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Genetic diversity of porcine reproductive and respiratory syndrome virus 1 in the United States of America from 2010 to 2018

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ABSTRACT

Porcine reproductive and respiratory syndrome virus 1 (PRRSV-1) was first detected in the United States of America (USA) in 1999, several strains were also recognized soon later, and these isolates are typically called North American (NA) PRRSV-1. However, few reports have characterized PRRSV-1 viruses in the USA. We explored the genetic characteristics and diversity of PRRSV-1 viruses circulating in the USA. PRRSV-1 PCR-positive samples collected from seven states in 2010–2018 (n = 27) were subjected to next-generation sequencing. The 27 PRRSV-1 viruses had 88.4–91.3% nucleotide identity to the PRRSV-1 Lelystad-virus strain (the type 1 prototype strain) and 87.4–89.8% to the previously reported NA PRRSV-1 viruses. Individual proteins had several unique genetic characteristics and only one of the 27 tested samples had the characteristic 17-amino acid (aa) deletion in Nsp2, a genetic marker of NA PRRSV-1 viruses described previously. Fourteen isolates displayed a 3-aa C-terminal truncation in the highly conserved Nsp12 gene; 16 samples had a 21- or 18-aa C-terminal truncation in GP3 gene; and one was observed with a 1-aa deletion at the overlapping region of GP3 and GP4. In addition, the GP5 protein in most isolates, excluding one exception, demonstrated similar genetic variation as other reported NA PRRSV-1 isolates. All tested isolates clustered within subtype 1 together with other available NA PRRSV-1 viruses. Collectively, our results provide up-to-date information on PRRSV-1 viruses circulating in the USA in the past 9 years although the number of PRRSV-1 isolates included in this study is limited. These PRRSV-1 viruses have undergone gradual genetic variation and exhibited some previously undescribed genetic characteristics and diversity, which complicates the diagnosis and control of NA PRRSV-1.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), characterized by reproductive failure in sows and respiratory disease in pigs of all ages, is the most economically important swine disease globally. PRRS virus (PRRSV), the causative agent of PRRS, is a single-stranded, positive-sense RNA virus that belongs to the family *Arteriviridae* (Cavanagh, 1997). The whole genome of PRRSV is approximately 15 kb in length and consists of a 5′ untranslated region (UTR), at least ten open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5a, and ORF5–ORF7), and a 3′ UTR. ORF1a and ORF1b occupy two-thirds of the genome and encode at least 16 nonstructural proteins (Nsps) involved in viral replication and transcription. ORFs located at the 3′ end of the genome encode structural proteins from a 3′-coterminal nested set of functionally monocistronic subgenomic mRNAs, including four glycoproteins (GP2, GP3, GP4, and GP5), two unglycosylated proteins (E and M), and a nucleocapsid (N) protein (Music and Gagnon, 2010; Snijder et al., 2013).

Historically, PRRSV is classified into two genotypes: the type 1 PRRSV (PRRSV-1) and type 2 PRRSV (PRRSV-2) (Collins et al., 1992; Wensvoort et al., 1991). Recently, PRRSV-1 and PRRSV-2 have been taxonomically classified into the species *Betaarterivirus suid 1* and *Betaarterivirus suid 2*, respectively. The Lelystad virus (LV) is a representative PRRSV-1, and ATCC VR-2332 is a representative PRRSV-2. In PRRSV-1, three subtypes (a pan-European subtype 1 and East European subtypes 2 and 3) are recognized, and a fourth subtype has been

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reported (Stadejek et al., 2006; Stadejek et al., 2008; Stadejek et al., 2013). These subtypes display a sharp geographical demarcation; subtype 1 (LV-like) circulates in Central and Western Europe and globally, whereas subtypes 2 and 3 are found only in Eastern European countries (Shi et al., 2010). To date, subtype 1 has been detected in Canada (Dewey et al., 2000), the United States of America (USA) (Fang et al., 2004; Fang et al., 2007; Ropp et al., 2004; Wasilk et al., 2004), China (Chen et al., 2011; Chen et al., 2017; Liu et al., 2017; Zhou et al., 2015), Korea (Nam et al., 2009; Nguyen et al., 2014), and Thailand (Amonsin et al., 2009; Jantafong et al., 2015). Sequence analysis of PRRSV-1 isolates from areas worldwide showed dramatic divergence, including genetic diversity, antigenic properties, and evolutionary trends, which has led to many obstacles in the diagnosis and control of PRRSV (Butler et al., 2014; Darwich et al., 2011; Murtaugh et al., 2010).

The European-like PRRSV-1 isolate, known as NA PRRSV-1, was first reported in swine herds in Canada and the USA in 1999 (Dewey et al., 2000; Ropp et al., 2004). Since the first full-length genomic sequence of NA PRRSV-1 (EuroPRRSV strain) in the USA was completed in 2004 (Ropp et al., 2004), several whole genomes of NA PRRSV-1 viruses have been sequenced, and many molecular characteristics were addressed (Fang et al., 2004; Fang et al., 2007). A distinct 51-nucleotide (nt) deletion in Nsp2 was found in most type 1 PRRSVs in the USA and was recognized as a genetic marker of NA PRRSV-1 viruses (Fang et al., 2004; Ropp et al., 2004). However, few reports have described PRRSV-1 viruses in the USA in the past decade, and their genetic diversity and evolution remain unknown.

Next-generation sequencing (NGS) is a powerful tool for pathogen detection and research because of its advantages of low cost per base, massive sequence output, and short turnaround time (Cortey et al., 2017; Fabre et al., 2012; Zhang et al., 2017). In this study, we utilized NGS to gain more information regarding the genetic diversity and evolution of NA PRRSV-1 viruses in the USA in recent years.

2. Materials and methods

2.1. Virus isolates and clinical specimens

Twenty-three PRRSV isolates were provided by Iowa State University Veterinary Diagnostic Laboratory (ISU VDL, Ames, Iowa), which were isolated from clinical samples collected from seven states (UT, IA, AR, NC, PA, MO, and IN), between 2010 and 2018. Four clinical samples (three lung samples and one serum sample) were collected from NC, IA, and UT in 2018 at ISU VDL. All isolates and clinical specimens were verified to be type 1 PRRSVs using PRRSV type-specific primers. Detailed information regarding the total samples is shown in Table 1. For convenience, we designated the samples as EU01–27.

2.2. Sample processing and genomic sequencing

To obtain whole-genome sequences, all 27 samples were subjected to NGS on a MiSeq platform (Illumina, San Diego, CA, USA), as previously described (Chen et al., 2018). Specifically, DNA extracted from these samples was cleared with an RNase-Free DNase Set (Qiagen, Valencia, CA, USA), and residual reagent was then removed from the remaining RNA with an Agencourt RNA Clean XP kit (Beckman Coulter, Indianapolis, IN, USA), according to the manufacturer's instructions. The library was prepared with a TruSeq Stranded Total RNA Library preparation kit (Illumina), according to the manufacturer's instructions. The normalized library was sequenced on the MiSeq platform (Illumina) with a 300-cycle MiSeq Reagent Micro Kit V2 (Illumina).

