.5	51.8	38.1	36.7	51.8	37.0				19.6	55.9	22.2	21.2	47.6	20.5	20.6	56.1	21.4
PoAstV2/USA/43/2010 (NC_023674)	35.0	52.2	38.4	34.6	52.5	38.4	33.5	50.8	36.7	40.5	59.5	37.7				21.0	48.7	15.5	32.6	59.5	26.4
PoAstV3/USA/US-MO123/2011 (JX556691)	32.6	48.9	34.3	33.2	48.6	34.0	32.0	48.6	33.5	36.1	52.6	35.6	37.6	54.2	32.2				20.7	49.1	16.8
PoAstV4/USA/35/2010 (JF713713)	32.1	51.7	38.8	31.2	50.0	38.3	29.7	50.6	36.5	35.6	57.3	35.4	42.4	60.2	38.0	33.1	51.6	32.4			

Table 8

Pairwise nucleotide (lower left) and amino acid (upper right (grey shades)) sequence identities (%) of complete CDS of ORF2 among PoAstV2s.

		lineage1									lineage2								
	Lineage	HgOg2-4	HgYa2-3	Iba-464-4-1	Ishi-Im3	Ishi-Ya8	CAN14-4	Bel-12	KNU14-07	LL-1	Bu5-10-1	HgOg2-1	Ishi-Ya4	Ishi-Ya6	43/2010)	CAN12-4	IA122	ExpPig-36	51/2010
PoAstV2/JPN/HgOg2-4/2015	1		80.8	74.1	79.8	56.5	60.9	72.9	73.7	73.3	58.1	57.2	53.2	53.9	57.2	65.4	57.1	56.7	56.7
PoAstV2/JPN/HgYa2-3/2015	1	78.0		71.2	66.6	57.1	61.3	75.3	75.7	75.7	58.0	59.5	55.7	55.6	57.1	58.3	57.5	56.8	58.1
PoAstV2/JPN/Iba-464-4-1/2015	1	70.6	70.2		69.3	54.4	63.4	69.9	72.2	72.5	58.7	59.9	56.9	52.9	59.5	61.1	59.8	59.1	59.0
PoAstV2/JPN/Ishi-Im3/2015	1	73.9	65.8	65.7		61.0	60.9	65.0	67.1	67.7	57.0	58.4	53.6	53.9	56.7	65.4	56.5	56.4	58.5
PoAstV2/JPN/Ishi-Ya8/2015	1	61.0	58.9	58.6	66.1		59.4	54.3	56.0	55.9	48.7	49.7	50.1	50.4	49.0	48.2	47.8	48.0	49.7
PoAstV2/PoAstV14-4/2006 (HM756260)	1	63.2	62.9	64.9	62.6	58.7		59.8	62.9	63.8	51.5	52.7	55.1	53.4	52.0	52.6	51.6	51.0	54.2
PoAstV2/BEL/Bel-12R021/ (KP982872)	1	72.1	72.1	69.2	64.4	58.0	62.6		84.2	84.9	58.1	57.4	53.6	54.8	56.1	56.7	56.2	56.5	57.2
PoAstV2/KOR/KNU14-07/2014 (KP759770)	1	72.0	72.9	70.4	65.8	59.8	65.0	79.0		97.6	58.8	59.1	55.2	55.1	57.6	57.9	58.6	57.2	58.6
PoAstV2/CHN/LL-1/2006 (KP747573)	1	71.7	72.8	70.6	66.4	59.5	65.4	79.5	95.2		58.8	59.2	55.5	55.2	57.6	58.2	58.3	57.2	58.7
PoAstV2/JPN/Bu5-10-1/2014	2	59.9	58.6	60.2	57.4	53.1	55.9	60.2	59.7	59.7		71.7	62.9	63.0	89.1	68.1	88.5	88.3	72.5
PoAstV2/JPN/HgOg2-1/2015	2	60.4	59.1	62.7	58.3	54.0	56.9	59.2	60.3	60.2	69.6		66.5	62.7	72.5	67.2	71.4	70.9	90.7
PoAstV2/JPN/Ishi-Ya4/2015	2	57.4	59.2	59.1	57.5	53.2	57.6	57.7	58.2	58.1	64.8	67.1		69.1	64.7	66.5	64.9	64.1	64.7
PoAstV2/JPN/Ishi-Ya6/2015	2	57.4	57.3	56.9	56.5	53.9	56.4	57.3	58.5	58.2	63.4	63.7	69.4		61.7	58.6	61.5	61.2	62.6
PoAstV2/USA/43/2010 (NC_023674)	2	59.7	58.5	60.1	58.1	52.8	55.7	58.9	58.8	58.5	85.0	69.4	64.6	61.7		68.2	94.9	96.0	71.5
PoAstV2/CAN/12-4/2006 (HM756259)	2	64.5	60.5	62.0	62.7	54.7	56.5	57.9	58.4	59.3	66.7	67.6	66.7	61.9	66.8		67.8	67.7	67.4
PoAstV2/USA/IA122/2011 (JX556690)	2	59.1	58.9	61.2	58.6	53.0	55.4	59.5	58.8	59.0	84.7	69.4	66.1	62.7	89.4	66.5		93.7	71.5
PoAstV2/USA/ExpPig-36 (KJ495986)	2	58.9	58.8	60.9	57.6	52.2	55.0	58.7	58.7	58.4	84.3	69.2	64.9	61.8	90.0	66.8	89.0		70.8
PoAstV2/USA/51/2010 (JF713712)	2	60.5	59.9	61.7	59.0	54.2	57.4	59.3	60.2	60.2	69.6	84.2	67.1	64.4	69.2	66.9	69.6	69.7	

contained dromedary AstV strains, 274/2013, 64/2013, reported by Woo et al. [93]. PoAstV4 strains were subdivided into four lineages (tentatively named PoAstV4 lineages 1 to 4 [PoAstV4 L1-L4]), with PoAstV4 L1 and L2 corresponding to *Mamastrovirus 26* and *27*, respectively. The identities in ORF 2 between PoAstV4 lineages was 55.4-64.7% nt and 45.1-59.7% aa, whereas the identities within each lineage were > 66.3% nt and > 63.0% aa (Table 9). Phylogenetic analyses based on ORF1a and ORF1b aa sequences showed that Japanese PoAstVs and PoAstVs from the DDBJ/EMBL/GenBank database, with the exception of PoAstV2 and PoAstV4, exhibit same topologies as the ORF2 tree (Fig. 5, 6); however, the clusters of PoAstV2 and PoAstV4 in ORF1a and ORF1b trees showed different topologies from that of the ORF2 tree. PoAstV2 L2 strains Bu5-10-1, Ishi-Ya4, and US-IA122 grouped with PoAstV2 L1 strains in ORF1a, and PoAstV2 L1 strain Iba-464-4-1 clustered with PoAstV2 L2 strains, whereas PoAstV2 L2 strain Ishi-Ya6 clustered with PoAstV2 L1 strains in ORF1b (Fig. 7) The topologies of the PoAstV4 phylogenetic trees showed significant difference in ORF1a, ORF1b, and ORF2 (Fig. 8).

Similarity plot analyses and recombination analyses

The whole genomes of PoAstVs were aligned using the ClustalW program in MEGA6.06 and used for similarity plot analyses and recombination analyses. The standard similarity plot analysis was conducted using SimPlot program with PoAstV2 L2 Bu5-10-1 (Fig. 9A), PoAstV2 L2 51/USA (Fig. 9C), PoAstV4 L4 Bu4-2-2 (Fig.

Table 9

Pairwise nucleotide (lower left) and amino acid (upper right (grey shades)) sequence identities (%) of complete CDS of ORF2 among PoAstV4s.

		lineage1		lineage2					lineage3				lineage4				
	Lineage	HUN/2007	PFP-25	MoI2-1-2	MoI2-2	MoI2-3-2	35/2010	US-IL135	HgTa2-3	15-14	15-12	15-13	Bu2-2	Bu4-6-1	MoI2-1-1	Ishi-Ya7-1	US-P2011-1
PoAstV4/HUN/2007 (GU562296)	1		65.1	59.7	59.7	59.7	54.6	54.6	46.4	47.3	47.4	47.3	45.1	45.1	45.4	45.1	46.3
PoAstV4/USA/PFP-25/2006 (KJ495993)	1	70.8		59.2	59.2	59.2	56.8	57.5	56.1	55.7	56.0	55.7	53.8	53.8	53.4	50.7	54.3
PoAstV4/JPN/MoI2-1-2/2015	2	64.7	63.1		100	100	78.6	78.7	56.0	52.0	52.4	52.0	53.8	53.8	54.3	49.6	52.2
PoAstV4/JPN/MoI2-2/2015	2	64.7	63.1	100		100	78.6	78.7	56.0	52.0	52.4	52.0	53.8	53.8	54.3	49.6	52.2
PoAstV4/JPN/MoI2-3-2/2015	2	64.7	63.1	100	100		78.6	78.7	56.0	52.0	52.4	52.0	53.8	53.8	54.3	49.6	52.2
PoAstV4/USA/35/2010 (NC_023675)	2	62.6	62.1	75.4	75.4	75.4		96.3	56.5	51.3	51.9	51.3	51.5	51.4	52.4	48.3	49.5
PoAstV4/USA/US-IL135/2011 (JX556692)	2	62.8	62.1	75.6	75.6	75.6	94.9		56.6	51.4	51.8	51.4	52.2	52.1	52.8	48.6	49.9
PoAstV4/JPN/HgTa2-3/2015	3	56.8	60.1	59.6	59.6	59.6	61.3	61.0		63.0	63.0	63.0	55.9	55.9	56.5	53.2	55.0
PoAstV4/USA/15-14/2015 (KU764485)	3	57.2	60.0	57.5	57.5	57.5	57.9	57.9	66.3		96.6	100	52.4	52.3	53.0	48.9	51.9
PoAstV4/USA/15-12/2015 (KU764486)	3	57.3	60.5	58.1	58.1	58.1	58.2	58.5	66.9	96.4		96.6	52.3	52.2	53.4	48.9	51.6
PoAstV4/USA/15-13/2015 (KU764484)	3	57.2	60.0	57.5	57.5	57.5	57.9	57.9	66.3	100.0	96.4		52.4	52.3	53.0	48.9	51.9
PoAstV4/JPN/Bu4-2-2/2014	4	55.4	58.4	58.8	58.8	58.8	56.6	56.8	60.0	58.9	59.2	58.9		99.5	70.1	75.2	65.9
PoAstV4/JPN/Bu4-6-1/2014	4	55.5	58.5	58.9	58.9	58.9	56.7	56.9	59.9	58.8	59.2	58.8	99.7		70.0	75.1	65.9
PoAstV4/JPN/MoI2-1-1/2015	4	55.8	58.4	60.8	60.8	60.8	57.6	57.8	59.7	57.3	57.6	57.3	71.4	71.2		69.4	67.2
PoAstV4/JPN/Ishi-Ya7-1/2015	4	56.0	56.7	57.0	57.0	57.0	55.5	56.2	57.4	55.6	55.7	55.6	73.4	73.4	69.0		67.5
PoAstV4/USA/US-P2011-1/2011	4	56.3	60.0	59.2	59.2	59.2	57.0	57.1	58.7	57.7	57.5	57.7	68.2	68.2	67.8	68.9	

