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Research paper

Complete genome sequencing and genetic characterization of porcine sapovirus genogroup (G) X and GXI: GVI, GVII, GX, and GXI sapoviruses share common genomic features and form a unique porcine SaV clade

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ABSTRACT

Sapoviruses (SaVs) are enteric viruses belonging to the family Caliciviridae that infect humans and animals, including pigs. To date, SaVs have been classified into 19 genogroups (G) based on complete VP1 sequences; however, complete genome sequences of some SaV Gs are not yet available. In this study, we determined the full genome sequences of four SaVs (two GX and two GXI SaVs) and analyzed them together with those of other SaVs. The complete genome sequences of GX and GXI SaVs, excluding the poly(A) tails, were 7124, 7142, 7170, and 7179 nucleotides, which were shorter than those of other SaVs, except for porcine GVI and GVII viruses. Genetic characterization revealed that GX SaVs and GXI SaVs shared common features with GVI and GVII viruses, such as the first 10 amino acid residues in the ORF1 coding region, a shorter ORF1 than that of the other genogroups, and the predicted secondary structure of the 5′ end of the genome and the starting region of non-structural protein/structural protein junction. Phylogenetic analyses showed that GX and GXI SaVs branched with porcine GVI, GVII, and GIX SaVs and formed a clade consisting of only porcine SaVs. These findings suggest that porcine GX and GXI SaVs together with porcine GVI, GVII, and possibly GIX SaVs, evolved from a common ancestor in the porcine population.

1. Introduction

The genus *Sapovirus* belongs to the family Caliciviridae together with the genera, *Bavovirus*, *Lagovirus*, *Minovirus*, *Nacovirus*, *Nebovirus*, *Norovirus*, *Recovirus*, *Salovirus*, *Valovirus*, and *Vesivirus* (http://www.ictvonline.org/virusTaxonomy.asp). Although sapoviruses (SaVs) do not have a more severe clinical impact compared to that of noroviruses (NoV), SaVs are human and animal pathogens that can cause symptoms of gastroenteritis in their respective hosts (Desselberger, 2019; Oka et al., 2015). Many SaVs have been detected in fecal samples of diarrheic and asymptomatic pigs in Brazil, Italy, Czech Republic, South Korea, China, Japan, Venezuela, Belgium, Slovenia, Denmark, Finland, Hungary, Spain, Slovakia, and the United States (Cunha et al., 2010; das Merces et al., 2014; Di Bartolo et al., 2014; Dufkova et al., 2013; Jeong et al., 2007; Keum et al., 2009; Kim et al., 2006; Liu et al., 2012; Liu et al., 2014; Kuroda et al., 2017; Martella et al., 2008; Martínez et al.,

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2006; Mauroy et al., 2008; Mijovski et al., 2010; Oka et al., 2014; Reuter et al., 2010; Salamunova et al., 2018; Scheuer et al., 2013; Valente et al., 2016; Wang et al., 2006; Zhang et al., 2014; Zhang et al., 2008). Since animal SaVs, especially porcine isolates, are closely related to human SaV isolates, a zoonotic potential of these viruses have been suggested in past studies (Bank-Wolf et al., 2010; Oka et al., 2016).

SaVs are non-enveloped viruses with a single-stranded, positive-sense RNA genome, approximately 7.1–7.7 kb in length with a poly-adenylated [poly (A)] tail at the 3′ end. The 5′ end of the genome is covalently linked to a small virus-encoded protein (VPg) (Alhatlani et al., 2015; Oka et al., 2016). The genomes of NoVs, vesiviruses, and recoviruses contain three open reading frames (ORFs) that encode the non-structural proteins (ORF1), the capsid protein VP1 (ORF2), and a minor structural protein VP2 (ORF3). However, the SaV genomes possess two ORFs (ORF1 and ORF2), because the SaVs VP1 coding region is fused to the non-structural protein coding region within the ORF1. As a result of an ORF1 cleavage event by a virus-encoded protease, six nonstructural proteins, NS1, NS2, NS3 (putative NTPase), NS4, NS5 (VPg), and NS6-NS7 [fused protease and RNA dependent RNA polymerase (RdRp)], together with VP1 are generated (Oka et al., 2015). Furthermore, sub-genomic RNA (sgRNA) encoding structural genes were experimentally expressed during replication of GIII SaV (Chang et al., 2004).

