

Molecular epidemiology of porcine reproductive and respiratory syndrome viruses isolated from 1991 to 2013 in Taiwan

Ming-Chung Deng¹ · Chia-Yi Chang¹ · Tien-Shine Huang¹ · Hsiang-Jung Tsai^{1,2} · Chieh Chang¹ · Fun-In Wang² · Yu-Liang Huang¹

Received: 22 April 2015 / Accepted: 17 July 2015 / Published online: 6 August 2015
© Springer-Verlag Wien 2015

Abstract Porcine reproductive and respiratory syndrome virus (PRRSV) was first identified in Taiwan in 1991, but the genetic diversity and evolution of PRRSV has not been thoroughly investigated over the past 20 years. The aim of this study was to bridge the gap in understanding of its molecular epidemiology. A total of 31 PRRSV strains were collected and sequenced. The sequences were aligned using the MUSCLE program, and phylogenetic analysis were performed by the maximum-likelihood method and the neighbor-joining method using MEGA 5.2 software. In the early 1990s, two prototype strains, WSV and MD001 of the North American genotype, were first identified. Over the years, both viruses evolved separately. The population dynamics of PRRSV revealed that the strains of the MD001 group were predominant in Taiwan. Evolution was manifested in changes in the nsp2 and ORF5 genes. In addition, a suspected newly invading exotic strain was recovered in 2013, suggesting that international spread is still taking place and that it is affecting the population dynamics. Overall, the results provide an important basis for vaccine development for the control and prevention of PRRS.

Introduction

Porcine reproductive and respiratory syndrome (PRRS), an economically devastating disease in swine production worldwide, is characterized by reproductive failure in sows and respiratory disease in pigs of all ages. The causative agent, PRRS virus (PRRSV), is an enveloped, single-stranded positive-sense RNA virus belonging to the order *Nidovirales*, family *Arteriviridae*, and genus *Arterivirus* [46].

The genome of PRRSV is approximately 15 kb in length [25] and contains at least 10 open reading frames (ORFs) [5, 13, 37, 41]. ORF1, which is divided into ORFs 1a and 1b, comprises almost 75 % of the genome, encoding non-structural proteins (nsps) for viral replication. ORF2a, 2b, 3, 4, 5a, 5, 6, and 7 encode seven structural proteins GP2, E, GP3, GP4, GP5, M and N, respectively. ORF5, encoding GP5, is one of the most variable regions of the PRRSV genome and is thus often used for phylogenetic analysis [11, 31, 42, 44]. In addition, nsp2 is considered an important region for studying the genetic variation and epidemiological evolution of PRRSV [4, 14].

Phylogenetically, PRRSV is divided into two distinct genotypes, the European (EU) type (type 1) and the North American (NA) type (type 2) [26, 29]. Viruses of both genotypes have significant genetic diversity, with only approximately 60 % nucleotide sequence identity [1, 29]. Since 2006, a highly pathogenic PRRSV (HP-PRRSV) of the NA type, characterized by causing high fever and high death rates in pigs of all ages, has emerged in China [20, 39].

In Taiwan, the first outbreak of PRRS was recorded in 1991. Although modified live vaccine (MLV) has been used on pig farms since the late 1990s, sporadic outbreaks continue to occur today. To further understand the genetic diversity and evolution of PRRSV in Taiwan, PRRSV strains collected from 1991 to 2013 were sequenced and analyzed.

✉ Chia-Yi Chang
cychang@mail.nvri.gov.tw

✉ Yu-Liang Huang
ylhuang@mail.nvri.gov.tw

¹ Animal Health Research Institute, Council of Agriculture, 376 Chung-Cheng Road, Tansui, New Taipei 25158, Taiwan

² School of Veterinary Medicine, National Taiwan University, No. 1, Section 4, Roosevelt Road, Taipei 10617, Taiwan

Materials and methods

Virus isolates

A total of 31 PRRSV strains (Table 1) from the lung and lymphoid tissues of pigs with porcine respiratory disease complex (PRDC) during 1991 to 2013 were isolated in MARC-145 cells, and these were all of the available isolates used in the analyses. These isolates were propagated in MARC-145 cells maintained in minimum essential medium supplemented with 5% fetal bovine serum and incubated at 37 °C in 5 % CO₂.

RNA extraction, reverse transcription PCR (RT-PCR) and nucleotide sequencing

The genomic RNAs of PRRSV isolates were extracted from 140 µl of the infected cell culture supernatant using a QIAamp[®] Viral RNA Mini Kit (QIAGEN) according to the manufacturer's recommendations. For amplification of the PRRSV genes, viral RNAs were reverse transcribed and amplified by one-step RT-PCR. The complete coding region of each PRRSV genome was sequenced and analyzed by fifteen reactions of RT-PCR (Table 2). The total reaction volume of 25 µl contained 2.5 µl of extracted

Table 1 PRRSV strains isolated from field outbreaks in Taiwan between 1991 and 2013

Strain	County	Year of isolation	CDS ^a lineage	ORF5 ^b lineages	nsp2 ^c type	Accession number
MD001	.. ^d	1991	VI	V	A	KP998431
WSV	Changhua	1992	I	I	V	KP998429
TD-1	Taitung	1997	VI	V	A	KP998425
TD/TP	Taitung	1998	VIII	VI	A	KP998422
Tsai	-	1999	VI	V	A	KP998423
TY1	Taoyuan	2000	I	V	V	KP998424
NT	Nantou	2000	I	V	V	KP998420
M1	-	2001	VIII	VI	V	KP998419
TY	Taoyuan	2002	I	I	B	KP998428
CH	Changhua	2002	VII	VI	D	KP998416
CH8V-J2	Changhua	2003	IX	VI	A	KP998426
TD-2	Taitung	2004	IX	VI	D	KP998427
HL	Hualien	2004	VIII	VI	D	KP998418
Q94-136	-	2005	VIII	VI	D	KP998421
CW46	Tainan	2005	I	I	B + C	KP998405
47-2	Tainan	2005	VIII	VI	A	KP998401
310	Changhua	2005	VII	VI	D	KP998413
312	Changhua	2005	VII	VI	D	KP998402
71-1	Nantou	2008	IX	VI	D	KP998412
660	-	2009	IX	VI	D	KP998414
338	Taoyuan	2011	IX	VI	D	KP998404
JM	Yunlin	2011	IX	VI	D	KP998410
PD119	Pingtung	2011	I	I	B	KP998411
HC120629	Changhua	2012	IX	VI	D	KP998430
1483	Nantou	2012	IX	VI	D	KP998403
HC120821-SH1	Yunlin	2012	IX	VI	D	KP998406
HC120821-SH2	Yunlin	2012	IX	VI	D	KP998407
HC120821-LL	Pingtung	2012	IX	VI	D	KP998408
HC120904-CHYL	Changhua	2012	IX	VI	D	KP998409
803	Nantou	2013	IX	VI	D	KP998415
319	Miaoli	2013	I	I	D	KP998417