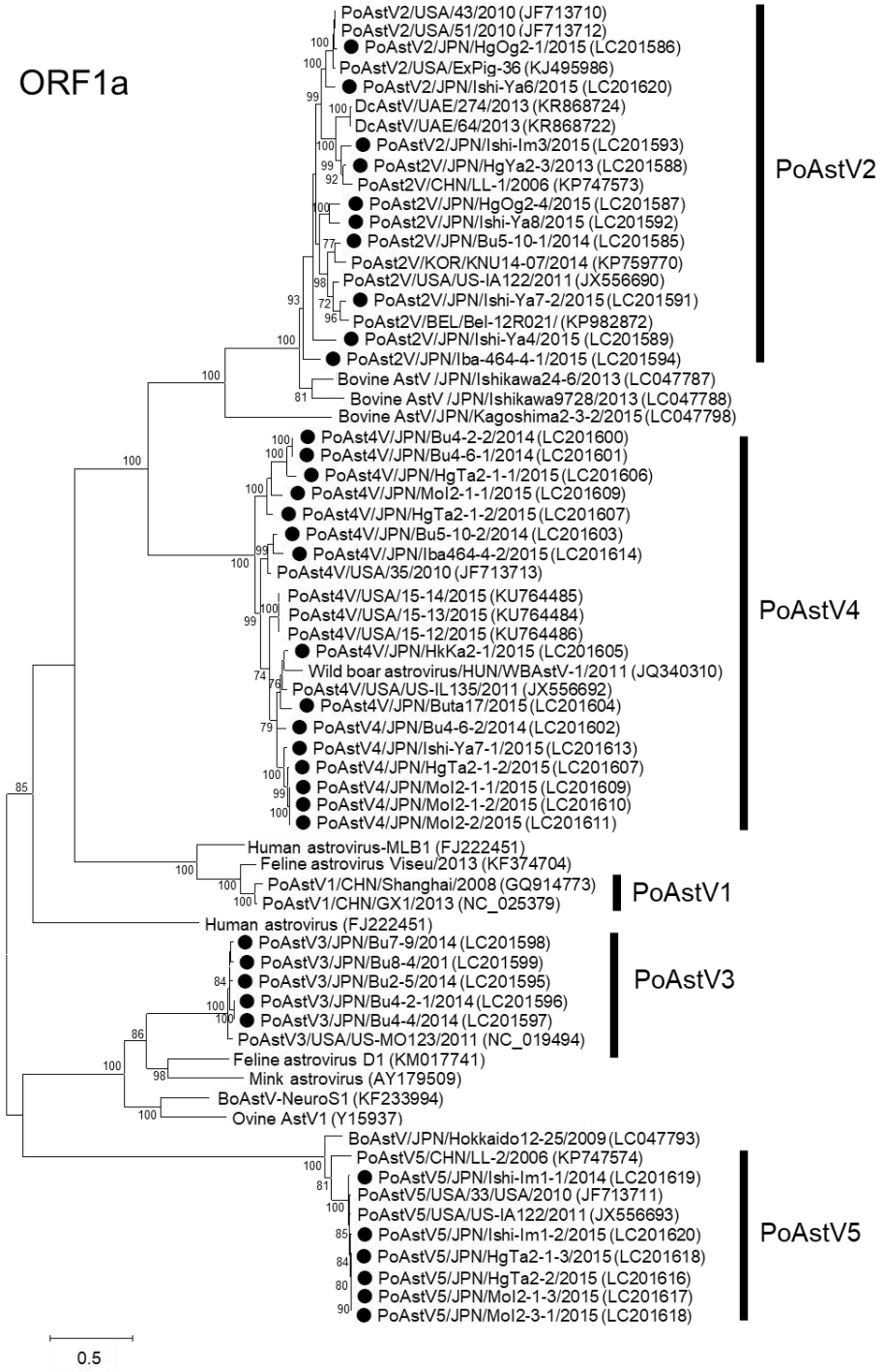

Fig. 5. Phylogenetic analysis based on deduced amino acid sequences of AstV ORF1a. Trees were constructed using the maximum likelihood method (JTT + G + I) in MEGA6.06 with 1,000 bootstrap values. The scale bar indicates amino acid substitutions per site. ● indicates PoAstV strains identified in this study. Percent bootstrap support is indicated by the value at each node, with values < 70 omitted.

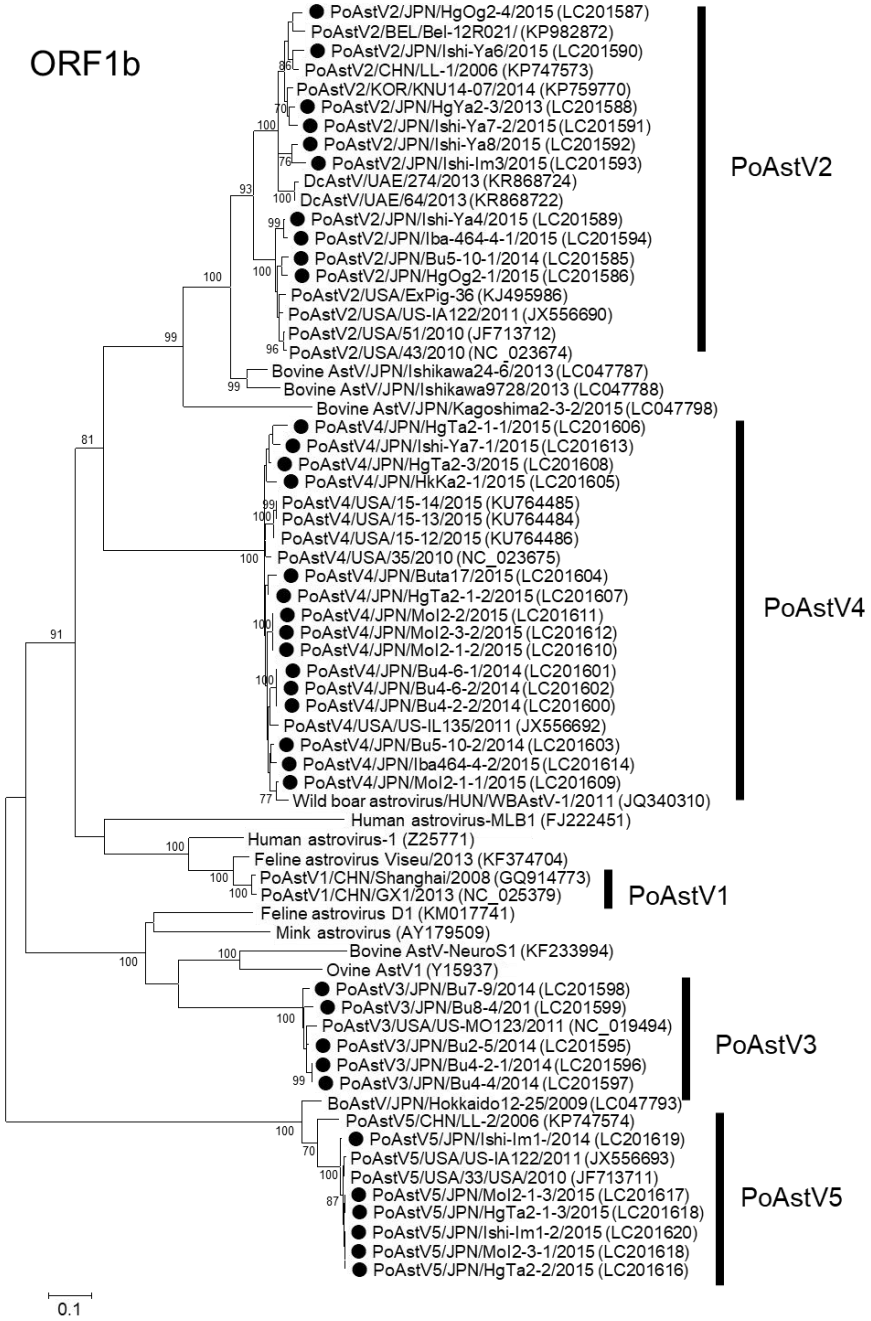

Fig. 6. Phylogenetic analysis based on deduced amino acid sequences of AstV ORF1b. Putative ORF1b codons were determined using human and animal AstV sequences deposited in DDBJ/EMBL/GenBank database, since the beginning of the translation of ORF1b is not well known due to the presence of the ribosomal frameshift. Trees were constructed using the maximum likelihood method (WAG + G + I) in MEGA6.06 with 1,000 bootstrap values. The scale bar indicates amino acid substitutions per site. ● indicates PoAstV strains identified in this study. Percent bootstrap support is indicated by the value at each node, with values < 70 omitted.