SaVs are classified into genogroups based on the VP1 sequences (Farkas et al., 2004; Scheuer et al., 2013). To date, 19 SaV genogroups have been reported, and a pairwise identity of < 57% amino acids (aa) in the VP1 sequence has been the criterion to define a different SaV genogroup (Oka et al., 2016; Yinda et al., 2017). Recently, Li et al. further classified SaVs into 51 genotypes within the 19 genogroups based on phylogenetic analyses using complete VP1 nucleotide (nt) sequences (Li et al., 2018). To date, including a novel genotype candidate GII.NA, at least 52 genotype sequences are available in DDBJ/EMBL/GenBank database (Diez-Valcarce et al., 2019). Among the 52 genotypes, 20 genotypes such as, GIII, GV.3, GV.5, GVI.1, GVI.2, GVII.1-GVII.6, GVIII.1, GVIII.2, GIX.1, GIX.2, GX.1, GX.2, and GXI.1-GXI.3, have been identified in porcine species (Li et al., 2018).

Extensive genetic characterization of the SaV genome has enabled the identification of a vast genomic diversity of SaVs even within the same genogroup or genotype. Therefore, phylogenetic analyses based on complete genome sequences are useful for comprehensive genetic characterization for SaVs. However, such sequences of some SaV genogroups, including SaV GX and GXI, are currently unavailable. In this study, we determined the complete genome sequences of GX and GXI SaVs identified in Japanese pigs and analyzed their phylogenetic properties together with other SaVs.

2. Materials and methods

2.1. Fecal samples, RNA extraction, and deep sequencing

Three porcine fecal samples used in a previous study (Kuroda et al., 2017), including SaV GX.1 HgTa2/2016, GX.2 HgTa3-2/2016, and GXI.3 Ishi-Im7-3/2016 (classification described in Li et al., 2018) were used. These samples were collected in 2016 in Tottori and Ishikawa Prefecture in Japan and stored in a − 80 °C freezer. One porcine fecal specimen collected in 2018 in Tottori Prefecture, from which a XI.3 SaV (HgYa/2018) was detected by deep sequencing, was also used in this study. Viral RNA was extracted from the fecal sample supernatants, using TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, USA), and was treated with DNase I (Takara Bio, Shiga, Japan). Extracted RNA samples were stored at −80 °C until use. Deep sequencing using a MiSeq bench-top sequencer (Illumina, San Diego, CA, USA) was carried out as described previously (Nagai et al., 2015).

Table 1
Complete genome characterization of sapoviruses.

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2.2. Sequencing of 5′ and 3′ terminals of genomes

The 5′ terminal nucleotide sequences of the SaV genomes were determined using the DNA-DNA ligation-based rapid amplification of cDNA end method (RACE) as described (Oka et al., 2017). Viral RNA was extracted from 200 µl supernatants of approximately 20% fecal samples in sterile phosphate-buffered saline using QIAGEN Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacture's recommendations. A modified method employing the QIAGEN Viral RNA kit was also carried out; yeast RNA (Invitrogen, Carlsbad, CA, USA) was added to AVL buffer instead of the carrier RNA supplied with the kit. Finally, RNA was eluted with 60 µl of DNase-and RNase-free water (Invitrogen). Synthesis of cDNA was performed using SuperScript IV reverse transcriptase (Invitrogen), and the 5′ terminal region of the GX or GXI sapovirus genome was amplified by semi-nested PCR using a forward primer complementary to the DNA anchor ligated to the end of the viral genomic sequence and gene specific reverse primers (supplementary Table S1) using KOD-Plus DNA polymerase. The 3′ end of a SaV genome was amplified by RT-PCR with a gene specific forward primer, and a reverse primer, TX30SXN (supplementary Table S1), using KOD-Plus DNA polymerase as described previously (Oka et al., 2016). The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen), and directly sequenced with a set of gene-specific primers using BigDye Terminator v3.1 cycle sequencing kit on an automated DNA sequencer (Seq Studio Genetic Analyzer, Applied Biosystems). Sequence editing and assembly were performed using the Sequencher program v5.4.6 (GeneCodes) and analyzed by Genetyx-Mac software v20.0.3 (Genetyx Corporation). Complete sequences of GX and GXI SaVs determined in this study have been deposited in the DDBJ/EMBL/GenBank database under accession number LC215896, LC215897, LC215899, and LC469052.

2.3. Genome analyses

The sequences of SaVs obtained in the current study and from the DDBJ/EMBL/GenBank database were aligned using ClustalW (Thompson et al., 1997) from the MEGA7 software (Kumar et al., 2016). Phylogenetic analysis was performed based on the nucleotide sequence analysis using the maximum likelihood (ML) method of MEGA7. The best-fit models in ML for phylogenetic tree construction were the GTR + G model for the 5′ genomic region (5′UTR and non-structural protein region), the GTR + G+ I model for the 3′ genomic region (structural protein and 3′UTR) and for the VP1 and partial VP2 region; the K2 + G model was used for the partial RdRp region. The trees were evaluated using bootstrap analysis with 1000 replicates (Felsenstein, 1985). The secondary structures of the viral RNA were predicted using the Mfold program available online (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) (Zuker, 2003).