Raw sequencing data were subjected to data cleaning by removing adapters, trimming low-quality ends, depleting sequences with lengths of less than 36 nt, and sequencing quality analysis with FastQC (Bolger et al., 2014). The taxonomy of cleaned reads was classified using Kraken v0.10.5-beta (Wood and Salzberg, 2014). Reads of particular viruses or viruses of interest were extracted from the kraken classification results as candidate reads of that taxon. In particular, PRRSV fragments were extracted and were *de novo* assembled with SPAdes (v 3.5.0) based on candidate reads (Robinson et al., 2011). The 27 near full-length genomic sequences were obtained, and the 5' and 3' ends were almost complete (Table S1).

2.3. Nucleotide sequence accession numbers

The 27 nt sequences of PRRSVs sequenced in the current study were deposited in GenBank (accession nos. MK359258-MK359284).

2.4. Sequence data analysis

In total, 73 PRRSV genomes were downloaded from the GenBank database, including 58 available full-length genomes of PRRSVs-1, one representative PRRSV-2 genome, and 14 representative PRRSV-1 ORF5 or ORF7 genes. Multiplex sequence alignments were generated using Muscle. The phylogenetic trees were constructed from aligned sequences by the neighbor-joining method with the maximum composite likelihood model in MEGA version 7 as previously reported (Yu et al., 2012). The robustness of the phylogenetic tree was evaluated by bootstrapping using 1000 replicates.

Sequence alignment was subjected to detection of homologous recombination using recombination detection program 4 (RDP4) (Martin et al., 2015). Seven methods embedded in RDP4 software package, including RDP, BootScan, GENECONV, Chimaera, Maxchi, SiScan, and 3Seq, were utilized to screen recombination events and breakpoints. The default settings were used for all methods, and the highest acceptable *P* value cut-off was set at 0.05 (Chen et al., 2013). The detected recombination events were further analyzed by SimPlot 3.5.1 software using Bootscan analysis to confirm the potential parental strains that were predicted by RDP4 software (Lole et al., 1999).

3. Results

3.1. Analysis of whole-genome sequences

To explore the genomic diversity of PRRSV-1 virus in swine herds in the USA, 23 type 1 PRRSV isolates and four clinical specimens positive for type 1 PRRSV were sequenced using NGS on an Illumina MiSeq platform. After using an in-house bioinformatics analysis pipeline, 27 nearly complete genomic sequences of PRRSV-1 viruses were obtained (Table S1). The complete genome nt homology of these 27 samples was 84.3–99.9%. Among these samples, EU03 and EU05; EU12 and EU17; EU13 and EU14; and EU07, EU08, and EU15 showed over 99% nt identity. Isolates showing high identity (over 99%) were collected from the same state and the same year with the exception of EU03 and EU05. The 27 samples had 88.4-91.3% nt identity to the PRRSV LV strain and 57.0-58.2% nt identity to the PRRSV VR-2332 strain, which further confirmed that all 27 samples belonged to PRRSV-1. The nt sequence identity varied for individual ORFs. ORF1a displayed 86.4–90.3% nt identity to the PRRSV-1 LV strain, whereas the identities of ORF1b and

Table 1
PRRSV strains used in this study.

No.	Virus strain (designation)	Geographic location	Isolation year	Accession no. (segment)
1	Lelystad virus	The Netherlands	1991	M96262 (complete genome)
2	Olot	Spain	1991	KF203132 (complete genome)
3	ESP-1991-Olot91	Denmark	1991	KC862570 (complete genome)
4	DK-1992-PRRS-111_92	Denmark	1992	KC862566 (complete genome)
5	DV	The Netherlands	1996	KF991509 (complete genome)
6	EuroPRRSV	USA	1999	AY366525 (complete genome)
7	MLV-DV	Spain	1999	KJ127878 (complete genome)
8	SD01-08	USA	2001	DQ489311 (complete genome)
9	01CB1	Thailand	2001	DQ864705 (complete genome)
10	DK-2003-6-5	Denmark	2003	KC862571 (complete genome)
11	DK-2003-7-2	Denmark	2003	KC862572 (complete genome)
12	SD03-15_P83	USA	2003	KU131560 (complete genome)
13	HK3	Hong Kong	2003	KF287129 (complete genome)
14	HK5	Hong Kong	2004	KF287130 (complete genome)
15	HK8	Hong Kong	2004	KF287128 (complete genome)
16	HK10	Hong Kong	2004	KF287131 (complete genome)
17	LV4.2.1	The Netherlands	2004	AY588319 (complete genome)
18	195-05	United Kingdom	2005	KU560579 (complete genome)
19	PRRS-FR-2005-29-24-1	France	2005	KY366411 (complete genome)
20	BJEU06-1[1]	China	2006	GU047344 (complete genome)
21	07V063	Belgium	2007	GU737264 (complete genome)
22	E38	South Korea	2007	KT033457 (complete genome)
23	KNU-07	South Korea	2007	FJ349261 (complete genome)
24	HKEU16	Hong Kong	2007	EU076704 (complete genome)
25	lena	Belarus	2007	JF802085 (complete genome)
26	DK-2008-10-5-2	Denmark	2008	KC862573 (complete genome)
27	GER09-613	Germany	2009	KT344816 (complete genome)
28	SHE	China	2009	GQ461593 (complete genome)
29	NMEU09-1	China	2009	GU047345 (complete genome)
30	SU1-Bel	Belarus	2010	KP889243 (complete genome)
31	DK-2010-10-10-3	Denmark	2010	KC862568 (complete genome)
32	14432/2011	Hungary	2011	KR296711 (complete genome)
33	DK-2011-05-23-9	Denmark	2011	KC862569 (complete genome)
34	DK-2011-05-11-14	Denmark	2011	KC862567 (complete genome)
35	GZ11-G1	China	2011	KF001144 (complete genome)
36	NVDC-FJ	China	2011	KC492506 (complete genome)
37	NVDC-NM1-2011	China	2011	JX187609 (complete genome)
38	NVDC-NM2	China	2011	KC492504 (complete genome)
39	NVDC-NM3	China	2011	KC492505 (complete genome)
40	9625/2012	Hungary	2012	KJ415276 (complete genome)
41	DK-2012-01-05-2	Denmark	2012	KC862574 (complete genome)
42	IVI-1173	Switzerland	2012	KX622783 (complete genome)
43	LNEU12	China	2012	KM196101 (complete genome)
44	13V091	Belgium	2013	KT159248 (complete genome)
45	13V117	Belgium	2013	KT159249 (complete genome)
46	AUT13-883	Austria	2013	KT326148 (complete genome)
47	FJEU13	China	2013	KP860912 (complete genome)
48	AUT14-440	Austria	2014	KT334375 (complete genome)
49	FJQEU14	China	2014	KP860913 (complete genome)
50	HLJB1	China	2014	KT224385 (complete genome)
51	Amervac	Spain	Vaccine	GU067771 (complete genome)
52	PRRS-FR-2014-56-11-1	France	2014	KY767026 (complete genome)
53	EuroViet-01	Vietnam	2016	MG251834 (complete genome)
54	EuroViet-02	Vietnam	2016	MG251833 (complete genome)
55	EuroViet-03	Vietnam	2016	MG251835 (complete genome)
56	15HEN1	China	2015	KX967492 (complete genome)
57	HENZMD-10	China	2016	KY363382 (complete genome)
58	CBNU0495	South Korea	2016	KY434183 (complete genome)
59	VR-2332	USA	1989	U87392 (complete genome)
60	Pyrsvac-187	Spain	Vaccine	DQ324681 (ORF5), DQ324712 (ORF7)
61	Porcilis	The Netherlands	Vaccine	DQ324678 (ORF5), DQ324710 (ORF7)
62	Sid	Lithuania	2000	DQ324682 (ORF5), AF438363 (ORF7)
63	Aus	Lithuania	2000	DQ324667 (ORF5), AF438362 (ORF7)
64	Sno-4	Belarus	2004	DQ324683 (ORF5), DQ324713 (ORF7)
65	Vas-2	Belarus	2005	DQ324689 (ORF5), DQ324722 (ORF7)
66	Zad-1	Belarus	2004	DQ324694 (ORF5), DQ324729 (ORF7)
67	Soz-6	Belarus	2004	DQ324686 (ORF5), DQ324719 (ORF7)
68	Vos	Belarus	2004	DQ324690 (ORF5), DQ324725 (ORF7)
69	Obu-1	Belarus	2005	DQ324676 (ORF5), DQ324707 (ORF7)
70	Okt-35	Belarus	2004	DQ324677 (ORF5)
71	PK	Belarus	2007	EU071229 (ORF5)
72	Okt-46	Belarus	2004	DQ324708 (ORF7)
73	Okt-47	Belarus	2004	DQ324709 (ORF7)
74	USA/ISU02609/2010 (EU01)	UT, USA	2010	MK359258 (complete genome)