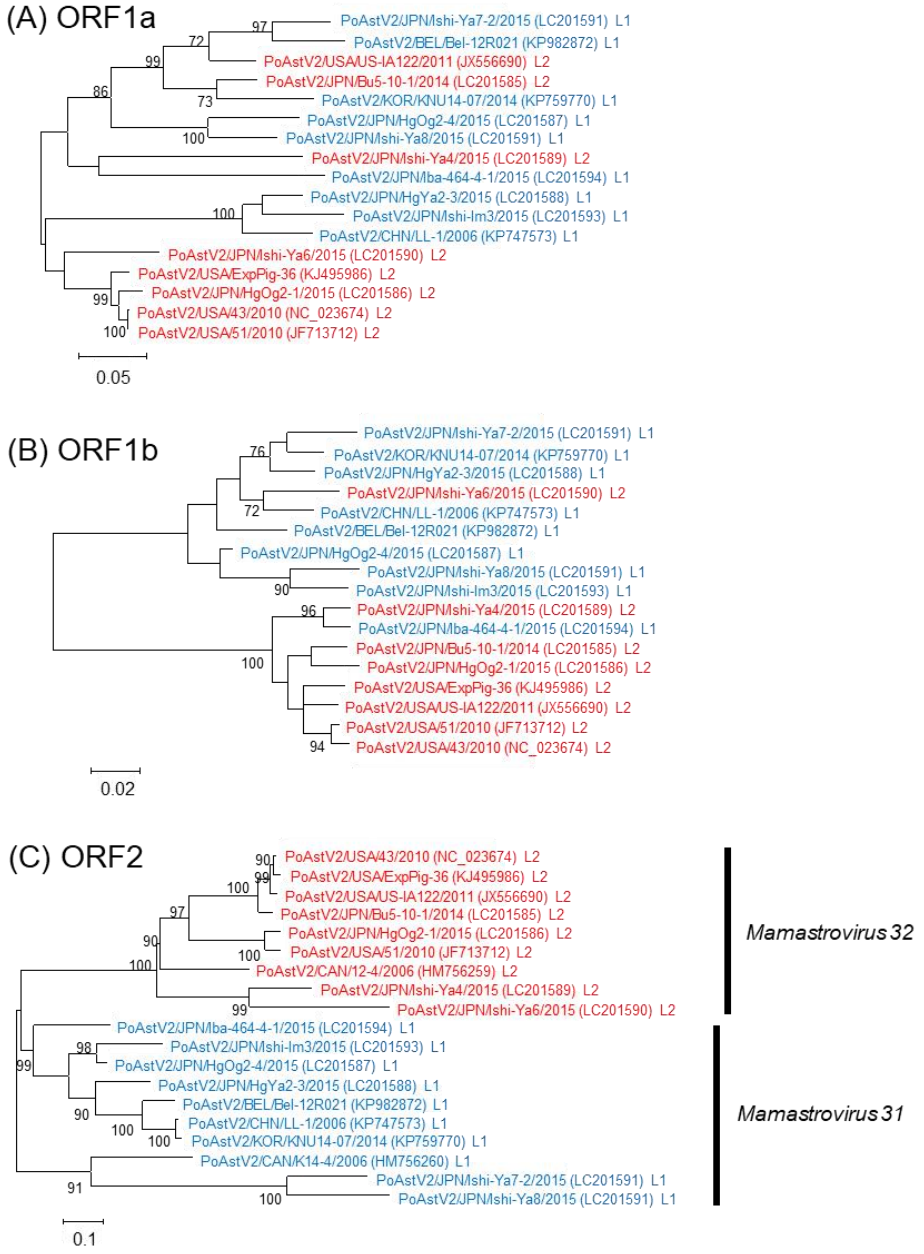

Fig. 7. Phylogenetic trees based on deduced amino acid sequences of ORF1a (A), ORF1b (B), and ORF2 (C) of PoAstV2 strains. Putative ORF1b codons were determined using human and animal AstV sequences deposited in DDBJ/EMBL/GenBank database, since the beginning of the translation of ORF1b is not well known due to the presence of the ribosomal frameshift. The trees were constructed using the maximum likelihood method (ORF1a: LG + G model, ORF1b: JTT + G model, ORF2: LG + G + I + F model) in MEGA6.06 with 1,000 bootstrap values. The scale bar indicates amino acid substitutions per site. Percent bootstrap support is indicated by the value at each node, with values < 70 omitted. PoAstV2 lineage 1 (L1) (*Mamastrovirus 31*) strains are presented in blue, whereas PoAstV2 lineage 2 (L2) (*Mamastrovirus 32*) strains are presented in red.

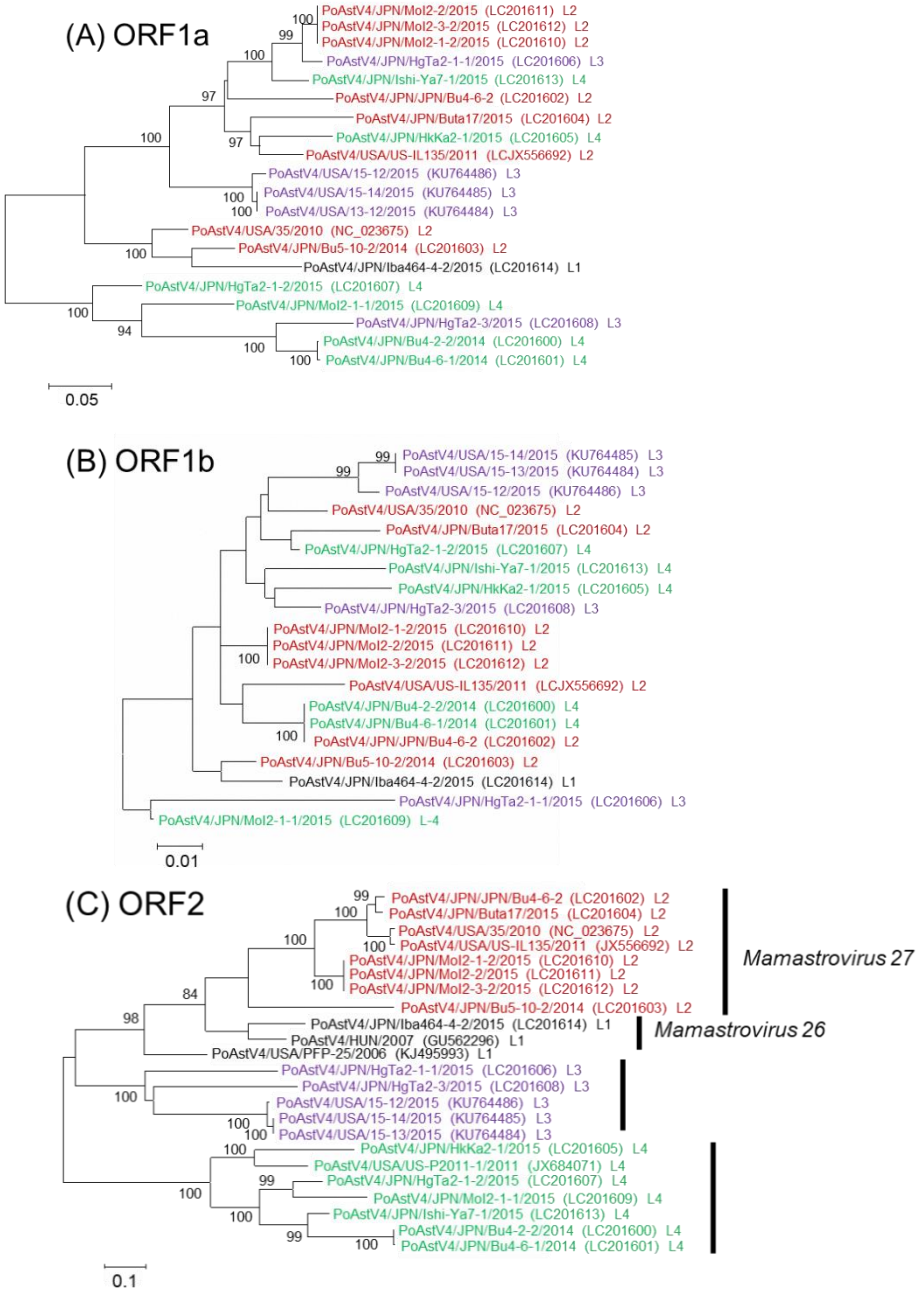

Fig. 8. Phylogenetic trees based on deduced amino acid sequences of ORF1a (A), ORF1b (B), and ORF2 (C) of PoAstV4 strains. Putative ORF1b codons were determined using human and animal AstV sequences deposited in DDBJ/EMBL/GenBank database, since the beginning of the translation of ORF1b is not well known due to the presence of the ribosomal frameshift. The trees were constructed using the maximum likelihood method (ORF1a: JTT + G + I model, ORF1b: JTT + G + I model, ORF2: LG + G + I + F model) in MEGA6.06 with 1,000 bootstrap values. The scale bar indicates amino acid substitutions per site. Percent bootstrap support is indicated by the value at each node, with values < 70 omitted. PoAstV4 lineage 1 (L1) (*Mamastrovirus 26*), lineage 2 (PoAstV2 L2) (*Mamastrovirus 27*), lineage 3 (PoAstV2 L3), and lineage 4 (PoAstV2 L4) strains are presented in black, red, purple, and green, respectively.

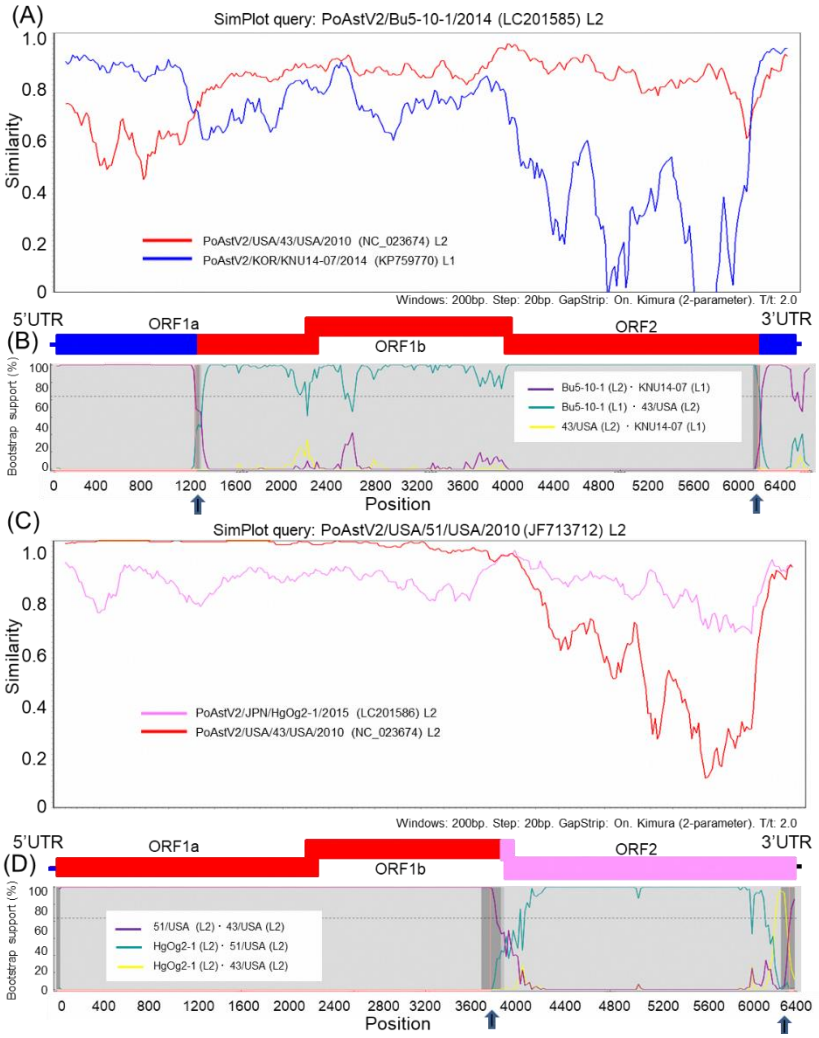

Fig. 9. Similarity plots of the entire genomes of (A) PoAstV2 lineage 2 (PoAstV2 L2) 43/USA (red line), PoAstV2 lineage 1 (PoAstV2 L1) KNU14-07 (blue line), and PoAstV2 L2 Bu5-10-1 as query sequences, (C) PoAstV2 L2 HgOg2-1 (pink line), PoAstV2 L2 43/USA (red line), and PoAstV2 L2 51/USA as query sequences, (E) PoAstV4 lineage 4 (PoAstV4 L4) Ishi-Ya7-1 (purple line), PoAstV4 lineage 3 (PoAstV4 L3) HgTa2-3 (green line), and PoAstV4 L4 Bu4-2-2 as query sequences, (G) PoAstV4 L4 HkKa2-1 (green line), PoAstV4 L2 43/USA (red line), and PoAstV4 L2 US-IL135 as query sequences, using a sliding window of 200 nt and a moving step size of 20 nt. Bootscan analysis of (B) Genome structure of PoAstV and recombination breakpoint. Blue part of genome indicates origin from PoAstV2 L1 and red part indicates origin from PoAstV2 L2. PoAstV2 L2 Bu5-10-1 vs. PoAstV2 L1 KNU14-07 (purple line), PoAstV2 L2 Bu5-10-1 vs. PoAstV2 L2 43/USA (blue line), and PoAstV2 L2 43/USA vs. PoAstV2 L1 KNU14-07 (yellow line), (D) Genome structure of PoAstV and recombination breakpoint. Red part of genome indicates origin from PoAstV2 L2 43/USA and pink part indicates origin from PoAstV2 L2 HgOg2-1. PoAstV2 L2 51/USA vs. PoAstV2 L2 43/USA (purple line), PoAstV2 L2 HgOg2-1 vs. PoAstV2 L2 51/USA (blue line), and PoAstV2 L2 HgOg2-1 vs. PoAstV2 L2 43/USA (yellow line), (F) Genome structure of PoAstV and recombination breakpoint. Green part of genome indicates origin from PoAstV4 L3, purple part indicates origin from PoAstV4 L4, and white part indicates unknown origin. PoAstV4 L4 Ishi-Ya7-1 vs. PoAstV4 L4 Bu4-2-2 (purple line), PoAstV4 L3 HgTa2-3 vs. PoAstV4 L4 Bu4-2-2 (blue line), and PoAstV4 L4 Ishi-Ya7-1 vs. PoAstV4 L3 HgTa2-3 (yellow line), and (H) Genome structure of PoAstV and recombination breakpoint. Green part of genome indicates origin from PoAstV4 L4, red part indicates origin from PoAstV4 L2, and white part indicates unknown origin. PoAstV4 L2 35/USA vs. PoAstV4 L2 US-IL135 (purple line), PoAstV4 L4 HkKa2-1 vs. PoAstV4 L2 US-IL135 (blue line), and PoAstV4 L4 HkKa2-1 vs. PoAstV4 L2 35 (yellow line). The cut-off value in the bootstrapping test (> 70%) is indicated by the breakpoint. Black arrows show putative recombination breakpoints.