3. Results

3.1. Genomic characteristics of SaV GX and GXI

Complete genomes of GX.1 HgTa2/2016, GX.2 HgTa3–2/2016, GXI.3 Ishi-Im7-3/2016, and GXI.3 HgYa/2018 were successfully obtained using deep sequencing and 5′ and 3′ RACE methods. The complete genome lengths, excluding the poly(A) tail, of HgTa2/2016, HgTa3-2/2016, Ishi-Im7-3/2016, and HgYa/2018 were 7142, 7124, 7170, and 7179 nucleotides (nt), respectively. The genome lengths of these viruses were shorter than those of the other SaV genogroups of

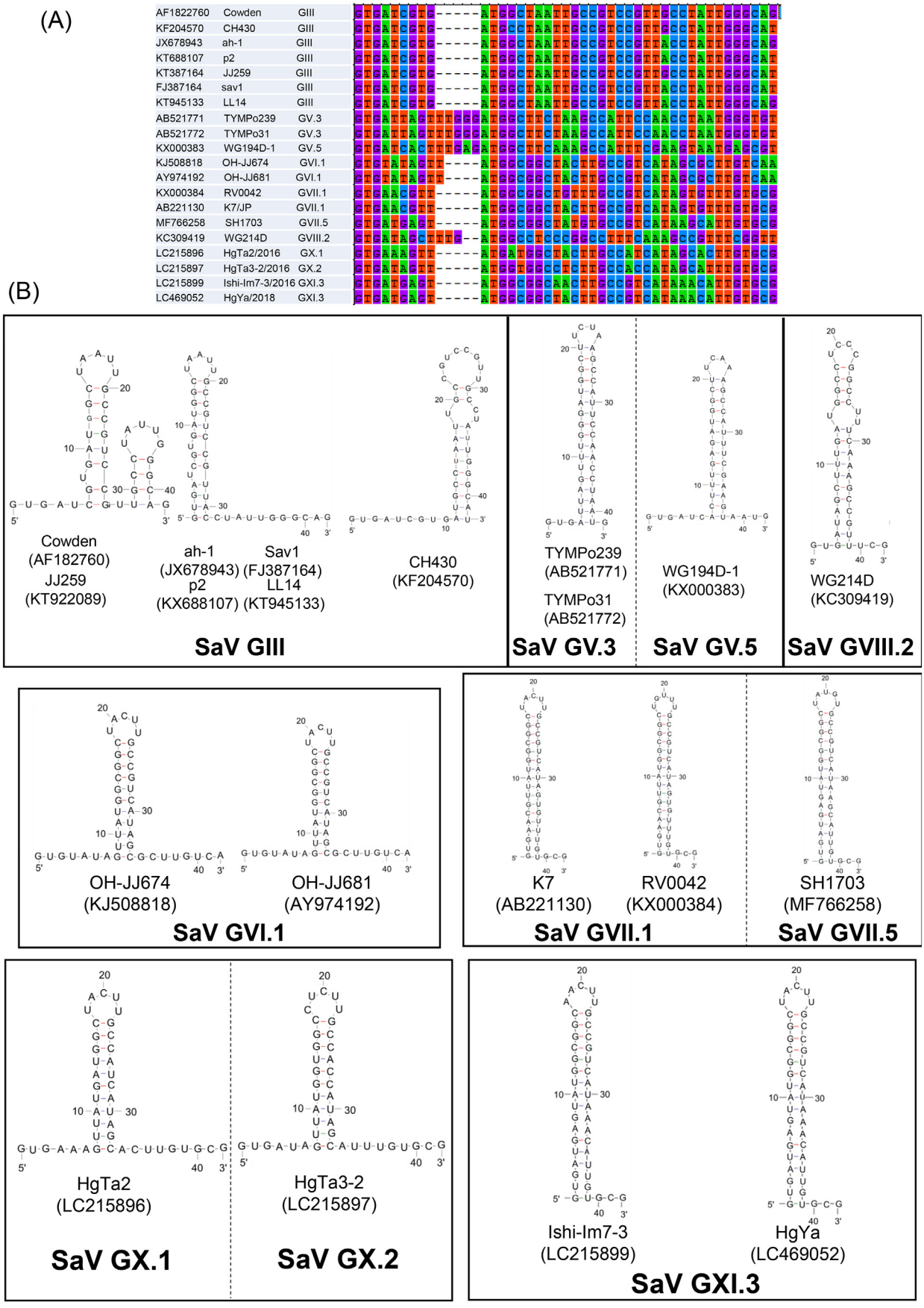

Fig. 1. Genome analysis of the 5′ end of genome of porcine SaVs. (A) Alignment of nt sequences of 5′UTR and ORF1 starting region. (B) Secondary structure prediction of the genome 5′ end nt sequences among porcine SaVs.