(*continued on next page*)

Table 1 (*continued*)

No.	Virus strain (designation)	Geographic location	Isolation year	Accession no. (segment)
75	USA/ISU01969/2011 (EU02)	IA, USA	2011	MK359259 (complete genome)
76	USA/ISU00731/2012 (EU03)	AR, USA	2012	MK359260 (complete genome)
77	USA/ISU43354/2012 (EU04)	NC, USA	2012	MK359261 (complete genome)
78	USA/ISU11271/2013 (EU05)	IA, USA	2013	MK359262 (complete genome)
79	USA/ISU78021/2015 (EU06)	PA, USA	2015	MK359263 (complete genome)
80	USA/ISU37369/2017 (EU07)	NC, USA	2017	MK359264 (complete genome)
81	USA/ISU41597/2017 (EU08)	NC, USA	2017	MK359265 (complete genome)
82	USA/ISU58061/2017 (EU09)	UT, USA	2017	MK359266 (complete genome)
83	USA/ISU79359/2017(EU10)	MO, USA	2017	MK359267 (complete genome)
84	USA/ISU85615/2017 (EU11)	UT, USA	2017	MK359268 (complete genome)
85	USA/ISU38853/2017 (EU12)	NC, USA	2017	MK359269 (complete genome)
86	USA/ISU39201/2017 (EU13)	NC, USA	2017	MK359270 (complete genome)
87	USA/ISU44362/2017 (EU14)	NC, USA	2017	MK359271 (complete genome)
88	USA/ISU41593/2017 (EU15)	NC, USA	2017	MK359272 (complete genome)
89	USA/ISU50779/2017 (EU16)	UT, USA	2017	MK359273 (complete genome)
90	USA/ISU55989/2017 (EU17)	NC, USA	2017	MK359274 (complete genome)
91	USA/ISU57212/2017 (EU18)	UT, USA	2017	MK359275 (complete genome)
92	USA/ISU79610/2017 (EU19)	UT, USA	2017	MK359276 (complete genome)
93	USA/ISU81436/2017 (EU20)	NC, USA	2017	MK359277 (complete genome)
94	USA/ISU82347/2017 (EU21)	NC, USA	2017	MK359278 (complete genome)
95	USA/ISU86456/2017 (EU22)	NC, USA	2017	MK359279 (complete genome)
96	USA/ISU00669/2018 (EU23)	IN, USA	2018	MK359280 (complete genome)
97	USA/ISU21886/2018 (EU24)	NC, USA	2018	MK359281 (complete genome)
98	USA/ISU21775/2018 (EU25)	IA, USA	2018	MK359282 (complete genome)
99	USA/ISU20054/2018 (EU26)	IA, USA	2018	MK359283 (complete genome)
100	USA/ISU60533/2018 (EU27)	UT, USA	2018	MK359284 (complete genome)

ORF2-7 were 89.9–92.3% and 90.3–92.2%, respectively. Compared to LV strain, several deletions were identified in Nsp2, Nsp12, GP3, and GP4 and one insertion was detected in Nsp2. Nsp1β, Nsp2, GP3, GP4, and GP5 displayed less than 90% nt identity to the LV strain with Nsp2 displaying the highest diversity. These highly varied regions were therefore studied in detail (Table S2).

3.2. Amino acid alterations in nonstructural proteins

Most of the Nsps were well conserved; however, Nsp1β and Nsp2 were the most variable proteins, displaying 81.3-88.1% and 79.0-84.2% aa identity to the LV strain, respectively. Nsp1β aa alignment revealed that mutations were scattered throughout the protease domain. All samples from the USA contained a glutamine (Q) instead of an arginine (R) and an asparagine (N) instead of an aspartic acid (D) in the LV strain at positions 34 and 74, respectively. The epitope site ES1 in Nsp1β at aa positions 70–86 showed high variation (Fig. 1A).