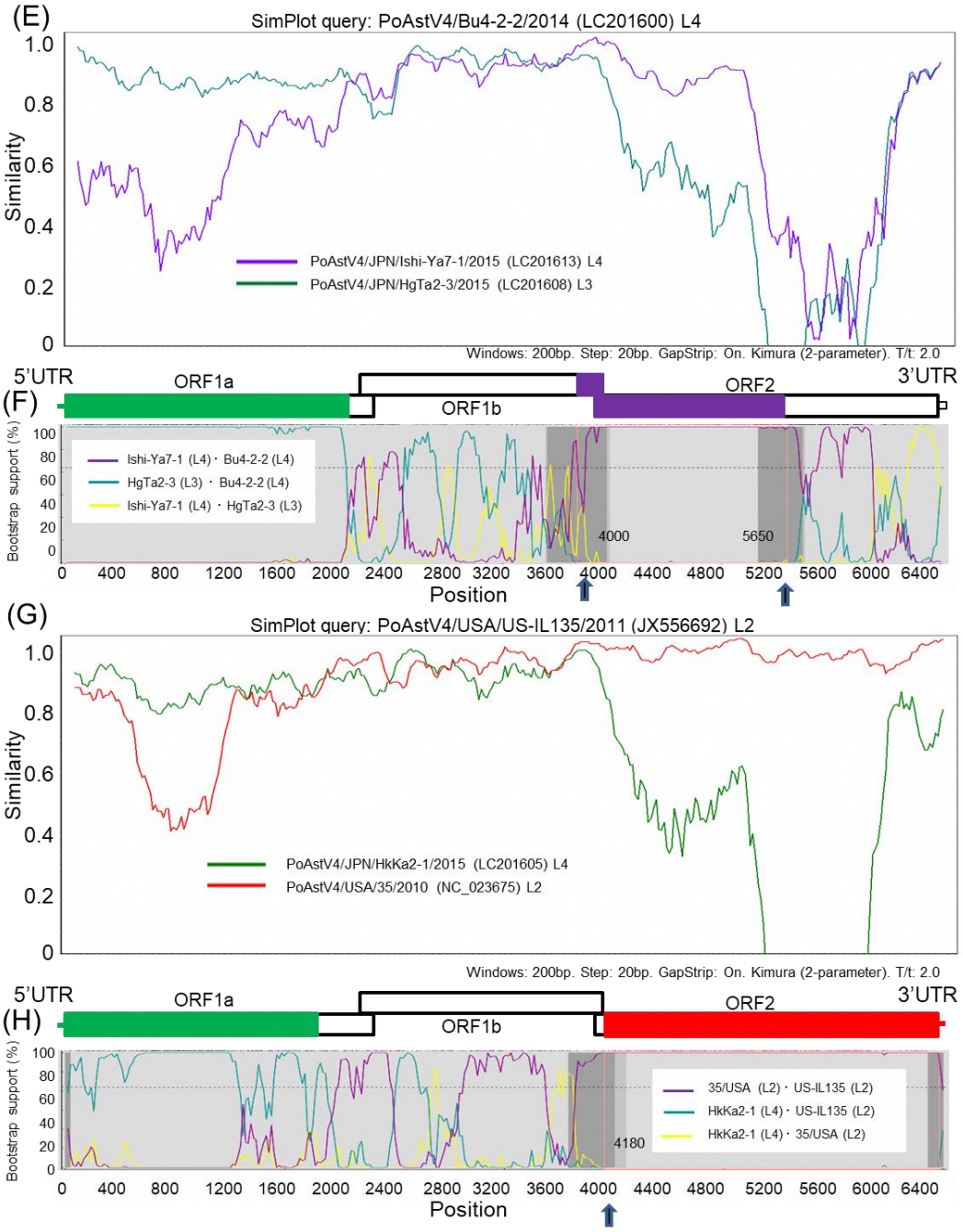

Fig. 9. (*continued*)

9E), and PoAstV4 L2 US-IL135 (Fig. 9G) sequences as separate queries. Bu5-10-1 had high nt sequence similarity with PoAstV2 L2 43/USA in the 3′ halves of ORF1a and ORF1b, whereas the 5′ UTR, 5′ half of ORF1a, 3′ end of ORF2, and 3′ UTR were highly similar to those of PoAstV2 L1 strain KNU14-07, suggesting an intra-genotypic recombination event (Fig. 9A). PoAstV2 L2 51/USA displayed significant sequence identity with PoAstV2 L2 43/USA in ORF1a and ORF1b, whereas 51/USA showed higher sequence similarity with PoAstV2 L2 HgOg2-1 than 43/USA in ORF2, suggesting an intra-lineage recombination event (Fig. 9C). PoAstV4 L4 Bu4-2-2 exhibited a high degree of similarity with PoAstV4 L3 HgTa2-3 in ORF1a, whereas Bu4-2-2 showed high identity with PoAstV4 L4 Ishi- Ya7-1 in the 5′ half of ORF2 (Fig. 9E). ORF1a of PoAstV4 L2 US-IL135 was highly similar to that of PoAstV4 L4 HkKa2-1; however, US-IL135 exhibited high identity with PoAstV4 L2 35/USA in ORF2 (Fig. 9G). To identify possible recombinant breakpoints, a bootscanning analysis was performed. Two putative recombination breakpoints were found at the center of ORF1a and the 3′ end of ORF2 in the Bu5-10-1 genome, at the 3′ end of ORF1b and the 3′ end of ORF2 in the 51/USA genome, and at the 3′ end of ORF1a and the 3′ of ORF2 in the Bu4-2-2 genome (Fig. 9B, D, F). One possible recombination breakpoint was identified at the 5′ end of ORF1b in the US-IL135 genome (Fig. 9H). A similarity plot analysis of strains PoAstV3 and PoAstV5 revealed that PoAstV3 and PoAstV5 exhibited high sequence identities within each genotype in all genomic regions; however, in ORF2, PoAstV3 Bu7-9 and PoAstV5 Ishi-Im1-1 exhibited low

sequence similarity with other PoAstV3 strains and PoAstV5 strains, respectively, suggesting possible recombination events (Fig. 10B, D). Although the recombination breakpoints were assumed to localize in the ORF2 region (Fig. 10C, E), recombinant counterparts of these strains were not identified in any database.

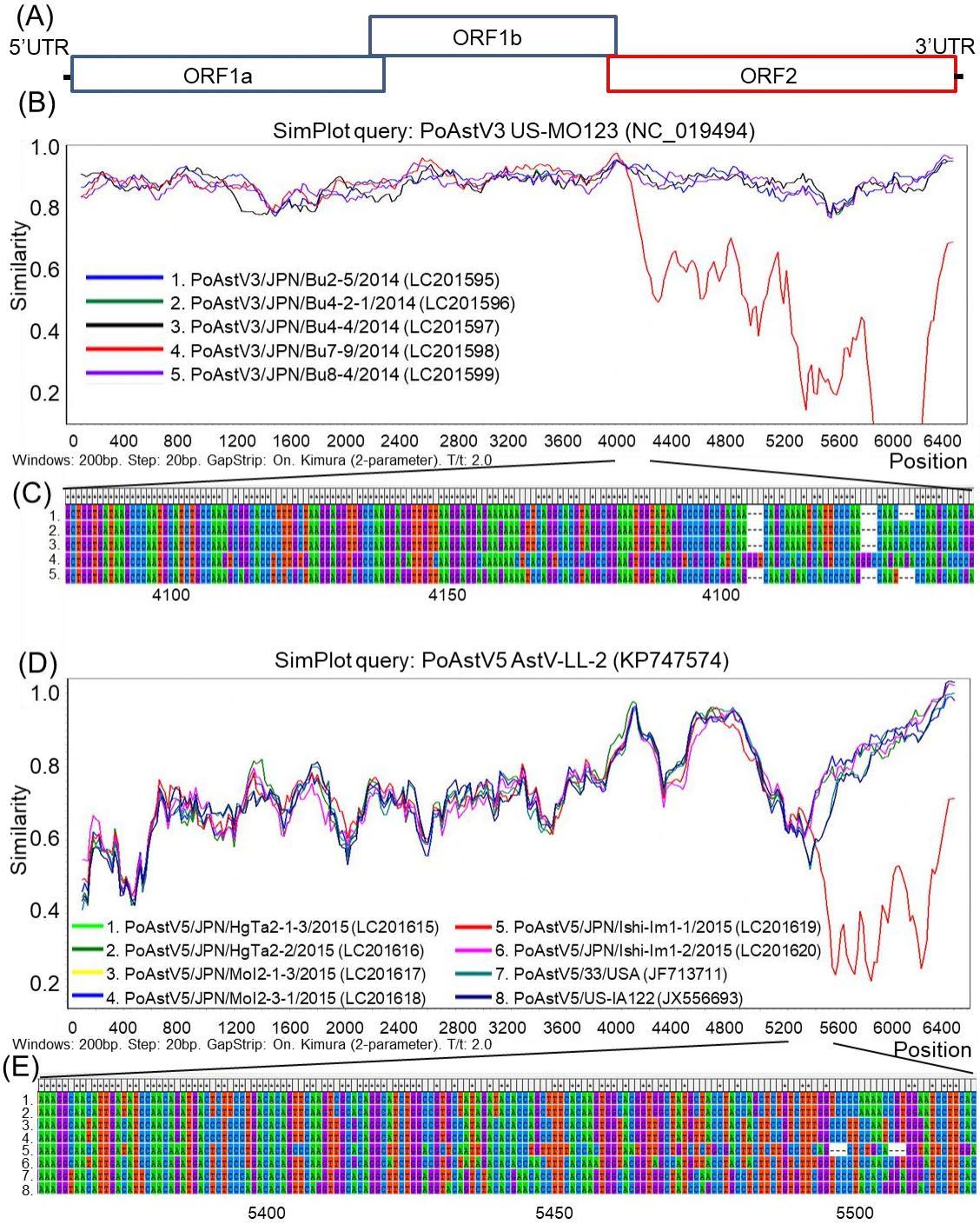

Fig. 10. (A) Genome organization of PoAstV. Similarity plot analyses of the entire genomes of (B) PoAstV3 strains (PoAstV3 US-MO123 as a query sequence) and (D) PoAstV5 strains (PoAstV5 AstV-LL-2 as a query sequence), using a sliding window of 200 nt and a moving step size of 20 nt. Sequence alignment at a putative recombination point of (C) PoAstV3 Bu7-9 and (E) PoAstV5 Ishi-Im1-1.