^a CDS = complete coding sequence = complete coding region. Refer also to Fig. 1

^b ORF = open reading frame. Refer also to Fig. 2

^c nsp2 = nonstructural protein 2. Refer also to Fig. 4

^d Unknown

nucleic acids, 1X DNA polymerase buffer, 4 units of recombinant RNase inhibitor (Promega, Madison, WI, USA), 1 unit of AMV reverse transcriptase (Promega), 1 unit of GoTaq[®] Flexi DNA polymerase (Promega), 0.2 μM deoxyNTP mixture, and 0.4 μM each primer. Cycling was carried out in a 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) as follows: 1 cycle of RT at 42 °C for 50 min and 94 °C for 3 min to inactivate transcriptase, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 45 s, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min before storage at 4 °C. Sizes of amplicons were verified by agarose gel electrophoresis in TAE buffer using known standards. The RT-PCR products were purified using a

QIAquick[®] PCR Purification Kit (QIAGEN) and sequenced using a 3700 XL DNA Analyzer and a BigDye[®] Terminator v3.1 Cycle Sequencing Kit.

Phylogenetic analysis

The nucleotide (nt) sequences of the complete coding region (marked as CDS), ORF5, and nsp2 genes from the 31 Taiwan strains were compared with each other and with PRRSV reference strains obtained from GenBank, National Center for Biotechnology Information. Virus sequences were aligned by MUSCLE in the MEGA 5.2 software. Pairwise sequence alignments were also performed with the MEGA 5.2 software to determine nt sequence similarities.

Table 2 Primers used to amplify different genes of PRRSV strains

Primer	Nucleotide sequence (5'-3') ^a	Position ^b (nucleotides)
F1	ATGA CGTA TAGG TGTT GGCT C	1-21
R1	GCCT CCGC TGTA GGTA CTTG	1120-1100
F2	TGTC CCWG AASGMAAC TGCT GG	984-1004
R2	CAAC ATYC CAAT YAAA GGAG GTGT C	2211-2187
F3	GARG AGGT CGCR GCAA AGAT TG	2089-2109
R3	CCGC CHGA GTCR ATGA TGGC TTG	3282-3260
F4	GTGT CATC AAGC AGCT CSYT GTC	3203-3225
R4	TTAC ACCT ACCT TGAG AAAG CAC	4485-4463
F5-1	CTGC TTGT GAGT TTGG ACTC GCC	4254-4275
F5-2	AGTT TGAC TCGC CAGA GTGT AG	4263-4284
R5	ACCA RCAA GGAA ACAC ACAA AGCA	5428-5405
F6	TCTY TGCA CGTC YAGA TTGT GCAT	5193-5217
R6	ATCA GRAC TTGC GCAG ACCA TG	6539-6518
F7	CTGG AAGG AGGY CTYT CCAC	6337-6356
R7	YTGG AAGG AGGY CTYT CCAC	7497-7477
F8	CATT GAGA CYAG RGTC CTTG C	7233-7259
R8	CTGT TTAG GGCA GTCA GGCC TAG	8499-8477
F9	ACTC CGGT GACY GATG GGCG CTC	8374-8396
R9	ACAR GCAC AGCT GTCC ATGA GTAT	9603-9580
F10	GACC TYGT GCTG TATG CCGA GTC	9334-9356
R10	TTCT TGCC AGGA CACC AACC G	10398-10377
F11-1	CCAA CYCA CCAG ACCA TGCT	10249-10268
F11-2	ARGG CTTT GGGG ACGT GCCG	10279-10298
R11	TCAA GRTC TGGG AGGT ACAC	11618-11596
F12	TATC TTGA TGAY CGGG AGCG AG	11401-11422
R12	AART GAGC CACC ACAT CCAA AC	12634-12615
F13	CARG CTGC CTGG AAAC AGGT GG	13561-12582
R13	AGRA TGGC AAAA AGAC AGGC TAAA AC	13858-13883
F14	TGGC AATG TGTC AGGC ATCG TG	13713-13734
R14	GGCC GGAC GACA AATG CGTG G	14904-14884
F15	TCGC GCTC ACTA TGGG AGCA G	14705-14725
R15	TTTT TTTT TTTT TTAA TTAC GGCC GCATG	Poly A tail

^a Primers were designed based on the alignment of multiple PRRSV sequences

^b The positions of primers are as located in the VR2332 strain (U87392)

Phylogenetic analysis of the aligned sequences was performed by the maximum-likelihood method and the neighbor-joining method, and by bootstrap analysis with 1,000 replicates using the MEGA 5.2 software [38]. The classification of lineages was based on the genetic distance, with sequences diverging more than 10 % from neighboring lineages divided into separate lineages [31].

Comparison and analysis of amino acid sequences of the GP5 protein

Amino acid sequences of GP5 proteins were compared by aligning all 31 sequences, using MUSCLE in the MEGA 5.2 software. The percentages of identity between the strains were calculated.