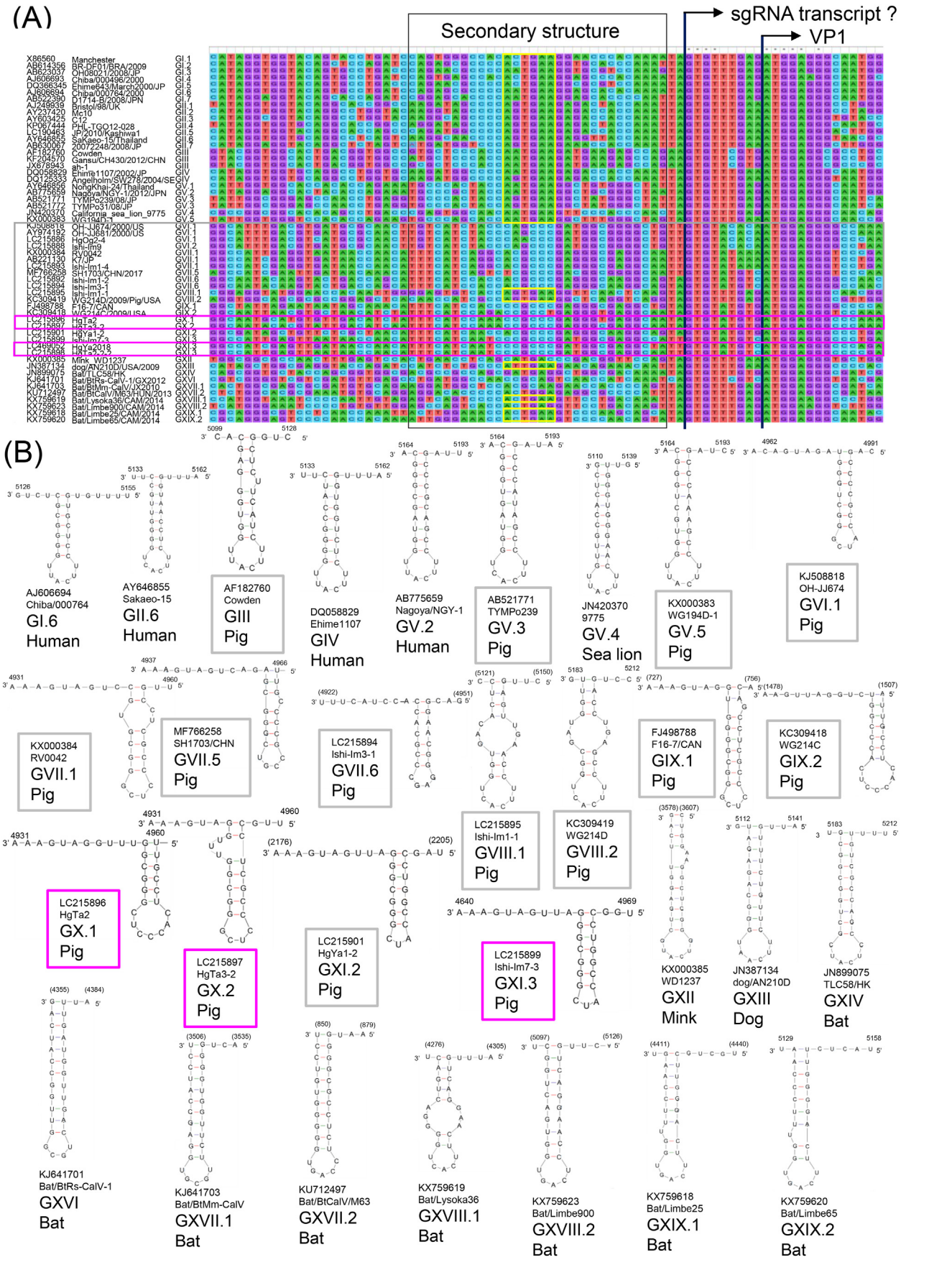

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Fig. 2. Genome analysis of the non-structural protein/structural protein junction site. (A) Alignment of nt sequences of GI-GXIX except GXV non-structural protein/structural protein (NS/S) junction. Conserved motifs are boxed by yellow. Porcine SaVs of which the sequences were already available and SaVs studied in this study are boxed by gray and pink, respectively. (B) Prediction of RNA structures upstream of the sgRNA transcript using anti-sense RNA sequences shown in a left to right direction from 3′ and 5′. The numbers indicated at 3′ and 5′ ends of sequences indicate the genome position of each virus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which the complete sequences are available [7320 nt (GIII), 7695 nt (GXIV)], excluding the GVI and GVII viruses (7144–7198 nt) (Table 1). The ORF1 lengths (2192 or 2205 aa) were shorter than those of the other SaVs [2254 aa (GIII), 2301 aa (GV)], except for GVI and GVII viruses (2198–2218 aa). Although there is limited data available, GX and GXI SaVs, and GVI and GVII viruses shared the first common aa residues of ORF1, MXAXCXHXXC, while the 5′UTRs of all the GX and GXI viruses were 9 nt long; however, the lengths of 3′UTR varied (33–72 nt) (Table 1).