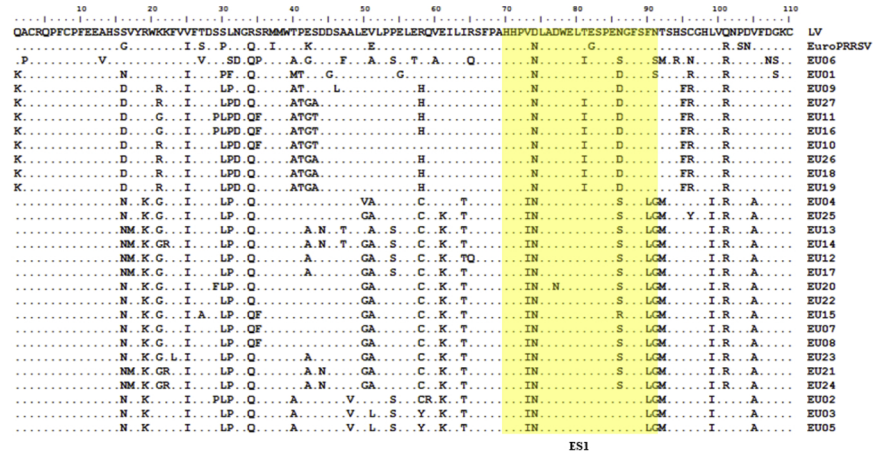
Most of the previously reported NA PRRSV-1 isolates had a distinct 17-aa deletion in Nsp2 at aa positions 349-365 in comparison to the LV strain. However, the aa alignment of 27 samples sequenced in this study demonstrated unexpected genetic diversity (Table 2). Only strain EU02 had the same 17-aa deletion at aa positions 349-365 as EuroPRRSV, the index strain of NA PRRSV-1 virus. When compared to the LV strain, besides the same 17-aa deletion, two strains (EU03 and EU05) had an additional deletion at aa positions 290-291, eleven strains (EU04, EU07-08, EU12-15, EU17, EU20, and EU22-23) contained an additional 5-aa deletion at aa positions 373-377, two strains (EU21 and EU24) had additional 5-aa deletion at aa position 373-377 and 6-aa deletion at aa position 341-346, and one strain (EU25) had additional 5-aa deletion at aa positions 373-377 and 3-aa deletion at aa positions 515-517 (Fig. 1B). In addition, nine strains (EU01, EU09-11, EU16, EU18-19, and EU26-27) contained a 13-aa deletion at aa positions 353-365 instead of the marked 17-aa deletion, a 5-aa deletion at aa positions 290-294, a 1-aa deletion at aa position 324, and a unique 1-aa insertion of leucine at position 378 (Fig. 1B). Notably, the isolate EU06 had unique deletions of 2 aa at positions 301-302 and 2 aa at positions 698-699, including deletion of two prolines, which had never been reported previously. Most of the deletions were located upstream of ES3 or between ES3 and ES4 (Fig. 1B).

NSP12 is highly conserved among all viral proteins. All 27 samples showed 92.1-96.7% aa identity to the LV strain. However, 14 samples (EU04, EU07, EU08, EU12-15, EU17, and EU20-25) displayed notable diversity at the end of the protein, particularly a 3-aa C-terminal truncation (Fig. 1C; Table 2), which was not found in previous reports.

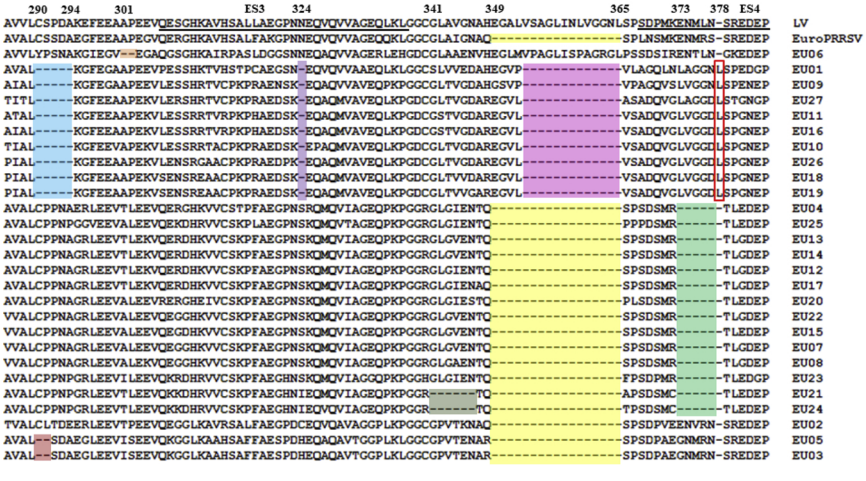
3.3. Genetic diversity analysis of structural proteins

ORF2a-7 encode viral structural proteins. Sequence alignment of this region showed that all 27 strains shared 90.3–92.2% nt identity and 80.8–99.2% aa identity to the LV strain. Among all structural proteins, GP3 was the most variable protein in this study and the GP3 in all 27 strains showed aa identity 80.8-89.8% to the LV strain, the lowest level of aa identity in all of the structural proteins. It was previously reported that there were mutational hotspots at aa positions 237-252 of GP3 and aa positions 60-67 of GP4 in the overlapping regions of GP3 and GP4 (Ropp et al., 2004). Sixteen strains had a premature stop codon resulting in a truncation from the C terminus. Among them, two strains (EU03 and EU05) had a 21-aa truncation and 14 strains (EU04, EU07-08, EU12-15, EU17, and EU20-25) had an 18-aa truncation (Fig. 2A and Table 2). The strain EU16 had two1-aa deletions: one was located at aa position 248 of GP3 (Fig. 2A) and the other located at aa position 66 of GP4 (Fig. 2B). However, seven potential N-glycosylation sites in GP3 were not affected.

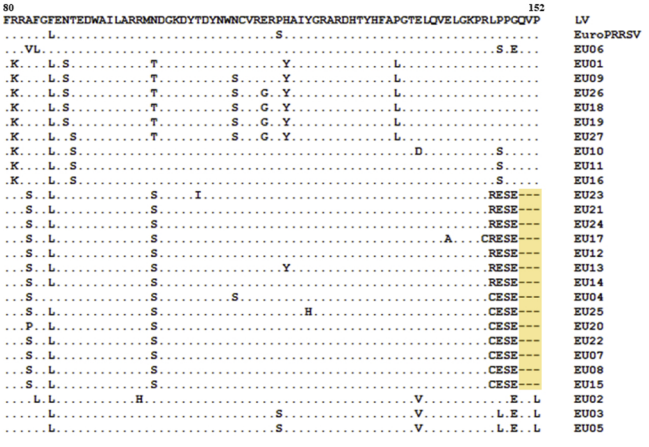
The GP5 protein shared 86.6–91.0% amino acid identity to the LV strain. In this study, most of the aa mutations were clustered in three hypervariable regions (Fig. 3). The two glycosylation sites of LV strain,



A



B



C

Fig. 1. The alignment of non-structural proteins aa sequences of PRRSV-1 strains. (A) Alignment of the partial Nsp1β aa sequences of PRRSV-1 strains. The B-cell epitope sites identified previously are shaded in yellow (Oleksiewicz et al., 2001). (B) Alignment of the partial Nsp2 aa sequences of PRRSV-1 strains. A 1-aa insertion at position 378 in Nsp2 is shown in the red box. The deletions are shaded in color. Underlined regions show B-cell epitope sites (ES) identified by Oleksiewicz et al. (2002). (C) Alignment of the partial Nsp12 aa sequences of PRRSV-1 strains. The 3-aa C-terminal truncation is shaded in orange.