Analysis of selection pressure in the ORF5 gene

The selection pressure and evolution rate are important indicators of evolutionary biology [15]. To study the evolutionary biology of Taiwan PRRSV strains, the intensities of selection pressure and evolution in the ORF5 gene were analyzed with the MEGA 5.2 software using the rates of non-synonymous substitution (dN) and synonymous substitution (dS) [28]. Ratio values equal to 1 indicate neutral evolution; <1, negative selection; and >1, positive selection. The comparisons between the WSV and MD001 groups were analyzed by Student's *t*-test. Statistical analysis was carried out using the Statistical Analysis System (SAS for Windows 6.12; SAS Institute Inc). A *P*-value <0.05 was considered statistically significant.

Comparison and analysis of amino acid sequences of nsp2 protein

Deletions and insertions in the nsp2 protein are important biomarkers for the highly pathogenic PRRSV in China. Therefore, the amino acid sequences of the nsp2 proteins of all 31 strains were aligned using MUSCLE in the MEGA 5.2 software. Based on the VR2332 reference strain, the divergence distance and deletion/insertion patterns between the isolates were analyzed.

Results

Nucleotide sequences and phylogenetic analysis of the complete coding region

To investigate the genetic diversity and evolution of PRRSV isolates in Taiwan, the complete coding regions (marked as CDS in Fig. 1) of 31 PRRSV strains collected

during the years 1991 to 2013 were analyzed. Phylogenetic analysis indicated that all Taiwan strains belonged to the NA genotype and could be further divided into a prototype WSV strain group of lineage I and a prototype MD001 strain group of lineage VI. The strains of the WSV group all clustered in lineage I and were collected from 1992 to 2013. The strains of the MD001 group were more evenly distributed in lineages VI to IX and were also collected from 1991 to 2013 (Fig. 1). Of the 11 recent strains

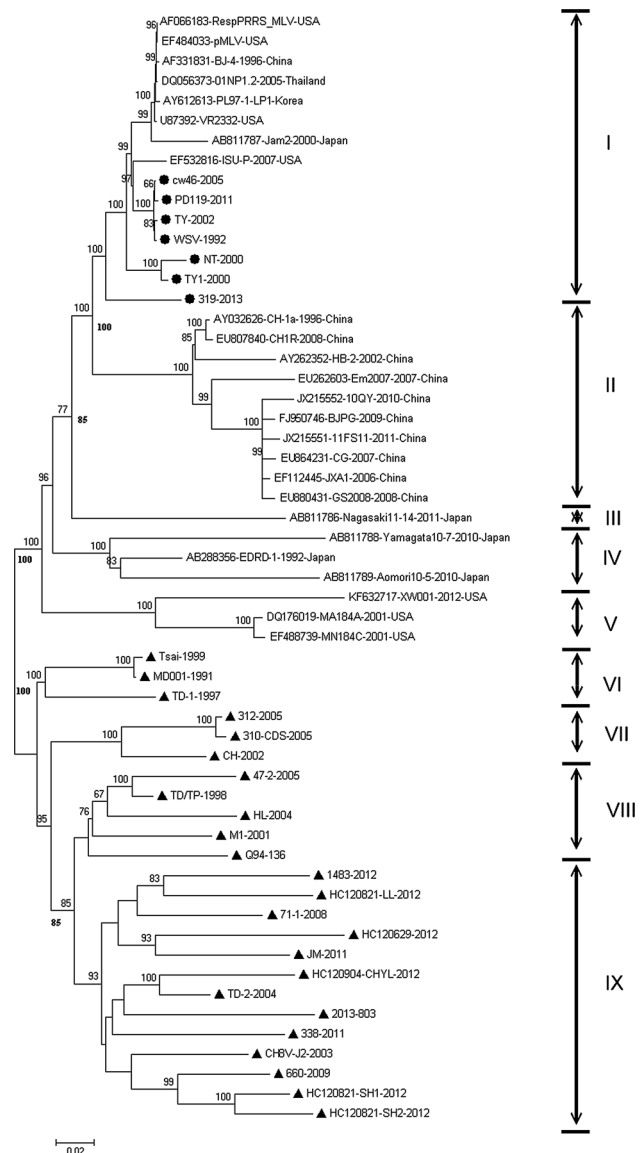


Fig. 1 Phylogenetic tree of the complete coding region (marked as CDS) of Taiwanese PRRSV strains analyzed by the maximum-likelihood method and the neighbor-joining method with 1,000 replicates of bootstrap analysis. The classification of lineages was based on the genetic distance, with isolates diverging by more than 10% from neighboring lineages divided into separate lineages [31]. The strains indicated by circles and triangles are Taiwanese strains of lineages I, VI, and VII-IX, respectively