3.2. Analysis of the secondary RNA structure of 5′ terminal of genomes and non-structural protein/structural protein junction sites

The secondary RNA structures of the 5′ terminal of the viral genomes were predicted using the first 42 nt by the Mfold program and were compared between the porcine SaVs (Fig. 1A). A single stem-loop structure was observed at the 5′ end of all the porcine SaV genomes. The stem-loop structures of GX SaVs were closely related to those of GVI viruses. In addition, the structures of GXI SaVs exhibited similarity to those of GVII viruses (Fig. 1B). The 5′ end of the sgRNA, where the starting region of non-structural protein/structural protein (NS/S) junction is predicted to be located, contains regions of RNA secondary structures that probably play a role in the viral life cycle of caliciviruses, including SaVs (Alhatlani et al., 2015; Simmonds et al., 2008). Using anti-sense RNA sequences, structurally related stem-loops were found at the NS/S junction of SaVs, and the terminal loop sequence was highly conserved between genogroups (Simmonds et al., 2008). Multiple alignment of nt sequences of the NS/S junction region revealed that the terminal loop sequences of the predicted stem-loops, AXTGAA, was conserved among SaV GI-GV, GVIII, GXIII, and GXVIII.1-GXIX sequences. In contrast, this motif was not found in the NS/S junction region of GVI, GVII, GIX-GXII, as well as GXIV, GXVI-GXVII, and GXVIII.2 sequences (Fig. 2A). Prediction of RNA structures upstream of the sgRNA transcript using anti-sense RNA sequences revealed a single stem-loop structure with a terminal unpaired region of three to seven nts (Fig. 2B). The terminal unpaired region of SaV GI-GV, GVIII, and GXIII contained a conserved motif. Besides, the secondary structure of GX.1 and GX.2 SaVs were similar to those of GIX.2 and GIX.1 viruses, respectively. The structures of GXI.2 and GXI.3 SaVs resembled those of GVII viruses; however, a terminal unpaired region of GXI.2 and GXI.3 contained 3 nt; in contrast, those of the GVII viruses contained 4 nt.

3.3. Phylogenetic analyses

Phylogenetic analyses using the complete genome nt sequences of GX and GXI SaVs with other SaVs, of which complete genome sequences are available, were performed (Fig. 3). GX and GXI SaVs, of which complete sequences were identified in this study, formed independent clusters in both the 5′ and 3′ genome region trees; however, they branched together with porcine SaV GVI and GVII viruses (Fig. 3A. and Fig. 3B). Although GIII, GV, and GVIII SaVs are of porcine origin, they branched at a distance from the GVI-GVII and GX-GXI clusters. All genogroups formed independent clusters in the 3′ region tree, whereas GVII.5 SaV was closely related to GXI viruses in the 5′ region tree. In the 5′ region tree, GII and GIV SaVs were not clearly divided. Furthermore, GV.4 SaV did not branch with the other GV viruses in the 5′ region, consistent with previous reports (Diez-Valcarce et al., 2018; Oka et al., 2015). GX and GXI SaVs generated a clade consisting of only porcine SaVs with GVI and GVII viruses in both the trees. Since complete genome sequences of GIX SaVs and several genotypes detected from pigs (e.g., GVI, GVII, GVIII, GX, and GXI) were not available in the DDBJ/EMBL/GenBank databases (Table 1), we reconstructed phylogenetic trees using either the partial RdRp region (765 nt) or VP1 and partial VP2 regions (2073 nt) of porcine SaVs (e.g., GIII, GV.3, GV.5, GVI.1, GVI.2, GVII.1, GVII.3, GVII.4, GVII.5, GVII.6, GVIII.1, GVIII.2, GIX.1, GIX.2, GX.1, GX.2, GXI.2, and GXI.3) (Fig. 4). Although porcine SaVs clustered according to genogroups in the VP1 and partial VP2 region trees (Fig. 4B), the clustering patterns of the RdRp tree were inconsistent with genogroups (Fig. 4A). Furthermore, GVII SaVs were subdivided into 3 groups, consisting of GVII.1 and GVII.4, GVII.5 and GXI.3, and GVII.3, GVII.6, GIX.1; GXI.2. GIX.2 WG214C clustered with GX SaVs. GVI, GVII, GIX, GX, and GXI SaVs clustered and were distantly related to GIII, GV, and GVIII porcine SaVs in both the phylogenetic trees (Fig. 4).