Table 2
The deletion/insertion in Nsp 2, 3'end truncation in Nsp12, and GP3 of all virus strains in the present study relative to LV.

Strain	State	Year	Position of amino acid deletion in Nsp2									Position of amino acid insertion in Nsp2	3' end tuncation in Nsp12	3' end tuncation in GP3
			290-291	290-294	301-302	324	341-346	349-365	353-365	373-377	515-517	698-699	378		
EU01	UT	2010		5 aa		1 aa			13 aa				1 aa		
EU02	IA	2011						17 aa							
EU03	AR	2012	2 aa					17 aa							21 aa
EU04	NC	2012						17 aa		5 aa				3 aa	18 aa
EU05	IA	2013	2 aa					17 aa							21 aa
EU06	PA	2015			2 aa							2 aa			
EU07	NC	2017						17 aa		5 aa				3 aa	18 aa
EU08	NC	2017						17 aa		5 aa				3 aa	18 aa
EU09	UT	2017		5 aa		1 aa			13 aa				1 aa		
EU10	MO	2017		5 aa		1 aa			13 aa				1 aa		
EU11	UT	2017		5 aa		1 aa			13 aa				1 aa		
EU12	NC	2017						17 aa		5 aa				3 aa	18 aa
EU13	NC	2017						17 aa		5 aa				3 aa	18 aa
EU14	NC	2017						17 aa		5 aa				3 aa	18 aa
EU15	NC	2017						17 aa		5 aa				3 aa	18 aa
EU16	UT	2017		5 aa		1-aa			13 aa				1 aa		
EU17	NC	2017						17 aa		5 aa				3 aa	18 aa
EU18	UT	2017		5 aa		1 aa			13 aa				1 aa		
EU19	UT	2017		5 aa		1 aa			13 aa				1 aa		
EU20	NC	2017						17 aa		5 aa				3 aa	18 aa
EU21	NC	2017					5 aa	17 aa		5 aa				3 aa	18 aa
EU22	NC	2017						17 aa		5 aa				3 aa	18 aa
EU23	IN	2018						17 aa		5 aa				3 aa	18 aa
EU24	NC	2018					5 aa	17 aa		5 aa				3 aa	18 aa
EU25	IA	2018						17 aa		5 aa	3 aa			3 aa	18 aa
EU26	IA	2018		5 aa		1 aa			13 aa				1 aa		
EU27	UT	2018		5 aa		1 aa			13 aa				1 aa		
EuroPRRSV	-	1999						17 aa							

46-NLT and 53-NGT, which were generally conserved in all PRRSV-1 and PRRSV-2 isolates, were unaffected, with the exception of the isolate EU23, which lost a potential glycosylation site as a result of an N-to-D mutation at aa 46. In addition, all samples acquired one or two new potential N glycosylation sites at aa positions 37 and 38 with the exception of the isolate EU06.

3.4. Phylogenetic analysis

The phylogenetic tree of whole-genome sequences showed that all 27 samples sequenced in this study belonged to PRRSV-1 and clustered together with the other three NA PRRSV-1 strains (EuroPRRSV, SD01-08, and SD03-15) reported previously. All 27 samples could be resolved into four subgroups, designated groups A-D. Each group contained viruses from geographically different regions. The isolate EU06 was well separated from other groups and occupied a basal position on the tree (Fig. 4A).

To further understand the genetic variation and evolution of NA PRRSV-1, Nsp2-, ORF5-, and ORF7-based phylogenetic trees were also constructed. Nsp2 is the most divergent protein of the nonstructural proteins. The phylogenetic tree of Nsp2 showed that group D and isolate EU06 were separated from other USA groups and formed a single group (Fig. 4B). ORF5, coding for the major envelope glycoprotein, is the most divergent protein of the structural proteins and is often used to study the diversity and evolution of PRRSV. Four subtypes were defined based on ORF5 sequence divergence (Stadejek et al., 2008). The ORF5-based phylogenetic tree showed that all US strains belonged to subtype 1 and clustered together, which was similar with the tree based on the full-length genome (Fig. 5A). According to the size and polymorphism of N protein, PRRSV-1 was divided into three subtypes: a pan-European subtype 1 and East European subtypes 2 and 3 with N protein sizes of 128, 125, and 124 aa, respectively (Stadejek et al., 2008). All PRRSV-1 strains in the USA contained a 128-aa ORF7 and belonged to pan-European subtype 1. However, the 27 PRRSVs separated from each other and scattered in the N protein tree, which was slightly different from the full-length genome- or ORF5-based phylogenetic tree (Fig. 5B). In order to detect potential recombination among NA PRRSV-1 strains, RDP4 and SimPlot analyses were performed based on the alignment of all available NA PRRSV-1 strains. However, no obvious recombination event was detected.

4. Discussion

Since the first full-length genome of NA PRRSV-1 strain was reported in 2004 (Ropp et al., 2004), few reports have described the genomic characteristics and diversity of NA PRRSV-1 viruses (Fang et al., 2004; Fang et al., 2007). In this study, 27 PRRSV-1 PCR-positive isolates and clinical samples collected from seven farms in the United States between 2010 and 2018 were sequenced using NGS followed by sequence analysis. The results identified several novel genetic