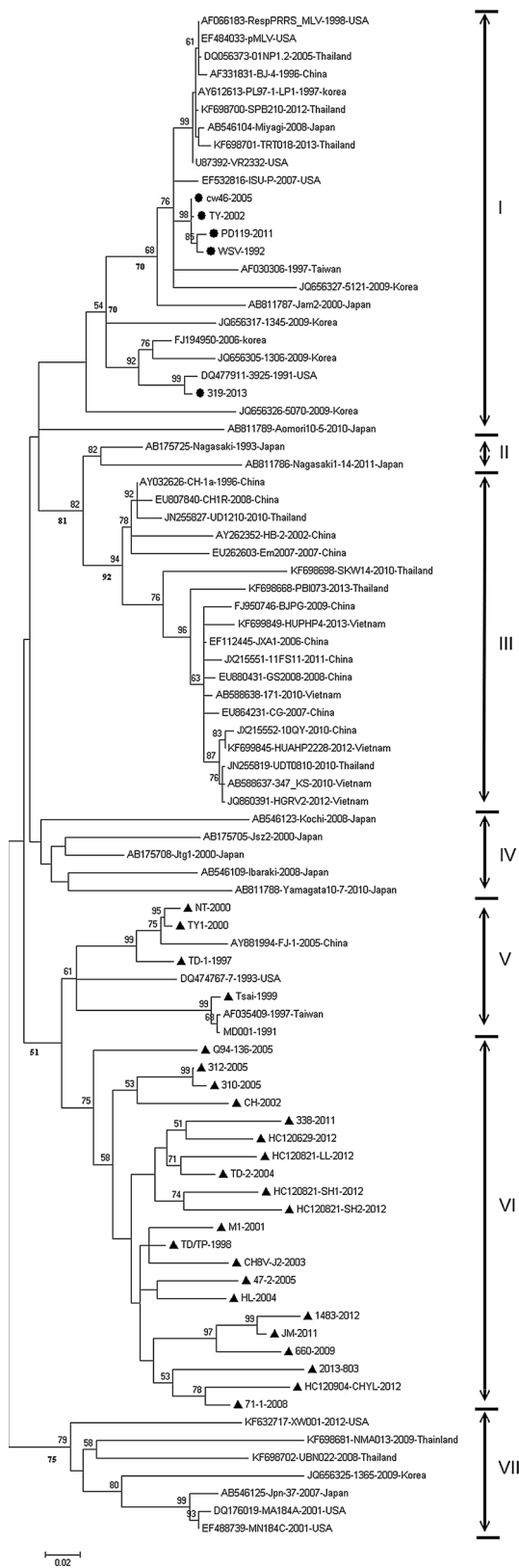


Fig. 2 Phylogenetic tree of the open reading frame 5 (ORF5) gene of Taiwanese PRRSV strains analyzed by the maximum-likelihood method and the neighbor-joining method with 1,000 replicates of bootstrap analysis. The classification of lineages was based on the genetic distance, with isolates diverging by more than 10 % from neighboring lineages divided into separate lineages [31]. The strains indicated by circles and triangles are Taiwanese strains of lineages I, V and VI, respectively

isolated from 2011 to 2013, nine were in the MD001 group, whereas only two were in the WSV group (Table 1). The nucleotide (nt) sequence divergence of Taiwan PRRSV isolates ranged from 0.3 to 18.3 %. The nt sequence divergence among the WSV and MD001 groups ranged from 0.2 to 7.4 % and from 0.9 to 18.3 %, respectively. The nt sequence divergence of the WSV and MD001 groups from the MLV strain ranged from 2.6 to 6.1 and from 11.1 to 17.4 %, respectively.

Sequences and phylogenetic analysis of ORF5

The results of ORF5 gene phylogeny (Fig. 2) were largely similar to those of the complete coding region (Fig. 1). However, it was noteworthy that two strains, NT-2000 and TY1-2000, originally characterized as the WSV group (Fig. 1), switched to the MD001 group (Fig. 2). Similar to the results in Fig. 1, most recent strains isolated from 2011 to 2013 belonged to the MD001 group. The nt sequence divergence of ORF5 in the Taiwanese PRRSV strains ranged from 0.02 to 24.8 %. The nt sequence divergence in the WSV and MD001 groups ranged from 0.2 to 11.0 % and from 1.4 to 19.0 %, respectively. The nt sequence divergence of ORF5 of the WSV and MD001 groups from the MLV strain ranged from 2.5 to 8.5 and from 12.2 to 22.3 %, respectively.

Amino acid sequence analysis of GP5 protein

The amino acid sequence divergence of the GP5 protein of the Taiwan PRRSV strains ranged from 1.1 to 24.5 %. Residues in GP5 related to virulence have been identified at positions 13 and 151 [2]. The 13th residue was R in the reference strain VR2332, Q in the MLV strain, R in the HP-PRRSV strains, and usually R (23/31) and sometimes Q (8/31) in the Taiwan strains (Fig. 3). The 151st residue was R in the VR2332 strain, G in the MLV strain, R in the HP-PRRSV strains, and usually R (29/31) and sometimes K (2/31) in the Taiwan strains. This indicated that most of the Taiwan strains were antigenically closer to the pathogenic strains than to the attenuated vaccine strain.

The GP5 protein has two major epitopes: the decoy epitope (DCE) located at residues 27 to 30 and the primary neutralizing epitope (PNE) located at residues 37 to 45 [32, 34] (Fig. 3). In the DCE, the residues were VLAN in the VR2332 and MLV strains and VLVN/VLAN in the HP-PRRSV strain. VLVN was the most common sequence of the DCE in lineages I and VI (Fig. 2) of the Taiwanese strains. However, the DCE in lineage V (Fig. 2) of the Taiwanese strains was ALVS/VLVS. In PNE, the residues were SHLQLIYNL in the VR2332, MLV, and HP-PRRSV strains. SHLQLIYNL was also the major sequence in lineage I of the Taiwan strains. However, two mutations were found in most of the Taiwan strains of lineages V and VI; they mutated from SHLQLIYNL to SYSQLIYNL (Fig. 3).

GP5 has four putative glycosylation sites (30, 32/33/34, 44 and 51) [27, 43]. The Taiwanese strains contained two conserved glycosylation sites at positions 44 and 51 (Fig. 3). The variable glycosylation site at position 30 was found only in the Taiwanese strains of lineages I and VI. The other variable glycosylation site of most Taiwanese strains (26/31) at position 34 was different from the site at position 33 of VR-2332 or the MLV strain (Fig. 3).

Selection pressure and evolution rate of ORF5

The dN/dS ratios in the WSV group strains ranged from -2.065 to 0.005 and averaged -0.781, indicating

that they evolved under negative selection. In contrast, the dN/dS ratios in the MD001 group ranged from 1.699 to 7.507 and averaged 5.483, indicating positive selection.

Sequences and phylogenetic analysis of nsp2

The nt divergence of the nsp2 gene of the Taiwanese PRRSV isolates ranged from 0.3 to 29.0 %. Based on insertions and deletions in the nsp2 gene, the Taiwan PRRSV strains were found to have five patterns: types V, A, B, C and D (Fig. 4; Table 1). Type V, represented by the VR2332 reference strain, had no deletions/insertions. Type A, represented by the TD-1997 strain, contained one insertion of 36 amino acids between positions 823 and 824. Type B, represented by the PD119-2011 strain, contained two deletions located between positions 583 and 643 and between positions 659 and 744. Type C, represented by the 319-2013 strain, contained one deletion of 125 amino acids between positions 303 and 427. The most common pattern of Taiwan strains was type D (Table 1), including the strains collected from 2002 to 2013, which contained two deletions located between positions 463 and 513 and between positions 530 and 549. The nsp2 types of all Taiwanese strains were different from that of the HP-PRRSV, which contains a 30-amino-acid deletion [39].