4. Discussion

Complete genome sequencing plays a critical role in understanding viral evolution and epidemiology. To date, complete genome sequences of GI-GVIII, GXIII, GXIV, and GXIX SaVs are available, including 7 GIII, 3 GV, 2 GVI, and 3 GVII complete genomes of porcine SaVs; however, complete sequences of GVI.2, GVII.2-GVII.4, GVII.6, GVIII.1, GIX.1, GIX.2, GX.1, GX.2, and GXI.1-GXI.3 have not been determined (Table 1). Improvement of the PCR primer sets used for SaV detection and a primer independent sequence technique developed recently has contributed to the discovery of new SaVs (Firth et al., 2014; Oka et al., 2015; Wu et al., 2016; Yinda et al., 2017). We have also detected nearly complete genomes of SaV from pig feces using a primer independent deep sequencing method (Kuroda et al., 2017). In the present study, we determined complete genome sequences of GX.1, GX.2, and GXI.3 porcine SaVs using a technique combining Illumina MiSeq sequencing and 5′ and 3′ RACE.

Complete genome length, excluding the poly(A) tail, of GX and GXI SaVs, were > 100 nt shorter than those of other genogroup SaVs, except for GVI and GVII viruses. However, the ORF2 lengths of GX and GXI SaVs were similar to the other SaVs, and the ORF1 length of GX and GXI as well as GVI and GVII viruses was shorter than that of the other SaVs. Additionally, the length of VP1 of GX SaVs (539 aa) and GXI SaVs (549 aa) were similar to, or in some cases, a little short compared to those of the other SaVs (545–572 aa) (data not shown). However, the lengths the 3′UTR region of SaVs were variable (28–233 nt) (Table 1). The genome lengths of porcine GIII SaV were similar to those of human SaVs, but the lengths of 3′UTR of GIII viruses were shorter than those of human SaVs. We also observed that the Bat SaVs, apart from GXVIII.1 and GXIX viruses, had a longer ORF2 than that of the other SaVs. The whole genome, ORF2, and 3′UTR length of GXIX SaVs were similar to those of human SaVs. GX and GXI SaVs also shared common genomic features with GVI and GVII viruses, such as a 5′UTR of short length (9 or 10 nt) and the initial aa residues of the ORF1. A profound suppression of the synonymous site variability at the 5′ end of genome and the initial region of the sgRNA transcript results in an underlying RNA structure that act as evolutionary constraints (Simmonds et al., 2008). Even though, in this study, there was little or no sequence conservation in the nt sequences forming the stem-loops at the 5′ end of genome and the starting region of the sgRNA transcript, similar stem-loops had been observed (Simmonds et al., 2008). Although the 5′UTR of caliciviruses is short and sequence conservation of this region is low, stem-loops are commonly present at the 5′ genome end of SaVs, including the coding