Discussion

In the present study, 10 strains (5 PoAstV2, 2 PoAstV3, and 3 PoAstV4) were isolated from diarrheic pigs, whereas 26 strains (5 PoAstV2, 3 PoAstV3, 12 PoAstV4, and 6 PoAstV5) were identified from pigs without diarrhea. Since PoAstVs are ubiquitously distributed to apparently healthy pig, the clinical significance of PoAstV infection has not been completely clarified [39]. The present result indicated that there seems to be no clear association of PoAstV infection with its disease.

PoAstVs are divided into five distinct genotypes, PoAstV1-PoAstV5 and distributed throughout the world. PoAstV1 has been reported in Canada [57], China [91], Croatia [94, 95], Germany [96] and Japan [91, 92]. Only PoAstV1 genotype has been reported in Japan; however, no PoAstV1 strains were identified in this study. This may indicate a shift in the predominant genotype or a difference in dominant type associated with geographical location. PoAstV2 and PoAstV4 are the predominant genotypes reported in Canada [57], Hungary (including a wild boar strain) [97-99], China [100-102], USA [90, 103], Czech Republic [104], South Korea [105, 106], Croatia [94, 95], Italy [107], Kenya [108,109], Austria, Germany, Spain, and Sweden [100]. In this study, it seems that PoAstV2 and PoAstV4 are also predominant in Japan. There was also no regional bias in the distribution of PoAstV2-PoAstV5 detected, but it may be due to the fact that origin of the detected strains were confined to the

mainland of Japan. However, this study did not aim to investigate the prevalence of PoAstV infection. Only one investigation has reported 39.1% PoAstV prevalence, based on a serological survey in Japan [56]. Further studies are needed to determine the PoAstV prevalence and genotypes circulating in Japan. PoAstV3 and PoAstV5 have been reported in Canada [57, 110], USA [90, 111], and Croatia [94, 95], whereas PoAstV3 alone has been reported in Kenya [108, 109] and PoAstV5 alone has been reported in China [101, 102]. To date, only one and three complete sequences of PoAstV3 and PoAstV5, respectively, have been reported. This study contributed to the second and fourth whole genome sequences of PoAstV3 and PoAstV5.

RNA viruses, including AstVs, showed high frequencies of recombination and evidence for positive selection, resulting in serotype differentiation [112]. There are many reports of genetic variety and recombination events in human AstVs [59-61, 113, 114]; however, only a few reports on recombination of animal AstVs, including a recombination between a human AstV and that of another animal, are available [115, 116]. In this study, although no recombination was found within PoAstV genotypes, a recombination analysis revealed evidence of several possible intra-genotypic recombination events. Because the PoAstV2 L1 and PoAstV2 L2 phylogenetic clades correspond to *Mamastrovirus 31* and *Mamastrovirus 32*, respectively, which are proposed as species of *Mamastrovirus* [38], recombination between PoAstV2 L1 and PoAstV2 L2 may also represent inter-genotypic recombination events. These analyses also identified putative recombination breakpoints within ORF1a and ORF2, including

the ORF1b/ORF2 junction, this region is a recombination hotspot [59-62]. Co-infections with multiple PoAstVs, identified in this study and reported previously [90, 109], may contribute to these recombination events and increase genetic diversity. Possible recombination events were also identified within ORF2 of PoAstV3 Bu7-9 and PoAstV5 Ishi-Im1-1; however, the origins of these recombinants are unknown, because the recombinant counterparts of these sequences were not found in the dataset obtained in this study or in the DDBJ/EMBL/GenBank database. Therefore, further investigations are required to obtain a better understanding of PoAstV genetics. Almost PoAstVs in this study were taken from pigs kept on a high-density farm. Owing to the current pig raising system, susceptible young pigs were continuously infected with multiple strains of PoAstVs and these circumstances may give the chance for occurrences of recombination events.

AstVs, including PoAstVs, are not easy to propagate in cell culture; thus, comparison for antigenic property of AstVs is difficult. However, since the capsid protein, which is encoded by ORF2, induces host immunity, serological property is speculated based on sequence similarity of ORF2. In the present study, Japanese PoAstVs showed < 28.4% aa identities among genotypes, < 65.4% aa identities between lineages within PoAstV2 and PoAstV4, and > 54.3% aa identities between strains in lineages of PoAstV2 and PoAstV4. Significant serological differences are expected because of the < 95% identity at the nt sequence level [39]. On the basis of these data, Japanese PoAstVs are believed to exhibit high serological variation, even

within the same genotype. This may allow co-infection of PoAstVs in a single host, facilitating recombination events and promoting the genetic diversity of PoAstVs.

Summary

PoAstVs are ubiquitous enteric virus of pigs that are distributed to several countries throughout the world. Since PoAstVs are detected in apparently healthy pigs, the clinical significance of the infection is unknown. However, AstVs have recently been associated with a severe neurological disorder in animals, including humans, and zoonotic potential has been suggested. To date, little is known about the epidemiology of PoAstVs among the pig population in Japan. In this study, an analysis of nearly complete genomes of 36 PoAstVs detected by a metagenomics approach in the feces of Japanese pigs was presented. Based on a phylogenetic analysis and pairwise sequence comparison, 10, 5, 15, and 6 sequences were classified as PoAstV2, PoAstV3, PoAstV4, and PoAstV5, respectively. Co-infection with two or three strains was found in individual fecal samples from eight pigs. The phylogenetic trees of ORF1a, ORF1b, and ORF2 of PoAstV2 and PoAstV4 showed differences in their topologies. The PoAstV3 and PoAstV5 strains shared high sequence identities within each genotype in all ORFs; however, one PoAstV3 strain and one PoAstV5 strain showed considerable sequence divergence from the other PoAstV3 and PoAstV5 strains, respectively, in ORF2. Recombination analysis using whole genomes revealed evidence of multiple possible intra-genotype recombination events in PoAstV2 and PoAstV4, suggesting that recombination might have contributed to the genetic

diversity and played an important role in the evolution of Japanese PoAstVs.

General Conclusion

The diagnosis of viral infections at livestock hygiene service centers depends on the isolation of viruses in cultured cells. However, many enteric viruses cannot be isolated using established methods; hence, diagnosis often depends on genetic testing. RNA viruses are thought to be a part of a genetically diverse quasispecies created by gene mutation and recombination. Isolated virus strains usually represent only one member of the quasispecies. Next generation sequencing (NGS), which directly determines genetic information of pathogens without using specific primers, has recently become popular. NGS makes the genetic identification of RNA viruses, which are difficult or impossible to propagate in cultured cells, possible in field diagnostic samples. In this study, NGS metagenomic analysis of RNA viruses in the feces of cattle and pigs collected for the diagnosis of infectious diseases was carried out to elucidate genetic diversity and interspecies transmission of viruses.

Bovine torovirus (BToV) belongs to the subfamily *Torovirinae* of the family *Coronaviridae* and cause intestinal disorders in cattle. In Chapter I, sequence analysis of the complete genome of two BToV strains and that of partial genomes of two other BToV strains and one PToV strain was carried out using field fecal samples and one isolated virus. Phylogenetic analysis and pairwise nucleotide sequence comparison revealed high similarities among BToVs in Japan. The S, M, and HE coding regions

showed high similarities with BToV Breda1. The ORF1a, ORF1b, N coding region, and untranslated region at 5' and 3' ends showed high similarities with PToV NPL/2014 and PToV SH1. Japanese BToVs are the interspecies recombinants of previously reported BToV and PToVs. On the basis of similarity plot analysis, recombinant breakpoints were predicted to be 3' ends of the ORF1b and HE regions. However, this recombination is not related to the serotype because the S coding region involved in the antigen properties is the same type as the BToV Breda1. This recombination also shows the possibility that BToV and PToV co-infect the same host in nature.

PoAstV belongs to the *Astroviridae* family and is a ubiquitous enteric virus of pigs in countries around the world. However, it is unknown whether PoAstV infection is clinically significant, as it is also frequently detected in healthy pigs. To date, the epidemiology of PoAstV in domestic pigs in Japan is not well understood. In Chapter II, partial genomic sequence analysis was carried out for 36 PoAstV strains using fecal samples. Phylogenetic analysis and pairwise nucleotide sequence comparison classified these strains as PoAstV2 (10 strains), PoAstV3 (5 strains), PoAstV4 (15 strains), and PoAstV5 (6 strains). In addition, 8 pigs were co-infected with 2 to 3 genotypes or lineages of viruses. From the phylogenetic trees of ORF2, PoAstV2 is further classified into two lineages, and PoAstV4 is classified into four lineages. However, in the phylogenetic trees of ORF1a and ORF1b of PoAstV2 and PoAstV4, topology was different from the phylogenetic trees of ORF2. From the results of the similarity plot analysis, multiple intra- and inter-genotype recombination events in

PoAstV2 and PoAstV4 were revealed. The PoAstV3 and PoAstV5 strains shared high sequence identities within each genotype in all ORFs. However, one PoAstV3 strain and one PoAstV5 strain showed considerable sequence divergence from the other strains in ORF2. In addition, the homology of the ORF2 gene involved in antigen properties is < 28.4% among the genotypes and < 65.4% among the lineages, and it is presumed to show high antigenic differences even within the same genotype. The antigenic variation was considered to allow simultaneous infection of multiple strains in the same host, facilitate recombination, and thus promote genetic diversity.

In conclusion, this study contributes to the elucidation of genetic diversity and interspecies transmission of the enteric RNA viruses, BToVs and PoAstVs.

Acknowledgments

The author would like to express sincere gratitude to Prof. Yoshihiro Sakoda (Laboratory of Microbiology, Graduate School of Veterinary Medicine, Hokkaido University), for valuable opinions and polite guidance in revising this dissertation.

The author would also like to be expired my special appreciation to Prof. Kazuhiko Ohashi (Laboratory of Infectious Diseases, Graduate School of Veterinary Medicine, Hokkaido University), Prof. Hiroaki Kariwa (Laboratory of Public Health, Graduate School of Veterinary Medicine, Hokkaido University), and Associate Prof. Masatoshi Okamatsu (Laboratory of Microbiology, Graduate School of Veterinary Medicine, Hokkaido University), for their valuable advice and suggestions of the manuscript.