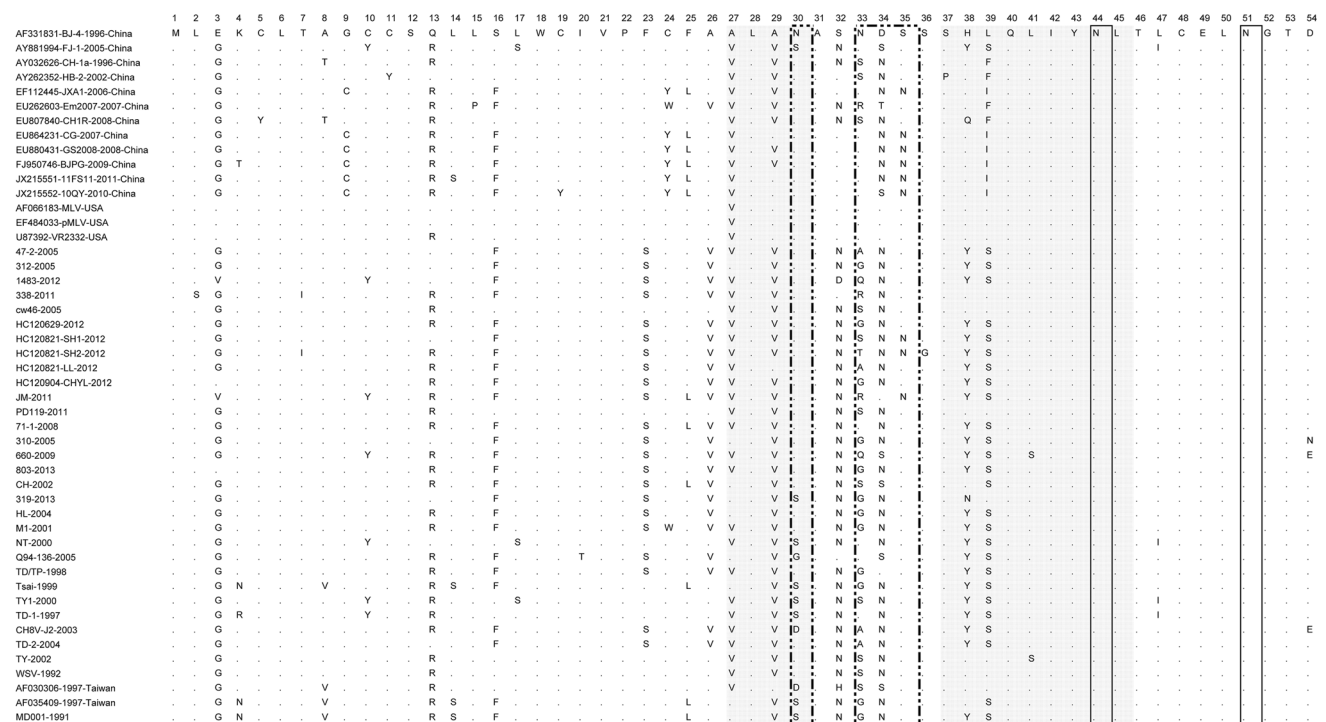
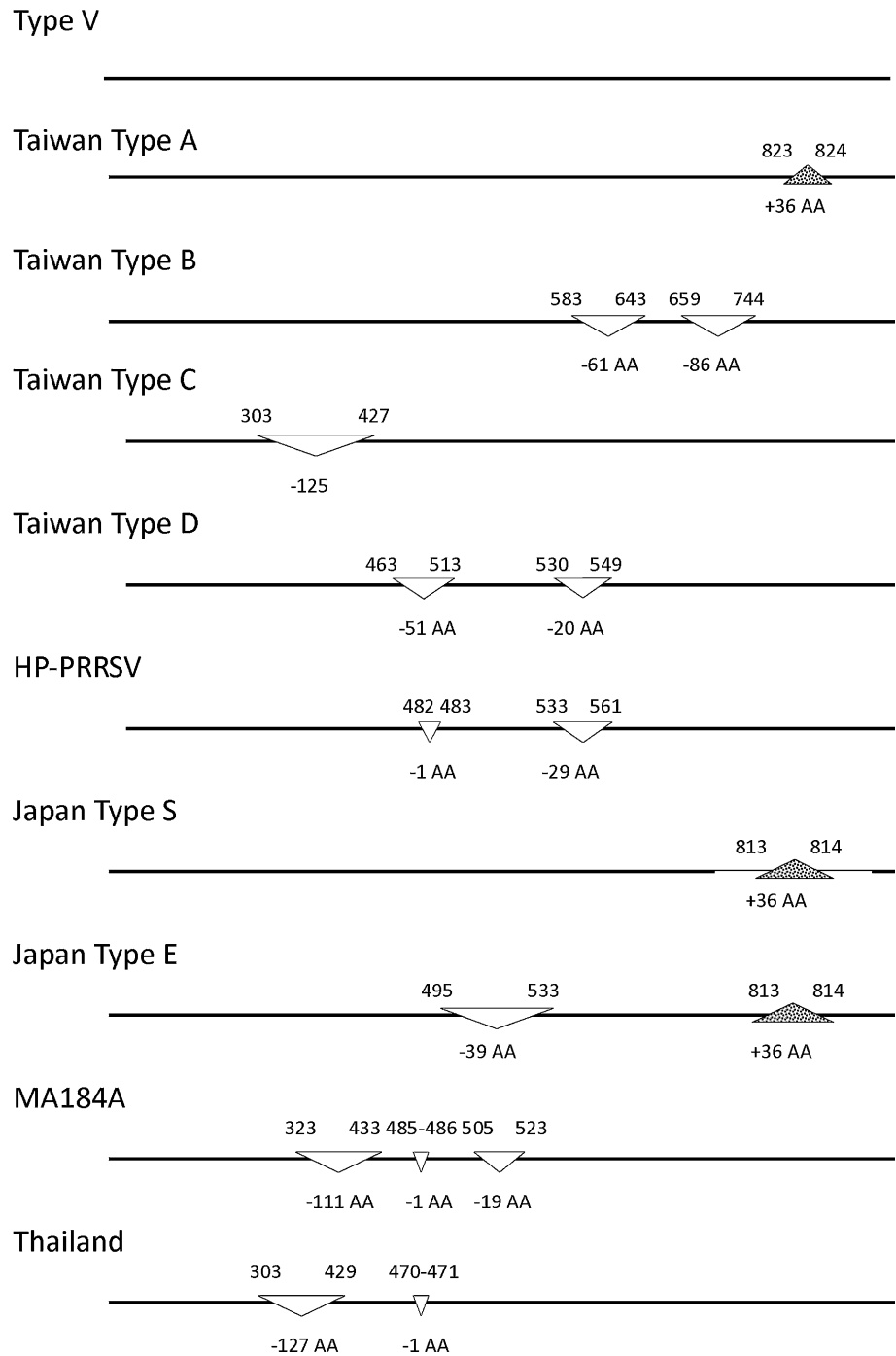