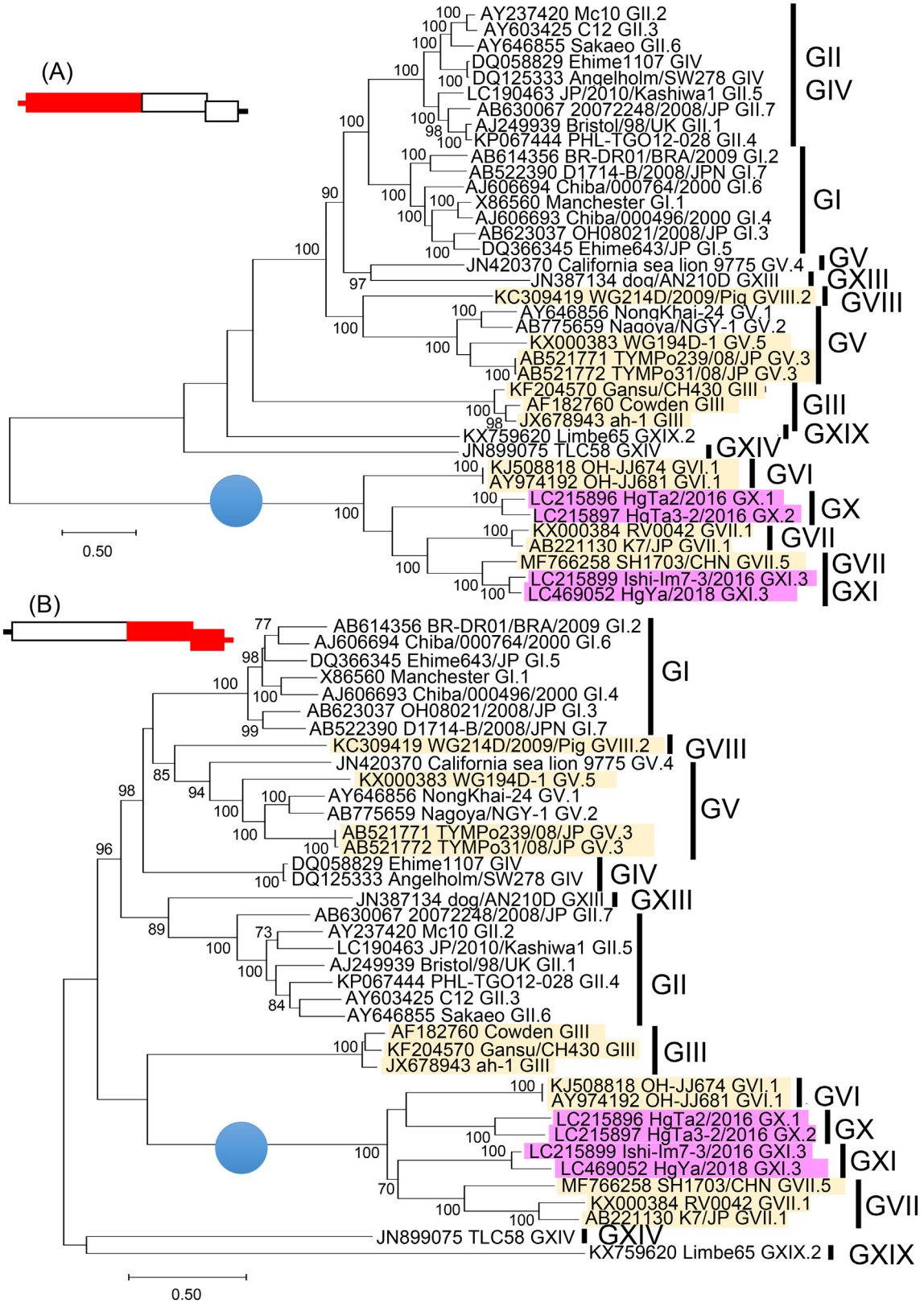

Fig. 3. Phylogenetic trees were constructed based on the nt sequences of 5′ of the genome region (5′UTR and nonstructural protein region) (A) and the 3′ of the genome region (structural proteins and 3′UTR) (B) of GX and GXI SaVs, the sequences of which were determined in this study, and 34 SaVs from the DDBJ/EMBL/GenBank database. The phylogenetic tree was constructed using the maximum likelihood method of MEGA 7, and bootstrap values (1000 replicates) above 70 are shown. The bar represents a corrected genetic distance. Porcine SaVs, and GX and GXI SaVs are shown in yellow and pink, respectively. Blue circles indicate the porcine SaV clades consisting of GVI, GVII, GX, and GXI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