The author expresses appreciation to Prof. Makoto Nagai (Ishikawa Prefectural University), Prof. Tetsuya Mizutani (Tokyo University of Agriculture and Technology), Dr. Kazuhiko Katayama (National Institute of Infectious Diseases), Dr. Kei Haga (National Institute of Infectious Diseases), Associate Prof. Hiroshi Aoki (Nippon Veterinary and Life Science University), and everyone at Research and Education Center for Prevention of Global Infectious Disease of Animal, Tokyo University of Agriculture and Technology, for their helpful suggestions, valuable comments and technical supports, throughout this study.

The author grateful thanks the person in charge of each prefecture who provided the fecal samples.

The author heartily thanks are also for colleagues at Ishikawa Prefectural Livestock Hygiene Service Center for their helpful and cordial encouragement.

The Japan Society for the Promotion of Science (JSPS) KAKENHI grant number 15K07718 and grants from the Ministry of Health, Labor, and Welfare of Japan and the Technology Research Partnership for Sustainable Development (SATREPS) supported this work.

References

1. Lai MMC. RNA Recombination in animal and plant viruses. Microbiol Rev 56, 61-79, 1992.
2. Simon-Loriere E, and Holmes EC. Why do RNA viruses recombine? Nat Rev Microbiol 9, 617-626, 2011.
3. Batty EM, Wong THN, Trebes A, Argoud K, Attar M, Buck D, Ip CLC, Golubchi T, Cule M, Bowden R, Manganis C, Klenerman P, Barnes E, Walker AS, Wyllie DH, Wilson DJ, Dingle KE, Peto TEA, Crook DW, and Piazza P. A modified RNA-Seq approach for whole genome sequencing of RNA viruses from faecal and blood samples. PLoS One 8, e66129, 2013.
4. Djikeng A, Halpin R, Kuzmickas R, DePasse J, Feldblyum J, Sengamalay N, Afonso C, Zhang X, Anderson NG, Ghedin E, and Spiro DJ. Viral genome sequencing by random priming methods. BMC Genomics 9:5, 2008.
5. Cavanagh D, and Horzinek MC. Genus torovirus assigned to the coronaviridae. Arch Virol 128, 395-396, 1993.
6. Sun H, Lan D, Lu L, Chen M, Wang C, and Hua X. Molecular characterization and phylogenetic analysis of the genome of porcine torovirus. Arch Virol 159, 773-778, 2014.
7. Draker R, Roper RL, Petric M, and Tellier R. The complete sequence of the

bovine torovirus genome. Virus Res 115, 56-68, 2006.

8. Smits SL, Lavazza A, Matiz K, Horzinek MC, Koopmans MP, and de Groot RJ. Phylogenetic and evolutionary relationships among torovirus field variants, evidence for multiple intertypic recombination events. J Virol 77, 9567-9577, 2003.

9. Cong Y, Zarlenga DS, Richt JA, Wang X, Wang Y, Suo S, Wang J, Ren Y, and Ren X. Evolution and homologous recombination of the hemagglutinin-esterase gene sequences from porcine torovirus. Virus Genes 47, 66-74, 2013.

10. Duckmanton L, Luan B, Devenish J, Tellier R, and Petric M. Characterization of torovirus from human fecal specimens. Virology 239, 158-168, 1997.

11. Ito T, Okada N, Okawa M, Fukuyama S, and Shimizu M. Detection and characterization of bovine torovirus from the respiratory tract in Japanese cattle. Vet Microbiol 136, 366-371, 2009.

12. Kroneman A, Cornelissen LA, Horzinek MC, de Groot RJ, and Egberink HF. Identification and characterization of a porcine torovirus. J Virol 72, 3507-3511, 1998.

13. Uziel Y, Laxer RM, and Petric M. Torovirus gastroenteritis presenting as acute abdomen. Clin Infect Dis 28, 925-926, 1999.

14. Vanopdenbosch E, Wellemans G, and Petroff K. Breda virus associated with respiratory disease in calves. Vet Rec 129, 203, 1991.

15. Woode GN, Reed DE, Runnels PL, HerrigMA, and Hill HT. Studies with an

unclassified virus isolated from diarrheic calves. Vet Microbiol 7, 221-240, 1982.

16. Weiss M, Steck F, and Horzinek MC. Purification and partial characterization of a new enveloped RNA virus (Berne virus). J Gen Virol 64, 1849-1858, 1983.

17. Hoet AE, Cho KO, Chang KO, Loerch SC, Wittum TE, and Saif LJ. Enteric and nasal shedding of bovine torovirus (Breda virus) in feedlot cattle. Am J Vet Res 63, 342-348, 2002.

18. Hoet AE, Nielsen PR, Hasoksuz M, Thomas C, Wittum TE, and Saif LJ. Detection of bovine torovirus and other enteric pathogens in feces from diarrhea cases in cattle. J Vet Diagn Invest 15, 205-212, 2003.

19. Pérez E, Kummeling A, Janssen MM, Jiménez C, Alvarado R, Caballero M, Donado P, and Dwinger RH. Infectious agents associated with diarrhoea of calves in the canton of Tilarán. Costa Rica Prev Vet Med 33, 195-205, 1998.

20. Haschek B, Klein D, Benetka V, Herrera C, Sommerfeld-Stur I, Vilcek S, Moestl K, and Baumgartner W. Detection of bovine torovirus in neonatal calf diarrhoea in Lower Austria and Styria (Austria). J Vet Med B Infect Dis Vet Public Health 53, 160-165, 2006.

21. Koopmans M, van Wuijckhuise-Sjouke L, Schukken YH, Cremers H, and Horzinek MC. Association of diarrhea in cattle with torovirus infections on farms. Am J Vet Res 52, 1769-1773, 1991.

22. Matiz K, Kecskeméti S, Kiss I, Adám Z, Tanyi J, and Nagy B. Torovirus detection in faecal specimens of calves and pigs in Hungary, short

communication. Acta Vet Hung 50, 293-296, 2002.

23. Ito T, Katayama S, Okada N, Masubuchi K, Fukuyama S, and Shimizu M. Genetic and antigenic characterization of newly isolated bovine toroviruses from Japanese cattle. J Clin Microbiol 48, 1795-1800, 2010.

24. Aita T, Kuwabara M, Murayama K, Sasagawa Y, Yabe S, Higuchi R, Tamura T, Miyazaki A, and Tsunemitsu H. Characterization of epidemic diarrhea outbreaks associated with bovine torovirus in adult cows. Arch Virol 157, 423-431, 2012.

25. Park SJ, Oh EH, Park SI, Kim HH, Jeong YJ, Lim GK, Hyun BH, and Cho KO. Molecular epidemiology of bovine toroviruses circulating in South Korea. Vet Microbiol 126, 364-371, 2008.

26. Penrith ML, and Gerdes GH. Breda virus-like particles in pigs in South Africa. J S Afr Vet Assoc 63, 102, 1992.

27. Duckmanton L, Carman S, Nagy E, and Petric M. Detecion of bovine torovirus in fecal specimens of calves with diarrhea from Ontario farms. J Clin Microbiol 36, 1266-1270, 1998.

28. Ito T, Okada N, and Fukuyama S. Epidemiological analysis of bovine torovirus in Japan. Virus Res 126, 32-37, 2007.

29. Kirisawa R, Takeyama A, Koiwa M, and Iwai H. Detection of bovine torovirus in fecal specimens of calves with diarrhea in Japan. J Vet Med Sci 69, 471-476, 2007.

30. Nogueira JS, Asano KM, de Souza SP, Brandão PE, and Richtzenhain LJ. First

detection and molecular diversity of Brazilian bovine torovirus (BToV) strains from young and adult cattle. Re Vet Sci 95, 799-801, 2013.

31. Anbalagan S, Peterson J, Wassman B, Elston J, and Schwartz K. Genome sequence of torovirus identified from a pig with porcine epidemic diarrhea virus from the United States. Genome Announc 2, e01291-14, 2014.

32. Shin DJ, Park SI, Jeong YJ, Hosmillo M, Kim HH, Kim HJ, Kwon HJ, Kang M I, Park SJ, and Cho KO. Detection, and molecular characterization of porcine toroviruses in Korea. Arch Virol 155, 417-422, 2010.

33. Méndez E, and Arias CF. Astroviruses. In: Knipe DM, and Howley PM. (Ed.), Fields Virology, Lippincott, vol. I. Williams and Wilkins, Philadelphia, PA, pp. 609-628, 2013.

34. Jiang B, Monroe SS, Koonin EV, Stine SE, and Glass RI. RNA sequence of astrovirus, distinctive genomic organization and a putative retrovirus-like ribosomal frameshifting signal that directs the viral replicase synthesis. Proc Natl Acad Sci USA 90, 10539-10543, 1993.

35. Koonin EV. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J Gen Virol 72, 2197-2206, 1991.

36. Méndez E, Murillo A, Velázquez R, Burnham A, and Arias CF. Replication Cycle of astroviruses. In: Schultz-Cherry S. (Ed.), Astrovirus Research, Essential Ideas, Everyday Impacts, Future Directions. Springer, New York, pp. 19-47, 2013.

37. Bosch A, Guix S, Krishna NK, Méndez E, Monroe SS, Pantin-Jackwood M, and

Schultz-Cherry S. Family Astroviridae. In: King AMQ, Lefkowitz E, Adams MJ, and Carstens EB. (Ed.), Virus taxonomy: classification and nomenclature of viruses, Ninth Report of the International Committee on the Toxonomy of Viruses. Elsevier Academic Press, New York, pp.953-959. 2012.

38. Guix S, Bosch A, and Pintó R. Astrovirus taxonomy. In: Schultz-Cherry S. (Ed.), Astrovirus Research, Essential Ideas, Everyday Impacts, Future Directions. Springer, New York, pp. 97-118, 2013.

39. De Benedictis P, Schultz-Cherry S, Burnham A, and Cattoli G. Astrovirus infections in humans and animals-molecular biology, genetic diversity, and interspecies transmissions. Infect Genet Evol 11, 1529-1544, 2011.

40. Brown JR, Morfopoulou S, Hubb J, Emmett WA, Ip W, Shah D, Brooks T, Paine SM, Anderson G, Virasami A, Tong CY, Clark DA, Plagnol V, Jacques TS, Qasim W, Hubank M, and Breuer J. Astrovirus VA1/HMO-C, an increasingly recognized neurotropic pathogen in immunocompromised patients. Clin Infect Dis 60, 881-888, 2015.

41. Cordey S, Vu DL, Schibler M, L'Huillier AG, Brito F, Docquier M, Posfay-Barbe KM, Petty TJ, Turin L, Zdobnov EM, and Kaiser L. Astrovirus MLB2, a new gastroenteric virus associated with meningitis and disseminated infection. Emerg Infect Dis 22, 846-853, 2016.

42. Frémond ML, Pérot P, Muth E, Cros G, Dumarest M, Mahlaoui N, Seilhean D, Desguerre I, Hébert C, Corre-Catelin N, Neven B, Lecuit M, Blanche S, Picard

C, and Eloit M. Next-generation sequencing for diagnosis and tailored therapy, a case report of astrovirus-associated progressive encephalitis. J Pediatric Infect Dis Soc 4, e53-e57, 2015.