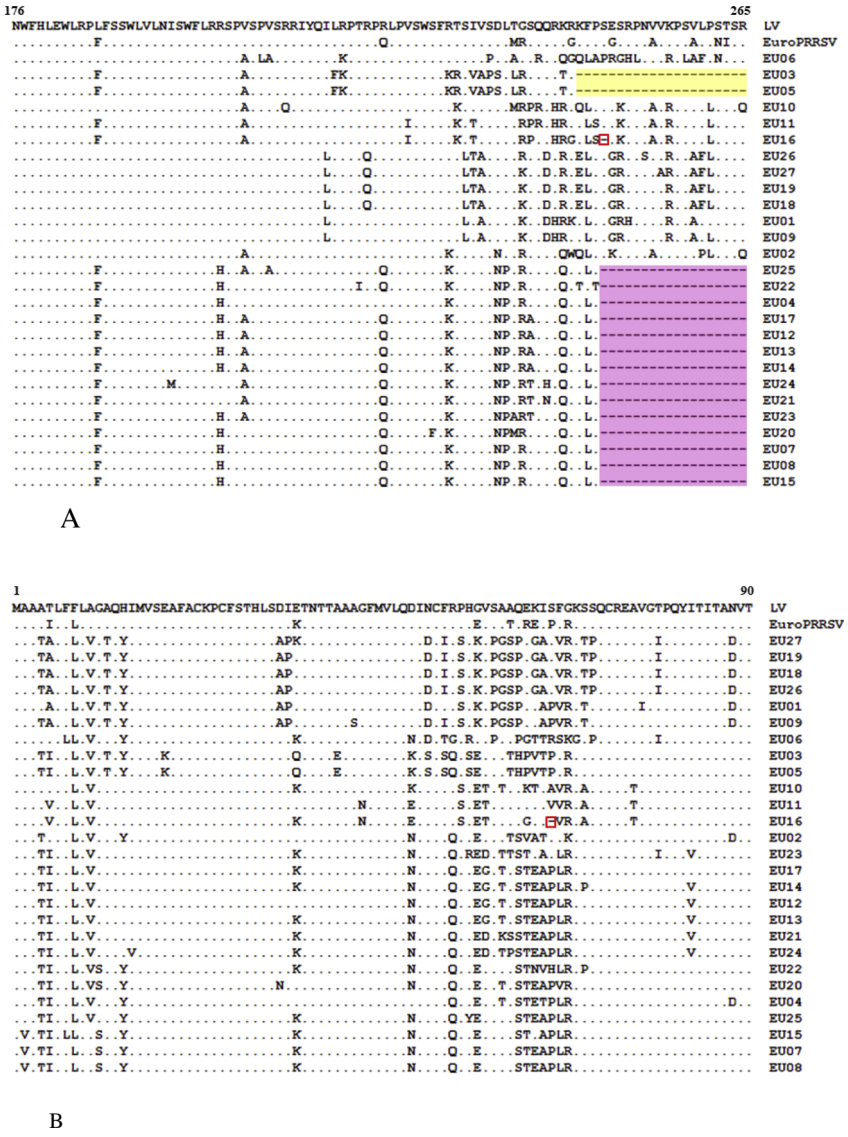


Fig. 2. The alignment of structural proteins aa sequences of PRRSV-1 strains. (A) Alignment of the partial GP3 aa sequences of PRRSV-1 strains. The 1-aa deletion is shown in a red box. The 21-and 18-aa C-terminal truncation are shaded in yellow or purple, respectively. (B) Alignment of the partial GP4 aa sequences of PRRSV-1 strains. The 1-aa deletion is shown in a red box.

characteristics and diversity, indicating that PRRSV-1 virus currently circulating in the USA had undergone gradual variation.

The full-length sequences of the 27 PRRSV-1 viruses from 2010 to 2018 in the current study showed 88.4-91.3% nucleotide identity to the LV strain. In contrast, three previously reported NA PRRSV-1 strains including EuroPRRSV isolated in 1999, SD01-08 in 2001, and SD03-15 in 2003 demonstrated much higher genetic identities to LV strain with 95.3%, 93.7%, and 93.5%, respectively (Fang et al., 2004; Fang et al., 2007; Ropp et al., 2004). The trend of decreasing PRRSV-1 nt identity observed in the current study suggests that the viruses accumulated substantial divergence after undergoing a 20-year interval of evolution. It was previously reported that Nsp2 was the most variable protein with deletions and insertions and had a 17-aa deletion serving as a genetic marker for NA PRRSV-1 viruses (Music and Gagnon, 2010). Interestingly, for all 27 PRRSVs in the current study, only one isolate (EU02) had the same 17-aa deletion as EuroPRRSV and 16 strains contained not only the 17-aa deletion but also other deletion(s). Whereas, nine strains showed a 13-aa deletion, a 5-aa deletion, a 1-aa deletion, and a new 1-aa insertion (Table 2), which had never been reported in NA PRRSV-1 isolates. In addition, one strain (EU06) had no 17-aa deletion at position 349-365, but two 2-aa deletions at positions 301-302 and 698-699. These data suggest that the 17-aa deletion in Nsp2 is no longer a consistent genetic marker for contemporary NA PRRSV-1 viruses and the presence of other hyper-variable deletions and insertions should be considered in Nsp2. Several linear B-cell epitopes have previously been identified in Nsp2 and the variability of peptide sequence in these epitope sites has been correlated with their antigenicity (Oleksiewicz et al., 2001). Less conserved epitopes in Nsp2

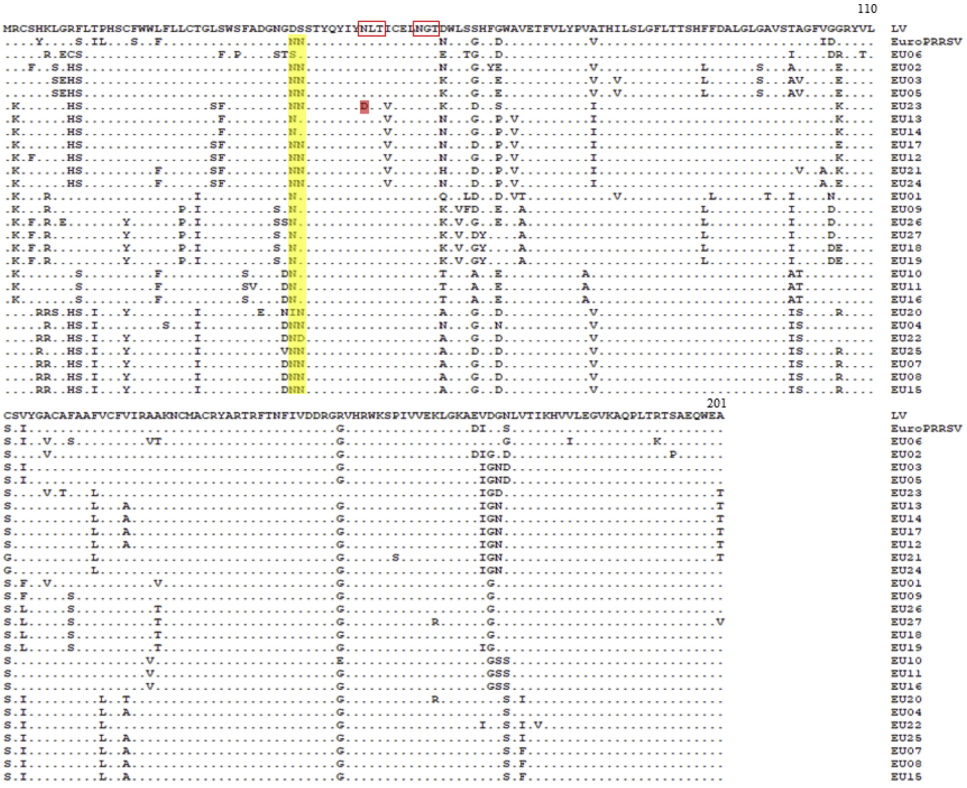


Fig. 3. Alignment of GP5 aa sequences of PRRSV-1 strains. The mutations at positions 37 and 38 are shaded in yellow. The mutation at position 46 (N46 to D46) is shaded in red. The N-glycosylation sites in GP5 are indicated in red boxes.

induced higher level of antibody response, while more conserved ones induced lower level responses (Wang et al., 2013). We found that most of the deletions in Nsp2 were located immediately upstream of epitope sites 3 or 4, which may alter the conformation of both epitopes. Whether these hypervariable epitopes within Nsp2 function as a decoy and redirect the immune response away from a conserved neutralizing epitope remain unknown. In addition, Nsp12, a well-conserved non-structural protein, exhibited unexpected diversity at the 3′ end in our study. It was recently reported that Nsp12 recruits cellular proteins, such as heat-shock protein 70, to maintain its own stability and is involved in virus replication (Dong et al., 2016). Further studies are required to determine the biological significance of the diversity at the 3' end of Nsp12.