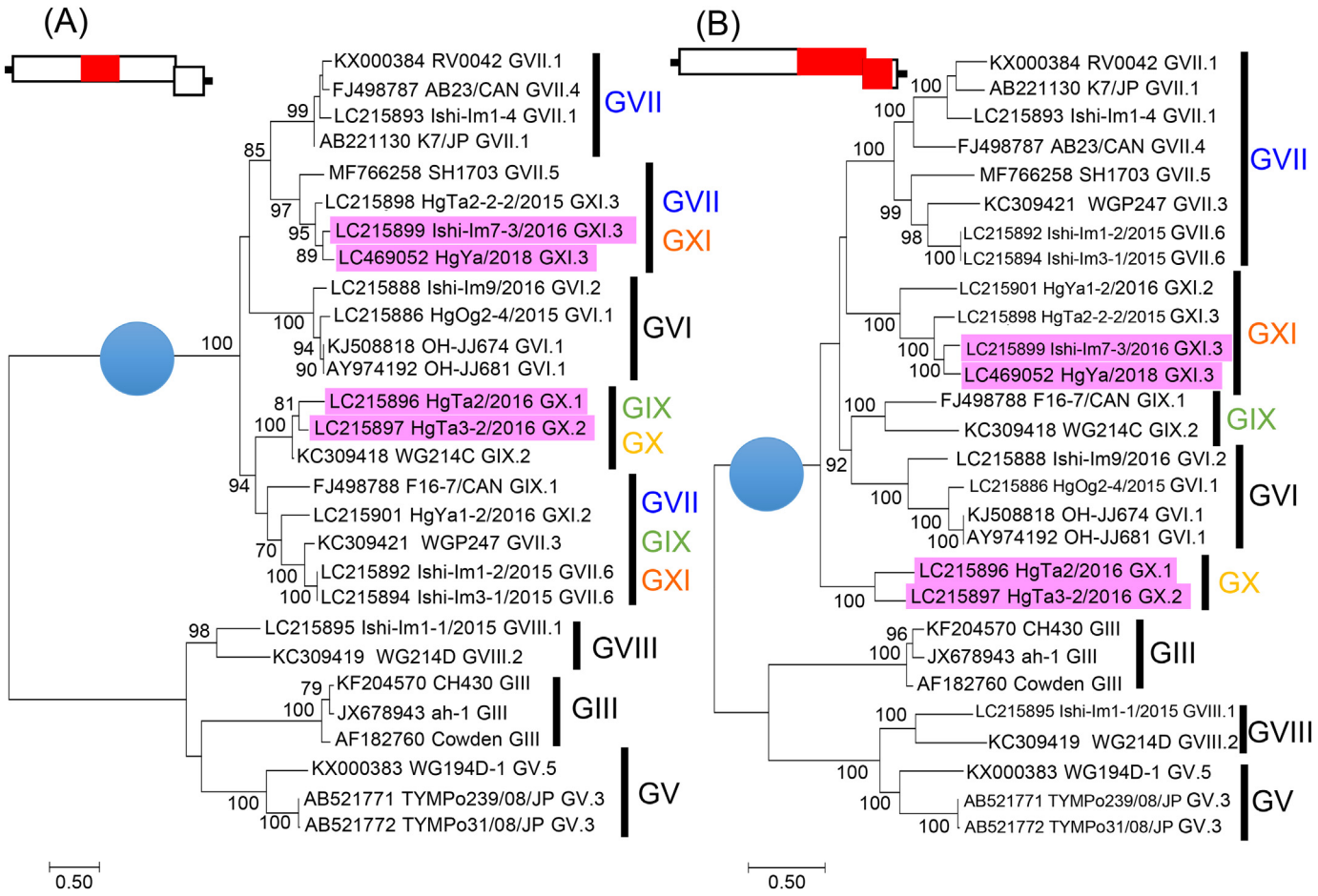

Fig. 4. A phylogenetic tree is constructed based on nt sequences of the partial RdRp region (A) and the VP1 and partial VP2 region (B) of GX and GXI SaVs, the sequences of which were determined in this study, and 24 porcine SaVs from the DDBJ/EMBL/GenBank database. The phylogenetic tree was constructed using the maximum likelihood method in MEGA 7, and bootstrap values (1000 replicates) above 70 are shown. The bar represents a corrected genetic distance. GX and GXI SaVs studied in this study are depicted in pink. Blue circles indicate a porcine SaV clade consisting of GVI, GVII, GIX, GX, and GXI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

region. Similar single stem-loop structures were identified at the 5′ end of genome region of SaVs (Hosmillo et al., 2016; Oka et al., 2017). The secondary RNA structures at the 5′ end of the genomes of GX and GXI SaVs were closely related to those of GVI and GVII viruses, respectively. A stem-loop with a terminal unpaired region with a highly conserved motif at the NS/S junction of SaVs has been previously reported (Simmonds et al., 2008). Sequence alignment of this region revealed that this conserved motif was not found in GX and GXI as well as in GVI and GVII SaVs. These findings suggest that GX-GXI and GVI-GVII, and probably GIX SaVs share common genomic features and are distantly related to other SaVs.

SaVs are known to exhibit significant genetic variability (Oka et al., 2016). Phylogenetic trees based on either the 3′ genome (VP1, VP2, and 3′UTR) or VP1 and partial VP2 clearly showed clustering patterns consistent with SaV genogroups and genotypes (Fig. 3B and Fig. 4B), whereas trees based on the 5′ genome (5′UTR and NS region) and partial RdRp displayed different topologies (Fig. 3A and Fig. 4A). This inconsistency in the phylogenetic tree clustering patterns of genomic regions has already been reported (Oka et al., 2015; Kuroda et al., 2017). The RdRp sequence-based classification is less reliable due to fewer available sequences with sufficient lengths compared to those of complete VP1 sequences (Oka et al., 2015). To better understand the genogroups and genotypes of SaVs and also to clarify the mechanism of replication and infection of SaVs, further accumulation of complete genome sequence data of SaVs is needed.

In conclusion, complete genome sequences of GX and GXI SaVs were determined by a combination of deep sequencing and 5′ and 3′ RACE methods. Genomic characterization and phylogenetic analyses revealed that GX and GXI SaV together with GVI, GVII, and probably GIX SaVs are closely related to each other and that these viruses form a unique clade consisting only of porcine SaVs. These findings suggest that these SaVs possess a common ancestor and have evolved in the porcine population. As GX and GXI SaVs in GVI and GVII viruses were detected in both asymptomatic or diarrheic pigs, the pathogenicity of these viruses is unknown. Further studies regarding the molecular biology and epidemiology of SaVs are warranted.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103959.

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Declaration of competing interests

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the

order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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