Fig. 3 Partial alignment of amino acid sequences of the PRRSV GP5 protein. The decoy epitope (DCE) located at residues 27 to 30 and the primary neutralizing epitope (PNE) located at residues 37 to 45 are in

shaded areas. The two conserved N-linked glycosylation sites at residues 44 and 51 are boxed in solid lines. The variable glycosylation sites are boxed in dotted lines. Dashes indicate identical bases

Fig. 4 Deletion/insertion patterns in the nsp2 gene, indicative of the evolution of PRRSVs in Taiwan. Type V indicates the standard strain of VR2332. All other strains were compared with the VR2332 strain by MUSCLE alignment. Open triangles with dots indicate insertions, while inverted open triangles indicate deletions



Discussion

This study clearly reveals the evolutionary trend and polymorphism of PRRSV in Taiwan. In the early 1990s, two groups of PRRSVs, prototyped by either WSV or MD001 strains, already existed in Taiwan. The WSV group was in lineage I, similar to the reference strain VR2332 (Fig. 1), while the MD001 group was in lineages VI to IX

(Fig. 1). Over the years, both groups of viruses have evolved separately, as also found in Japan [10] and China [19]. In the early 1990s, two subtype strains of the NA genotype of PRRSV were already detected in both countries. These results suggest that the first PRRSV cases in the Netherlands and the United States in 1989 were just the earliest detection with PRRSV in swine. The earliest infection point of PRRSV in swine was inferred to be about

1880 by analysis of molecular evolution [7]. After that point, PRRSV extended rapidly among domesticated pigs and evolved in the 1980s [9].

Over the last 20 years, the population dynamics of PRRSV in Taiwan have changed. The number of WSV group members has decreased, while that of MD001 group members has increased, the virus becoming predominant in the most recent decade (Table 1). The important question is, what led to this change in PRRSV population dynamics? The answer may partially be associated with the use of MLV, which began in the year 1999. First, the genetic diversity of PRRSV affected the efficacy of MLV, especially in humoral immunity, which offered better protection against antigenically homologous strains than against heterologous strains [8, 17, 18, 22, 24]. However, the protection of cellular immunity between homologous and heterologous strains was depended on the number of virus-specific interferon- γ producing cells [3, 22, 23, 33, 47]. In Taiwan, three kinds of PRRSV vaccines have been used, including two MLV and one subunit vaccine. The first commercial vaccine, Ingelvac[®] PRRSV MLV, is derived from the prototypic American strain VR2332. The ORF5 sequences of Taiwanese strains and the Ingelvac[®] PRRSV MLV strain showed a 2.5–8.5 % and 12.2–22.3 % divergence from the WSV group and the MD001 group, respectively. These suggested that the protection of Ingelvac[®] PRRSV MLV against the WSV group may be better than against the MD001 group. It may thus reduce the infection and spread of WSV-group viruses in Taiwan. Second, antigenic variation is an important mechanism of virus evolution and survival [6, 12]. The results of PRRSV evolution in the WSV and MD001 groups showed that the WSV group was under negative selection but the MD001 group was under positive selection. This indicated that the antigenicity of members of the MD001 group gradually changed over time and possibly favored the spread of strains of the MD001 group. Third, the predominance of a given PRRSV strain might be related to the invasive time of the strain. The strains of the MD001 group identified earlier than those of the WSV group may have adapted to Taiwan's pig population earlier and thus led to the prevalence of the MD001 group. To test this hypothesis, more information of PRRSV isolates is essential. The above may partially explain why the MD001 group is predominant today in Taiwan, while the WSV group is subsiding.

Glycoprotein 5 (GP5), encoded by the ORF5 gene, is important for the interaction between swine and PRRSV. It is associated with the induction of host immunity [32, 34, 39], with immune evasion, and the virulence of PRRSV [14, 16, 41]. The amino acid sequence divergence of GP5 among Taiwanese PRRSV strains ranged from 1.1 to 24.5 %, and the divergence gradually increased over time. For induction of host immunity, the sequences of epitopes

DEC and PNE were mostly VLVN and SYSQLIYNL (Fig. 3), and the two T-cell epitopes [40] were LAA-LICFVIRLAKNC and KGRLYRWRSPVIEK (data not shown), respectively. Such amino acid divergence among the Taiwan strains could affect the cross-protection among Taiwan strains. This speculation is supported by the findings of a negative correlation between divergence of GP5 and activity of neutralizing antibody [31]. These antigenic variations were also most likely driven by the positive selection imposed by the use of MLV (see above).

This study also revealed a possible invasion by a new exotic strain in the year 2013. Phylogenetically, strain 319 belongs to a distant branch of lineage I (Figs. 1, 2). This strain has 99 % similarity in its ORF5 gene to an exotic PRRSV-3925 strain (DQ477911) that originated in the USA, suggesting that it did not evolve from the WSV strain but rather represents a newly invading strain in Taiwan. This international spread has also occurred in the USA [36], Thailand [30, 31] and Japan [11], revealing that transnational transmission of PRRSV is still taking place.