GP3 protein is highly glycosylated and possesses seven well conserved potential N-linked oligosaccharides (Music and Gagnon, 2010). Consistent with previous studies, epitope site 11 located in the N-terminus of GP3 was relatively conserved, whereas epitope site 12 located in the overlapping region of GP3 and GP4 showed high divergence (Costers et al., 2010; Ropp et al., 2004). Importantly, ORF3 was truncated by a premature stop codon in 16 strains with either 18- or 21-aa truncation. The 18-aa truncation at the C terminus was previously reported in PRRSV-1 in North America (Ropp et al. (2004)) and also in Europe (Forsberg et al., 2001). To our best knowledge, our study is the first one that reports the 21-aa truncation at the C terminus of GP3 in PRRSV-1 viruses. One epitope of GP3 was found in the truncated region (Oleksiewicz et al., 2001) and C-terminus antigenicity of GP3 varied greatly among the different PRRSV-1 isolates. It remains unknown whether these truncations modify the antigenicity of GP3.

GP5, an important virus structural protein exposed on the surface of the virion, is one of the most variable proteins in PRRSVs. It is critical in cell recognition and binding, and virus neutralization (Music and Gagnon, 2010) and glycosylation of GP5 plays an important role in host immune escape by an N-glycan-shielding mechanism (Diaz et al., 2009). Almost all 27 strains acquired one or two N-glycosylation site(s) at positions 37 and (or) 38 with the exception for EU06, which was similar with previous reports. Glycosylation sites 46-NLT and 53-NGT are highly conserved in almost all PRRS -1 and PRRSV-2 viruses (Ansari et al., 2006) with very rare exceptions. It serves as a genetic switch to adjust immune system interactions in the host and is important for infectious virion production (Stadejek et al., 2006; Wissink et al., 2004). In our study, 26 out of 27 NA PRRSV-1 viruses were conserved in 46-N glycosylation site. The only one exception was the isolate EU2 from Indiana, which displayed a mutation from N to D at the N-46 glycosylation site. Variability in the highly conserved N-46 glycosylation site was first reported in the Belarusian PRRSV strains (Chen et al., 2000; Mateu et al., 2006) and a similar mutation was then reported in NA PRRSV-1 isolate, in which a glycosylation site was lost as a result of an N-to-D mutation at aa 46 (Ropp et al., 2004).

The phylogenetic analysis based on the full-length genome showed that all NA PRRSV-1 isolates clustered together and formed a monophyletic group. This result supports the notion that there was limited introduction of PRRSV isolates from Europe (Fang et al., 2007; Ropp et al., 2004). The US PRRSV-1 isolates could be divided into four subgroups. The three previously reported isolates EuroPRRSV, SD01-08,

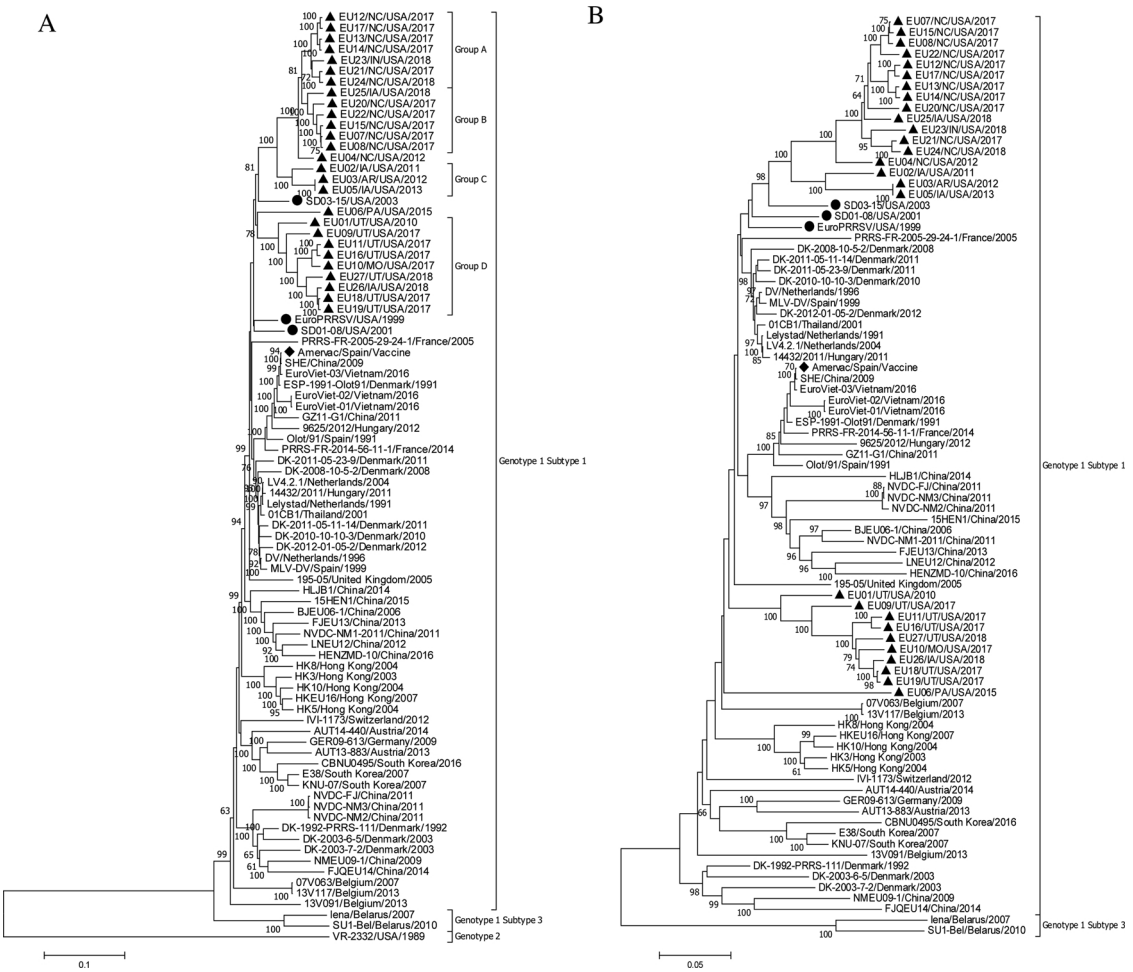


Fig. 4. Phylogenetic analysis of the nt sequences of the full-length genome (A) and Nsp2 (B) of PRRSV strains. The phylogenetic trees were constructed by the neighbor-joining method in MEGA 7.0. Bootstrap values from 1000 replicates are indicated for each node. Each isolate is presented by the isolate name, country, and isolation year. The 27 isolates in this study were designated EU01-27. The isolates from this study are indicated by dark triangle symbols. The other three NA PRRSV-1 strains are marked by black dot symbols. The vaccine strain is marked by a black diamond symbol.

and SD03-15 together with the isolate EU06, could not be placed into any subgroup. Each subgroup contained viruses from geographically different regions, while all isolates from Utah clustered within subgroup D. Thus, the virus diversity showed certain geographic characteristics, which might be a result of interstate transmission of pigs. Combining the fact that NA PRRSV-1 strains EuroPRRSV, SD01-08, and SD03-15 isolated before 2003 had a higher nucleotide identity to the LV strain compared to all isolates studied in this report, our results suggest the genetic diversification and evolution of PRRSV-1 in the USA appear to be geographical and temporal.