43. Naccache SN, Peggs KS, Mattes FM, Phadke R, Garson JA, Grant P, Samayoa E, Federman S, Miller S, Lunn MP, Gant V, and Chiu CY. Diagnosis of neuroinvasive astrovirus infection in an immunocompromised adult with encephalitis by unbiased next-generation sequencing. Clin Infect Dis 60, 919-923, 2015.

44. Quan PL, Wagner TA, Briese T, Torgerson TR, Hornig M, Tashmukhamedova A, Firth C, Palacios G, Baisre-De-Leon A, Paddock CD, Hutchison SK, Egholm M, Zaki SR, Goldman JE, Ochs HD, and Lipkin WI. Astrovirus encephalitis in boy with X-linked agammaglobulinemia. Emerg Infect Dis 16, 918-925, 2010.

45. Sato M, Kuroda M, Kasai M, Matsui H, Fukuyama T, Katano H, and Tanaka-Taya K. Acute encephalopathy in an immunocompromised boy with astrovirus-MLB1 infection detected by next generation sequencing. J Clin Virol 78, 66-70, 2016.

46. Wunderli W, Meerbach A, Güngör T, Berger C, Greiner O, Caduff R, Trkola A, Bossart W, Gerlach D, Schibler M, Cordey S, McKee TA, Van Belle S, Kaiser L, and Tapparel C. Astrovirus infection in hospitalized infants with severe combined immunodeficiency after allogeneic hematopoietic stem cell transplantation. PLoS One 6, e27483, 2011.

47. Blomström AL, Widén F, Hammer AS, Belák S, and Berg M. Detection of a novel astrovirus in brain tissue of mink suffering from shaking mink syndrome by use of viral metagenomics. J Clin Microbiol 48, 4392-4396, 2010.

48. Bouzalas IG, Wüthrich D, Walland J, Drögemüller C, Zurbriggen A, Vandevelde M, Oevermann A, Bruggmann R, and Seuberlich T. Neurotropic astrovirus in cattle with nonsuppurative encephalitis in Europe. J Clin Microbiol 52, 3318-3324, 2014.

49. Li L, Diab S, McGraw S, Barr B, Traslavina R, Higgins R, Talbot T, Blanchard P, Rimoldi G, Fahsbender E, Page B, Phan TG, Wang C, Deng X, Pesavento P, and Delwart E. Divergent astrovirus associated with neurologic disease in cattle. Emerg. Infect Dis 19, 1385-1392, 2013.

50. Schlottau K, Schulze C, Bilk S, Hanke D, Höper D, Beer M, and Hoffmann B. Detection of a novel bovine astrovirus in a cow with encephalitis. Transbound Emerg Dis 63, 253-259, 2016.

51. Seuberlich T, Wüthrich D, Selimovic-Hamza S, Drögemüller C, Oevermann A, Bruggmann R, and Bouzalas I. Identification of a second encephalitis-associated astrovirus in cattle. Emerg Microbes Infect 5, e5, 2016.

52. Blomström AL, Ley C, and Jacobson M. Astrovirus as a possible cause of congenital tremor type AII in piglets? Acta Vet Scand 16, 82, 2014.

53. Padmanabhan A, and Hause BM. Detection and characterization of a novel genotype of porcine astrovirus 4 from nasal swabs from pigs with acute

respiratory disease. Arch Virol 161, 2575-2579, 2016.

54. Bridger JC. Detection by electron microscopy of caliciviruses, astroviruses and rotavirus-like particles in the faeces of piglets with diarrhoea. Vet Rec 107, 532-533, 1980.

55. Shirai J, Shimizu M, and Fukusho A. Coronavirus-, calicivirus-, and astrovirus-like particles associated with acute porcine gastroenteritis. Jpn J Vet Sci 47, 1023-1026, 1985.

56. Shimizu M, Shirai J, Narita M, and Yamane T. Cytopathic astrovirus isolated from porcine acute gastroenteritis in an established cell line derived from porcine embryonic kidney. J Clin Microbiol 28, 201-206, 1990.

57. Luo Z, Roi S, Dastor M, Gallice E, Laurin MA, and L'homme Y. Multiple novel and prevalent astroviruses in pigs. Vet Microbiol 149, 316-323, 2011.

58. van Hemert FJ, Lukashov VV, and Berkhout B. Different rates of (non-)synonymous mutations in astrovirus genes; correlation with gene function. Virol J 4, 25, 2007.

59. Babkin IV, Tikunov AY, Sedelnikova DA, Zhirakovskaia EV, and Tikunova NV. Recombination analysis based on the HAstV-2 and HAstV-4 complete genomes. Infect Genet Evol 22, 94-102, 2014.

60. Martella V, Medici MC, Terio V, Catella C, Bozzo G, Tummolo F, Calderaro A, Bonura F, Di Franco M, Bányai K, Giammanco GM, and De Grazia S. Lineage diversification and recombination in type-4 human astroviruses. Infect Genet

Evol 20, 330-335, 2013.

61. Walter JE, Briggs J, Guerrero ML, Matson DO, Pickering LK, Ruiz-Palacios G, Berke T, and Mitchell DK. Molecular characterization of a novel recombinant strain of human astrovirus associated with gastroenteritis in children. Arch Virol 146, 2357-2367, 2001.

62. De Grazia S, Medici MC, Pinto P, Moschidou P, Tummolo F, Calderaro A, Bonura F, Banyai K, Giammanco GM, and Martella V. Genetic heterogeneity and recombination in human type 2 astroviruses. J Clin Microbiol 50, 3760-3764, 2012.

63. Gallagher TM, and Buchmeier MJ. Coronavirus spike proteins in viral entry and pathogenesis. Virology 279, 371-374, 2001.

64. Kuwabara M, Wada K, Maeda Y, Miyazaki A, and Tsunemitsu H. First isolation of cytopathogenic bovine torovirus in cell culture from a calf with diarrhea. Clin Vaccine Immunol 14, 998-1004, 2007.

65. Ito M, Ichikawa Y, Shimoike K, Matsuda T, Uechi M, and Shintani E. Outbreak of bovine torovirus diarrhea in Ishikawa Prefecture. J Jpn Vet Med Assoc 65, 350-354, 2012. (In Japanese)

66. Nagai M, Omatsu T, Aoki H, Otomaru K, Uto T, Koizumi M, Minami-Fukuda F, Takai H, Murakami T, Masuda T, Yamasato H, Shiokawa M, Tsuchiaka S, Naoi Y, Sano K, Okazaki S, Katayama Y, Oba M, Furuya T, Shirai J, and Mizutani T. Full genome analysis of bovine astrovirus from fecal samples of cattle in Japan,

identification of possible interspecies transmission of bovine astrovirus. Arch Virol 160, 2491-2501, 2015.

67. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, and Higgins DG. The CLUSTAL_X windows interface, flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25, 4876-4882, 1997.

68. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S. MEGA5, molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28, 2731-2739, 2011.

69. Felsenstein J. Confidence limits on phylogenies, an approach using the bootstrap. Evolution 39, 783-791, 1985.

70. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkani SS, Novak NG, Ingersoll R, Sheppard HW, and Ray SC. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73, 152-160, 1999.

71. Martin D, and Rybicki E. RDP; detection of recombination amongst aligned sequences. Bioinformatics 16, 562-563, 2000.

72. Martin DP, Posada D, Crandall KA, and Williamson C. A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. AIDS Res Hum Retroviruses 21, 98-102, 2005.

73. Smits SL, Gerwig GJ, van Vliet AL, Lissenberg A, Briza P, Kamerling JP, Vlasak R, and de Groot RJ. Nidovirus sialate-O-acetylesterases, evolution and substrate specificity of coronaviral and toroviral receptor-destroying enzymes. J Biol Chem 280, 6933-6941, 2005.

74. Graham RL, and Baric RS. Recombination, reservoirs, and the modular spike, mechanisms of coronavirus cross-species transmission. J Virol 84, 3134-3146, 2010.

75. Herrewegh AA, Smeenk I, Horzinek MC, Rottier PJ, and de Groot RJ. Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. J Virol 72, 4508-4514, 1998.

76. Hewson KA, Noormohammadi AH, Devlin JM, Browning GF, Schultz BK, and Ignjatovic J. Evaluation of a novel strain of infectious bronchitis virus emerged as a result of spike gene recombination between two highly diverged parent strains. Avian Pathol 43, 249-257, 2014.

77. Lau SK, Feng Y, Chen H, Luk HK, Yang WH, Li KS, Zhang YZ, Huang Y, Song ZZ, Chow WN, Fan RY, Ahmed SS, Yeung HC, Lam CS, Cai JP, Wong SS, Chan JF, Yuen KY, Zhang HL, and Woo PC. Severe acute respiratory syndrome (SARS) coronavirus ORF8 protein is acquired from SARS-related coronavirus from greater horseshoe bats through recombination. J Virol 89, 10532-10547, 2015.

78. Song JE, Jeong WG, Sung HW, and Kwon HM. Sequencing, phylogenetic analysis, and potential recombination events of infectious bronchitis viruses isolated in Korea. Virus Genes 46, 371-374, 2013.

79. Terada Y, Matsui N, Noguchi K, Kuwata R, Shimoda H, Soma T, Mochizuki M, and Maeda K. Emergence of pathogenic coronaviruses in cats by homologous recombination between feline and canine coronaviruses. PLoS One 9, e106534, 2014.

80. Tian PF, Jin YL, Xing G, Qv LL, Huang YW, and Zhou JY. Evidence of recombinant strains of porcine epidemic diarrhea virus, United States. 2013. Emerg Infect Dis 20, 1735-1738, 2014.

81. Wang Y, Liu D, Shi W, Lu R, Wang W, Zhao Y, Deng Y, Zhou W, Ren H, Wu J, Wang Y, Wu G, Gao GF, and Tan W. Origin and possible genetic recombination of the Middle East respiratory syndrome coronavirus from the first imported case in China, phylogenetics and coalescence analysis. mBio 6, e01280-15, 2015.

82. Zhang T, Han Z, Xu Q, Wang Q, Gao M, Wu W, Shao Y, Li H, Kong X, and Liu S. Serotype shift of a 793/B genotype infectious bronchitis coronavirus by natural recombination. Infect Genet Evol 32, 377-387, 2015.

83. Zhang Y, Li J, Xiao Y, Zhang J, Wang Y, Chen L, Paranhos-Baccalà G, Ren L, and Wang J. Genotype shift in human coronavirus OC43 and emergence of a novel genotype by natural recombination. J Infect 70, 641-650, 2015.

84. Zhao F, Zou N, Wang F, Guo M, Liu P, Wen X, Cao S, and Huang Y. Analysis of

a QX-like avian infectious bronchitis virus genome identified recombination in the region containing the ORF 5a, ORF 5b, and nucleocapsid protein gene sequences. Virus Genes 46, 454-464, 2013.

85. Hurst KR, Ye R, Goebel SJ, Jayaraman P, and Masters PS. An interaction between the nucleocapsid protein and a component of the replicase-transcriptase complex is crucial for the infectivity of coronavirus genomic RNA. J Virol 84, 10276-10288, 2010.

86. Krishna NK. Identification of structural domains involved in astrovirus capsid biology. Viral Immunol 18, 17-26, 2005.