The nsp2 gene is polymorphic, and the insertion/deletion patterns accompanying PRRSV evolution change. In the early 1990s, different patterns of insertions and deletions in nsp2 were found in various PRRSV strains [14, 21, 45]. Each insertion/deletion of nsp2 was also accompanied by individual evolution over time. This trend was also found in the Taiwanese strains for which the strains of the WSV group changed from pattern V to pattern B or B + C, and strains of the MD001 group changed from pattern A to D (Fig. 4; Table 1). Although the nsp2 gene is not directly involved in the induction of host immunity, it is, however, involved in viral replication. Whether the change in pattern affects the function of nsp2 is dependent on the location and distribution of insertions and deletions. In addition, the polymorphism of nsp2 is a potential marker to differentiate infected from vaccinated animals (DIVA) by serology.

The cell type plays an important role in PRRSV isolation. Porcine alveolar macrophages (PAM) and the MARC-145 cell line, originally from kidney cells of an African green monkey, were mostly used to isolate and culture PRRSV. PAM cells have higher susceptibility to type EU and NA PRRSV isolates, whereas MARC-145 cells may bias toward isolation of type NA PRRSV isolates [46]. As the present study used MARC-145 cells to isolate PRRSV from the tissues of PRDC-affected pigs, the loss of type EU PRRSV isolates may be misleading. However, in recent studies of PRRSV epidemiology in Taiwan, only type NA isolates were detected, without any type EU PRRSV isolates [35, 42]. We presented similar findings in our monitoring reports during 2013 to 2014, with a total of 67 cases in 225 PRDC-affected pigs from 92 farms that were PRRSV positive by RT-PCR, all of which were identified as type NA PRRSV (data not shown). These results suggest

that the fact that no type EU PRRSV was isolated was not due to the methods in the present study.

In conclusion, the present study clearly reveals the evolutionary trends and polymorphisms of PRRSV in Taiwan. In the early 1990s, two prototypic strains, WSV and MD001, were first identified in Taiwan. Over the years, both strains have evolved separately, but the strains of the MD001 group were predominant in Taiwan. Evolution was manifested in changes in the *nsp2* and ORF5 genes. In addition, in the year 2013, a suspected exotic strain, strain 319, was recovered, and its effect on the population dynamics of PRRSV remains to be determined. Overall, these results provide an important basis for vaccine development for the control and prevention of PRRSV.

Acknowledgments This work was supported by Grant 102AS-10.1.1-HI-H1 from the Council of Agriculture (COA). We thank for Dr. Ling-Ling Chueh for sharing the MD001 strain.

References

- Allende R, Lewis TL, Lu Z, Rock DL, Kutish GF, Ali A, Doster AR, Osorio FA (1999) North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. *J Gen Virol* 80:307–315
- Allende R, Kutish GF, Laegreid W, Lu Z, Lewis TL, Rock DL, Friesen J, Galeota JA, Doster AR, Osorio FA (2000) Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. *Arch Virol* 145:1149–1161
- Diaz I, Darwich L, Pappaterra G, Pujols J, Mateu E (2006) Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. *Virology* 351:249–259
- Fang Y, Schneider P, Zhang WP, Faaberg KS, Nelson EA, Rowland RR (2007) Diversity and evolution of a newly emerged North American Type 1 porcine arterivirus: analysis of isolates collected between 1999 and 2004. *Arch Virol* 152:1009–1017
- Firth AE, Zevenhoven-Dobbe JC, Wills NM, Go YY, Balasuriya UB, Atkins JF, Snijder EJ, Posthuma CC (2011) Discovery of a small arterivirus gene that overlaps the GP5 coding sequence and is important for virus production. *J Gen Virol* 92:1097–1106
- Flint SJ, Enquist LW, Krug RM, Racaniello VR, Skalka AM (2000) Principles of virology: molecular biology, pathogenesis, and control. American Society for Microbiology, Washington, DC
- Forsberg R (2005) Divergence time of porcine reproductive and respiratory syndrome virus subtypes. *Mol Biol Evol* 22:2131–2134
- Geldhof MF, Vanhee M, Van Breedam W, Van Doorselaere J, Karniyuchuk UU, Nauwynck HJ (2012) Comparison of the efficacy of autogenous inactivated porcine reproductive and respiratory syndrome virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. *BMC Vet Res* 8:182
- Hanada K, Suzuki Y, Nakane T, Hirose O, Gojobori T (2005) The origin and evolution of porcine reproductive and respiratory syndrome viruses. *Mol Biol Evol* 22:1024–1031
- Iseki H, Takagi M, Kawashima K, Shibahara T, Kuroda Y, Tsunemitsu H (2012) Type I porcine reproductive and respiratory syndrome virus emerged in Japan. In: In 22nd International Pigs Veterinary Society Congress. International Pigs Veterinary Society, pp 978
- Iseki H, Takagi M, Miyazaki A, Katsuda K, Mikami O, Tsunemitsu H (2011) Genetic analysis of ORF5 in porcine reproductive and respiratory syndrome virus in Japan. *Microbiol Immunol* 55:211–216
- Ito T, Couceiro JNSS, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y (1998) Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J Virol* 72:7367–7373
- Johnson CR, Griggs TF, Gnanandarajah J, Murtaugh MP (2011) Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. *J Gen Virol* 92:1107–1116
- Kedkovid R, Nuntawan S, Ayudhya N, Amonsin A, Thanawongnuwech R (2010) NSP2 gene variation of the North American genotype of the Thai PRRSV in central Thailand. *Virol J* 7:340
- Kosakovsky-Pond SL, Poon AFY, Frost SDW (2009) The phylogenetic handbook, 2nd edn. Cambridge University Press, Cambridge
- Kwon B, Ansari IH, Pattnaik AK, Osorio FA (2008) Identification of virulence determinants of porcine reproductive and respiratory syndrome virus through construction of chimeric clones. *Virology* 380:371–378
- Labarque G, Reeth KV, Nauwynck H, Drexler C, Van Gucht S, Pensaert M (2004) Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. *Vaccine* 22:4183–4190
- Lager KM, Mengeling WL, Brockmeier SL (1999) Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. *Am J Vet Res* 60:1022–1027
- Li B, Fang L, Liu S, Zhao F, Jiang Y, He K, Chen H, Xiao S (2010) The genomic diversity of Chinese porcine reproductive and respiratory syndrome virus isolates from 1996 to 2009. *Vet Microbiol* 146:226–237
- Li Y, Wang X, Bo K, Tang B, Yang B, Jiang W, Jiang P (2007) Emergence of a highly pathogenic porcine reproductive and respiratory syndrome virus in the Mid-Eastern region of China. *Vet J* 174:577–584
- Liu JK, Wei CH, Yang XY, Hou XL, Dai AL, Li XH, Wei MK, Pan XZ (2013) Genetic diversity and evolutionary characterization of Chinese porcine reproductive and respiratory syndrome viruses based on NSP2 and ORF5. *Arch Virol* 158:1811–1816
- Mateu E, Diaz I (2008) The challenge of PRRS immunology. *Vet J* 177:345–351
- Martelli P, Gozio S, Ferrari L, Rosina S, De Angelis E, Quintavalla C, Bottarelli E, Borghetti P (2009) Efficacy of a modified live porcine reproductive and respiratory syndrome (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: Clinical protection and cell-mediated immunity. *Vaccine* 27:3788–3799
- Mengeling WL, Lager KM, Vorwald AC, Koehler KJ (2003) Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. *Vet Microbiol* 93:13–24
- Meulenbergh JJM, Hulst MM, de Meijer EJ, Moonen PLJM, den Besten A, de Kluyver EP, Wensvoort G, Moormann RJM (1993) Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. *Virology* 192:62–72