The Nsp2-based phylogenetic analysis showed that strain EU06 and subgroup D separated from other NA PRRSV-1 viruses and formed a single group. The Nsp2 alignment demonstrated that subgroup D viruses contained a 5-aa deletion at positions 290-294, a 13-aa deletion at positions 353–365, and a 1-aa insertion at position 378. Moreover, the EU06 strain contained two 2-aa deletions at positions 301-302 and 699-700, whereas all other viruses contained a 17-aa deletion, a well-known marker deletion in NA PPRSV-1 viruses (Fang et al., 2004). The results of Nsp2-based phylogenetic analysis together with Nsp2 alignment suggested that the *Nsp2* gene in NA PRRSV-1 underwent a hyperdivergent evolution and the 17-aa deletion is no longer a consistent marker for contemporary NA PRRSV-1 viruses. Additionally, the results of ORF5- and ORF7-based phylogenetic analyses suggests that all NA PRRSV-1 belong to pan-Europe subtype 1, consistent with previously reported results (Ropp et al., 2004; Shi et al., 2010).

Although PRRSV-1 viruses have been extensively studied in Europe, there have been very few studies describing the genetic diversity and evolution of PRRSV-1 in the United States in the last decade. Our study, though with a limited number of strains, still clearly showed that PRRSV-1 viruses in the USA had undergone gradual variations and exhibited greater genetic divergence than previously thought, making the prevention and control of PRRSVs in the USA more complicated. To further understand the PRRSV-1 epidemiology and evolution of PRRSV-1, with the eventual goal to improve the prevention and control of PRRSV-1 infection in the United States, more comprehensive studies including more strains from different geographical regions and different times are required in the future.

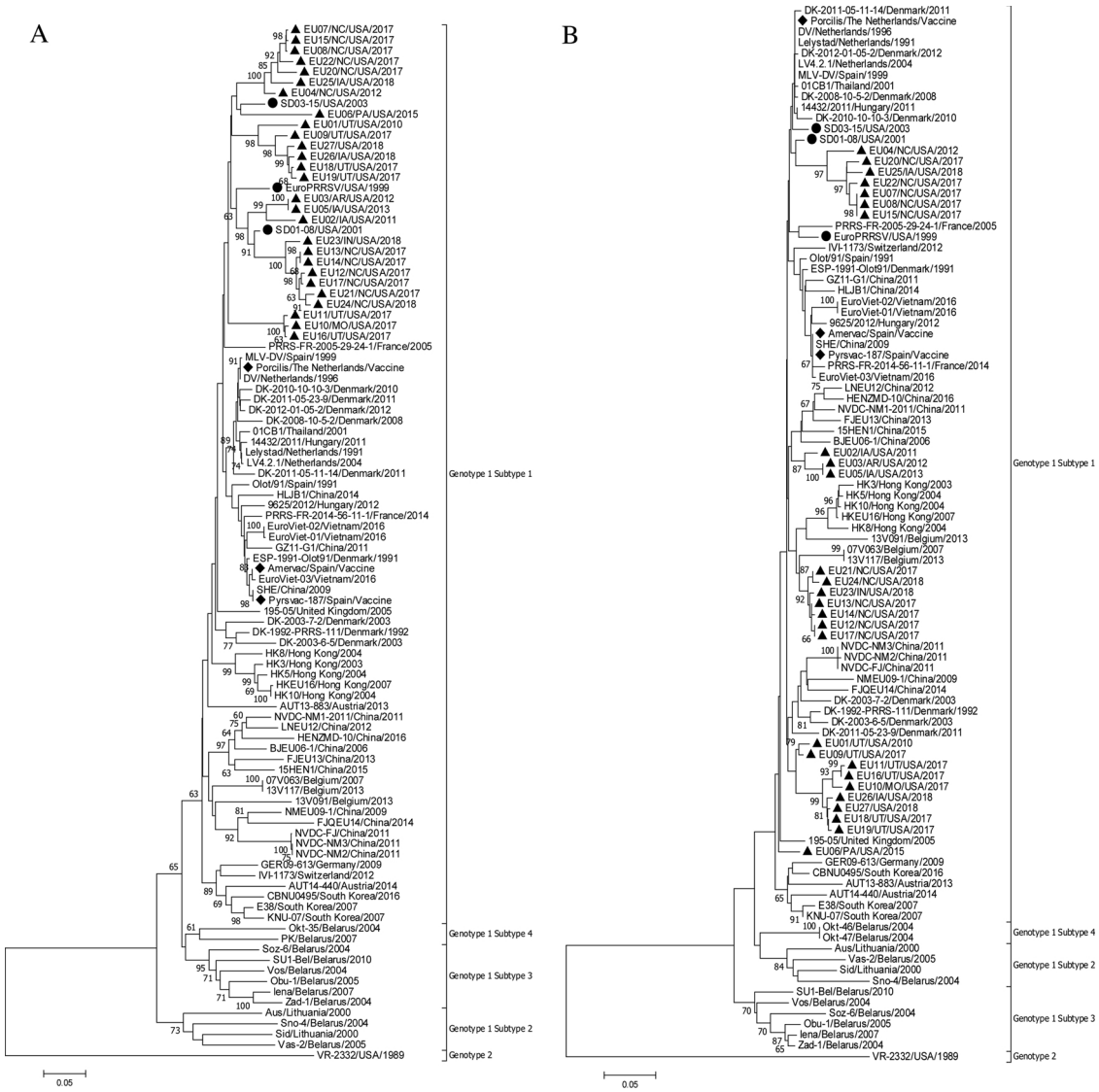


Fig. 5. Phylogenetic analysis of the nt sequences of ORF5 (A) and ORF7 (B) of PRRSV strains. The phylogenetic trees were constructed by the neighbor-joining method in MEGA 7.0. Bootstrap values from 1000 replicates are indicated for each node. Each isolate is presented by the isolate name, country, and isolation year. The 27 isolates in this study were designated EU01-27. The isolates from this study are indicated by dark triangle symbols. The other three NA PRRSV-1 strains are marked by black dot symbols. The vaccine strains are marked by black diamond symbols.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108486.

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