87. Dong J, Dong L, Méndez E, and Tao Y. Crystal structure of the human astrovirus capsid spike. Proc Natl Acad Sci USA 108, 12681-12686, 2011.

88. Matsui M, Ushijima H, Hachiya M, Kakizawa J, Wen L, Oseto M, Morooka K, and Kurtz JB. Determination of serotypes of astroviruses by reverse transcription-polymerase chain reaction and homologies of the types by the sequencing of Japanese isolates. Microbiol Immunol 42, 539-547, 1998.

89. Strain E, Kelley LA, Schultz-Cherry S, Muse SV, and Koci MD. Genomic analysis of closely related astroviruses. J Virol 82, 5099-5103, 2008.

90. Xiao CT, Giménez-Lirola LG, Gerber PF, Jiang YH, Halbur PG, and Opriessnig T. Identification and characterization of novel porcine astroviruses (PAstVs) with high prevalence and frequent co-infection of individual pigs with multiple PAstV types. J Gen Virol 94, 570-582, 2013.

91. Shan T, Wang C, Tong W, Zheng H, Hua X, Yang S, Guo Y, Zhang W, and Tong G. Complete genome of a novel porcine astrovirus. J Virol 86, 13820-13821, 2012.

92. Wang QH, Kakizawa J, Wen LY, Shimizu M, Nishio O, Fang ZY, and Ushijima H. Genetic analysis of the capsid region of astroviruses. J Med Virol 64, 245-255, 2001.

93. Woo PC, Lau SK, Teng JL, Tsang AK, Joseph S, Xie J, Jose S, Fan RY, Wernery U, and Yuen KY. A novel astrovirus from dromedaries in the Middle East. J Gen Virol 96, 2697-2707, 2015.

94. Brnić D, Prpić J, Keros T, Roić B, Starešina V, and Jemeršić L. Porcine astrovirus viremia and high genetic variability in pigs on large holdings in Croatia. Infect Genet Evol 14, 258-264, 2013.

95. Brnić D, Jemeršić L, Keros T, and Prpić J. High prevalence and genetic heterogeneity of porcine astroviruses in domestic pigs. Vet J 202, 390-392, 2014.

96. Machnowska P, Ellerbroek L, and Johne R. Detection and characterization of potentially zoonotic viruses in faeces of pigs at slaughter in Germany. Vet Microbiol 168, 60-68, 2014.

97. Reuter G, Pankovics P, and Boros A. Identification of a novel astrovirus in a domestic pig in Hungary. Arch Virol 156, 125-128, 2011.

98. Reuter G, Nemes C, Boros A, Kapusinszky B, Delwart E, and Pankovics P. Astrovirus in wild boars (Sus scrofa) in Hungary. Arch Virol 157, 1143-1147,

2012.

99. Zhou W, Ullman K, Chowdry V, Reining M, Benyeda Z, Baule C, Juremalm M, Wallgren P, Schwarz L, Zhou E, Pedrero SP, Hennig-Pauka I, Segales J, and Liu, L. Molecular investigations on the prevalence and viral load of enteric viruses in pigs from five European countries. Vet Microbiol 182, 75-81, 2016.

100. Lan D, Ji W, Shan T, Cui L, Yang Z, Yuan C, and Hua X. Molecular characterization of a porcine astrovirus strain in China. Arch Virol 156, 1869-1875, 2011.

101. Cai Y, Yin W, Zhou Y, Li B, Ai L, Pan M, and Guo W. Molecular detection of Porcine astrovirus in Sichuan Province, China. Virol J 6, 6, 2016.

102. Li JS, Li MZ, Zheng LS, Liu N, Li DD, and Duan ZJ. Identification and genetic characterization of two porcine astroviruses from domestic piglets in China. Arch Virol 160, 3079-3084, 2015.

103. Mor SK, Chander Y, Marthaler D, Patnayak DP, and Goyal SM. Detection and molecular characterization of Porcine astrovirus strains associated with swine diarrhea. J Vet Diagn Invest 24, 1064-1067, 2012.

104. Dufkova L, Scigalkova I, Moutelikova R, Malenovska H, and Prodelalova J. Genetic diversity of porcine sapoviruses, kobuviruses, and astroviruses in asymptomatic pigs, an emerging new sapovirus GIII genotype. Arch Virol 158, 549-558, 2013.

105. Lee MH, Jeoung HY, Park HR, Lim JA, Song JY, and An DJ. Phylogenetic

analysis of porcine astrovirus in domestic pigs and wild boars in South Korea. Virus Genes 46, 175-181, 2013.

106. Lee S, Jang G, and Lee C. Complete genome sequence of a porcine astrovirus from South Korea. Arch Virol 160, 1819-1821, 2015.

107. Monini M, Di Bartolo I, Ianiro G, Angeloni G, Magistrali CF, Ostanello F, and Ruggeri FM. Detection and molecular characterization of zoonotic viruses in swine fecal samples in Italian pig herds. Arch Virol 160, 2547-2556, 2015.

108. Amimo JO, Okoth E, Junga JO, Ogara WO, Njahira MN, Wang Q, Vlasova AN, Saif LJ, and Djikeng A. Molecular detection and genetic characterization of kobuviruses and astroviruses in asymptomatic local pigs in East Africa. Arch Virol 159, 1313-1319, 2014.

109. Amimo JO, El Zowalaty ME, Githae D, Wamalwa M, Djikeng A, and Nasrallah GK. Metagenomic analysis demonstrates the diversity of the fecal virome in asymptomatic pigs in East Africa. Arch Virol 161, 887-897, 2016.

110. Laurin MA, Dastor M, and L'homme Y. Detection and genetic characterization of a novel pig astrovirus, relationship to other astroviruses. Arch Virol 156, 2095-2099, 2011.

111. Xiao CT, Halbur PG, and Opriessnig T. Complete genome sequence of a newly identified porcine astrovirus genotype 3 strain US-MO123. J Virol 86, 13126, 2012.

112. Simmonds P. Recombination and selection in the evolution of picornaviruses and

other Mammalian positive-stranded RNA viruses. J Virol 80, 11124-11140, 2006.

113. Medici MC, Tummolo F, Martella V, Banyai K, Bonerba E, Chezzi C, Arcangeletti MC, De Conto F, and Calderaro A. Genetic heterogeneity and recombination in type-3 human astroviruses. Infect Genet Evol 32, 156-160, 2015.

114. Wolfaardt M, Kiulia NM, Mwenda JM, and Taylor MB. Evidence of a recombinant wild-type human astrovirus strain from a Kenyan child with gastroenteritis. J Clin Microbiol 49, 728-731, 2011.

115. Rivera R, Nollens HH, Venn-Watson S, Gulland FM, and Wellehan JF Jr. Characterization of phylogenetically diverse astroviruses of marine mammals. J Gen Virol 91, 166-173, 2010.

116. Ulloa JC, and Gutiérrez MF. Genomic analysis of two ORF2 segments of new porcine astrovirus isolates and their close relationship with human astroviruses. Can J Microbiol 56, 569-577, 2010.

Summary in Japanese（和文要旨）

家畜保健衛生所で実施するウイルス感染症の診断は、培養細胞を用いてウイルスを分離することを基本とする。しかし、腸内ウイルスは分離方法が確立されてないものが多く、遺伝子検査に依存することが多い。また、RNA ウイルスは遺伝子の変異や組換えによる遺伝的多様な集団（quasispecies）と考えられており、分離されたウイルス株は quasispecies を代表する 1 株にすぎない。近年、特異的なプライマーを使用せず、病原体の遺伝子情報を直接決定する次世代シークエンスの技術が普及してきた。この技術を用いることにより、野外診断材料に含まれる培養細胞で増殖が困難または不可能な RNA ウイルスの遺伝学的な同定が可能となってきた。本研究では次世代シークエンス技術を用いて、感染症の診断のために収集された牛及び豚の糞便に含まれる RNA ウイルスのメタゲノム解析を行い、ウイルス遺伝子の多様性やウイルスの種間伝播の解明を目指した。

本研究は 2 章から成る。牛トロウイルスは、コロナウイルス科トロウイルス亜科に属し、牛の腸疾患の原因となる。第 I 章では、分離ウイルス 1 株を含む野外糞便材料由来の、牛トロウイルス 2 株の全ゲノム及び 2 株の部分ゲノム、豚トロウイルス 1 株の部分ゲノムのシークエンス解析を実施した。塩基配列の比較及び系統樹解析では、日本の牛トロウイルス間に高い相同性が認められ、S、M 及び HE コード領域は牛トロウイルス Breda1 株と、ORF1a、ORF1b、N コード領域、非翻訳領域の 5’ 端及び 3’ 端は豚トロウイルス NPL/2014 株及び SH1 株と相同性が高く、日本の牛トロウ

イルスは、既報の牛及び豚トロウイルスの、種間組換え体であると考えられた。また、相同性プロット解析により、組換えを生じている箇所（ブレイクポイント）を、ORF1b 領域の 3’ 端及び HE コード領域の 3’ 端と予測した。この組換えは、抗原性状に関与する S コード領域が牛トロウイルス Breda1 株と同じ型であるため血清型に関わらないこと、また、自然界で牛トロウイルスと豚トロウイルスが同一宿主に重感染したことを示している。

豚アストロウイルスはアストロウイルス科に属し、世界中の豚に遍在する腸内ウイルスである。健康な豚からもウイルスが頻繁に検出されるため、感染の臨床的意義は不明である。そのため、国内の豚における豚アストロウイルスの疫学も、現在までほとんど知られていない。第Ⅱ章では、野外糞便材料を由来とする豚アストロウイルス 36 株の、ほぼ全ゲノムのシークエンス解析を実施した。塩基配列の比較及び系統樹解析により、10 株が豚アストロウイルス 2 型に、5 株が豚アストロウイルス 3 型に、15 株が豚アストロウイルス 4 型に、6 株が豚アストロウイルス 5 型に分類された。また、8 頭の豚で、2 ないし 3 つの型のウイルスに重感染していた。ORF2 領域の系統樹解析から、豚アストロウイルス 2 型はさらに 2 系統に、4 型は 4 系統に分類される。豚アストロウイルス 2 型及び 4 型の、ORF1a 及び ORF1b 領域の系統樹のトポロジーは、ORF2 領域の系統樹と異なっており、相同性プロット解析から、2 型及び 4 型内において遺伝子型内及び遺伝子型間の組換えが複数発見された。さらに、豚アストロウイルス 3 型及び 5 型内のそれぞれ 1 株で、ORF2 領域の塩基配列に多様性が認められた。しかし比較可能なシークエンス情報の不足により、これ以上は言及できなかった。また、抗原性状に関与する ORF2 領域の相同性は、各遺伝子型

間で 28.4%以下、系統間で 65.4%以下であり、同じ遺伝子型であっても抗原性に差があることが示唆された。このことは、同一宿主に複数株の同時感染を可能とし、組換えを容易にさせ、遺伝的多様性を促進するものと考えられた。

以上、本研究で明らかになった知見は、腸内 RNA ウイルスである牛トロウイルス及び豚アストロウイルスの遺伝子多様性や種間伝播の解明に寄与するものである。