26. Murtaugh MP, Elam MR, Kakach LT (1995) Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Arch Virol* 140:1451–1460
27. Music N, Gagnon CA (2010) The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis. *Animal Health Res Rev* 11:135–163
28. Nei M, Gojobori T (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol Biol Evol* 3:418–426
29. Nelsen CJ, Murtaugh MP, Faberg KS (1999) Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. *J Virol* 73:270–280
30. Nilubol D, Tripipat T, Hoonsuwan T, Kortheerakul K (2012) Porcine reproductive and respiratory syndrome virus, Thailand, 2010–2011. *Emerg Infect Dis* 18:2039–2043
31. Nilubol D, Tripipat T, Hoonsuwan T, Tipsombatboon P, Piriyaopongsa J (2013) Genetic diversity of the ORF5 gene of porcine reproductive and respiratory syndrome virus (PRRSV) genotypes I and II in Thailand. *Arch Virol* 158:943–953
32. Ostrowski M, Galeota JA, Jar AM, Platt KB, Osorio FA, Lopez OJ (2002) Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. *J Virol* 76:4241–4250
33. Park C, Seo HW, Han K, Kang I, Chae C (2014) Evaluation of the efficacy of a new modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Fostera PRRS) against heterologous PRRSV challenge. *Vet Microbiol* 172:432–442
34. Plagemann PG (2004) GP5 ectodomain epitope of porcine reproductive and respiratory syndrome virus, strain Lelystad virus. *Virus Res* 102:225–230
35. Shen SY, Ma WJ, Yu CY, Chang CC (2010) Genetic variation of porcine reproductive and respiratory syndrome viruses in Taiwan. *Taiwan Vet J* 36:305–314
36. Shi M, Lemey P, Brar MS, Suchard MA, Murtaugh MP, Carman S, D’Allaire S, Delisle B, Lambert ME, Gagnon CA, Ge L, Qu Y, Yoo D, Holmes EC, Leung FCC (2013) The spread of type2 porcine reproductive and respiratory syndrome virus (PRRSV) in North America: a phylogeographic approach. *Virology* 447:146–154
37. Snijder EJ, Meulenber JJ (1998) The molecular biology of arteriviruses. *J Gen Virol* 79:961–979
38. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
39. Tian K, Yu X, Zhao T, Feng Y, Cao Z, Wang C, Hu Y, Chen X, Hu D, Tian X, Liu D, Zhang S, Deng X, Ding Y, Yang L, Zhang Y, Xiao H, Qiao M, Wang B, Hou L, Wang X, Yang X, Kang L, Sun M, Jin P, Wang S, Kitamura Y, Yan J, Gao GF (2007) Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One* 2:e526
40. Vashisht K, Goldberg TL, Husmann RJ, Schnitzlein W, Zuckermann FA (2008) Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American genotype of porcine reproductive and respiratory syndrome virus. *Vaccine* 26:4747–4753
41. Vu HLX, Kwon B, Yoon KJ, Laegreid WW, Pattnaik AK, Osorio FA (2011) Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein as well as glycoprotein 3. *J Virol* 85:5555–5564
42. Wang C, Lee F, Huang TS, Pan CH, Jong MH, Chao PH (2008) Genetic variation in open reading frame 5 gene of porcine reproductive and respiratory syndrome virus in Taiwan. *Vet Microbiol* 131:339–347
43. Wei Z, Lin T, Sun L, Li Y, Wang X, Gao F, Liu R, Chen C, Tong G, Yuan S (2012) N-linked glycosylation of GP5 of porcine reproductive and respiratory syndrome virus is critically important for virus replication in vivo. *J Virol* 86:9941–9951
44. Wu WH, Fang Y, Farwell R, Steffen-Bien M, Rowland RR, Christopher-Hennings J, Nelson EA (2001) A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. *Virology* 287:183–191
45. Yoshii M, Okinaga T, Miyazaki A, Kato K, Ikeda H, Tsunemitsu H (2008) Genetic polymorphism of the nsp2 gene in North American type-porcine reproductive and respiratory syndrome virus. *Arch Virol* 153:1323–1334
46. Zimmerman JJ, Benfield DA, Dee SA, Murtaugh MP, Stadejek T, Stevenson GW, Torremorell M (2012) Porcine reproductive and respiratory syndrome virus (Porcine Arterivirus). In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW (eds) *Disease of Swine*, 10th edn. Blackwell Publishing Ltd, Oxford, pp 461–486
47. Zuckermann FA, Garcia EA, Luque ID, Christopher-Hennings J, Doster A, Brito M, Osorio F (2007) Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccine based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. *Vet Microbiol* 123:69–85