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ORIGINAL ARTICLE

Genetic diversity and evolutionary characteristics of type 2 porcine reproductive and respiratory syndrome virus in southeastern China from 2009 to 2014

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Abstract The objective of this study was to assess the genetic diversity of porcine reproductive and respiratory syndrome virus circulating in Fujian province (southeastern China). Based on 53 ORF5 nucleotide sequences collected from nine sites, both highly pathogenic (sublineage 8.7) and lineage 1 strains were circulating in Fujian in 2009-2014 along with lineages 3 and 5.1. Notably, the lineage 1 strains were closely related to the NADC30 strain circulating in North America and were the predominant strains in 2014. In addition, we found that nonstructural protein 2 (NSP2) was the most variable nonstructural protein in Fujian isolates, with a 36-amino-acid (aa) insertion and seven different deletions detected in the 53 sequences examined. Similarly, analysis of GP5 amino acid sequences showed that the isolates were highly variable in primary neutralizing epitopes. Interesting, FJ3.2 and FJ7-2 strains have the mutation N44K, but they exhibited high replication and high titers in MARC-145 and PAM cells. The complete genome sequences determined for 12 type 2 isolates were 82.1-99.3% identical and were 15,016-15,407 nucleotides (nt), in length excluding the poly(A) tail. The strains also shared 88.2-99.4% identity with strain VR2332 (the prototype North American strain), 83.4-99.2% identity with strain JXA1 (the prototype high-pathogenicity Chinese strain), 88.2-97.1% identity with strain CH-1a (the prototype classical Chinese strain), and 82.9-97.1% identity with strain NADC30 (the prototype NADC30-like strain). Strikingly, phylogenetic and molecular evolutionary analyses indicated that strain FJW05 is a spontaneous recombinant between a circulating lineage 1 virus and the vaccine strain JXA1-R, which is derived from the highly pathogenic strain JXA-1. Collectively, the data highlight the epidemiology of porcine reproductive and respiratory syndrome in Fujian and may aid in selecting a suitable vaccine for use on pig farms.

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) has become one of the most important infectious diseases in pigs since PRRS emerged in Europe and in the US in the early 1990s. In 2006, highly pathogenic PRRSV (HP-PRRSV) emerged in China and caused substantial economic losses for the swine industry in China [1].

Porcine reproductive and respiratory syndrome virus (PRRSV), the causative agent of PRRS, is an enveloped, positive-sense, single-stranded RNA virus belonging to the family *Arteriviridae* [2, 3], with a genome that contains 10 open reading frames (ORFs) in approximately 15 kb [4, 5]. ORF1a and ORF1b encode nonstructural proteins (NSPs/

nsps) involved in viral replication and transcription, while ORF2a, ORF2b, ORF5a, and ORF3-7 encode eight viral structural proteins [4, 6]. Generally, PRRSV isolates are classified into two major genotypes, namely type 1 (European-like) and type 2 (North American-like), which are identical in about 50-70% of their nucleotides and in 50-80% of their amino acids [7].

Now, multiple PRRSV types co-exist in Chinese swine herds, and HP-PRRSVs have been the predominant isolates since 2006 in China [8–13]. Genome analysis has shown that HP-PRRSV strains contain a unique discontinuous deletion of 30 amino acids (aa) (at positions 481 and 533 to 561) in NSP2, which has become a gene marker of HP-PRRSV [1, 10, 14, 15]. Most studies on the genetic diversity of PRRSV have been conducted by examining NSP2 and ORF5 [5, 8–13, 15, 16]. In addition, the number of live pigs in Fujian Province (southeastern China) is over 20 million. In order to explore the epidemic status of PRRS and the genetic diversity of PRRSV in Fujian Province, we analyzed 12 complete genome sequences and 53 NSP2 and 53 ORF5 nucleotide sequences of PRRSV isolates from the period of 2009-2014.

Materials and methods

Ethics statement

Sampling procedures were performed in accordance with the guidelines of the South China Agricultural University Institutional Animal Care and Use Committee (SCAU-AEC-2014-10) and approved by an animal ethics committee of South China Agricultural University. However, the manuscript does not contain any studies with animals performed by any of the authors.

Sample collection and virus isolation

A total of 480 tissue samples (lungs, kidneys, livers, and lymph nodes) and 2,200 serum samples were collected from 90 Fujian pig farms (Table 1). The farms were located in different districts, collectively covering a geographic area of about 120,000 km^2 between the latitudes 23°30′ and 28°22′N and the longitudes 115°50′ and 120°40′E. In particular, samples were collected from 40 farms in Longyan (776 serum samples, 105 tissue samples), 15 farms in Zhangzhou (308 serum samples, 84 tissue samples), eight farms in Xiamen (225 serum samples, 59 tissue samples), five farms in Fuzhou (149 serum samples, 51 tissue samples), four farms in Ningde (141serum samples, 39 tissue samples), five farms in Nanping (159 samples serum samples, 41 tissue samples), four farms in Sanming (138 serum samples, 38 tissue samples), five farms in Quanzhou (147 serum samples, 38 tissue samples), and four farms in Putian (157 samples serum samples, 25 tissue samples) between 2009 and 2014 (see Table S1 in the supplemental material). Serum samples were collected randomly from gilts, sows, and growers from each farm. PRRSV was isolated from infected samples using PRRSV-free primary porcine alveolar macrophages or MARC-145 cells.

RT-PCR and nucleotide sequencing

RNA was extracted from virus isolates using an HP Total RNA Kit (OMEGA, USA), and reverse transcribed using Superscript III reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions. cDNA was constructed using Superscript III reverse transcriptase (Invitrogen, USA) as follows: a mixture, including 1 μg of RNA, 1 μl of 10 mM dNTPs, 1 μl of oligo(dT20) (50 μM) and 4.5 μl of nuclease-free water, was incubated for 5 min at 65 °C and then for 2 min on ice. Then, 4 of μl 5× first-strand buffer, 1 μl of 0.1 M DTT, 1 μl of RNaseOUT Recombinant RNase Inhibitor (40 units/μl), and 1 μl of SuperScript III RT (200 units/μl) were added, and the mixture was incubated at 50 °C for 60 min. The reaction was then inactivated the by heating at 70 °C for 15 minutes and chilled on ice. The obtained cDNA was then used in specific PCRs designed to amplify overlapping segments of the viral genome of type-2 PRRSV, NSP2 and ORF5 as described previously [9–13, 16]. The cDNA was used as the template in the subsequent PCR in a final volume of 50 μl that included 5 μl of 10× PCR buffer with $MgSO_4$, 1.0 μl of 10 mM dNTP mix, 2 μl of cDNA, 0.8 μM each PRRSV-specific primer, and 1 μl of PrimeSTAR polymerase (TaKaRa Co. Dalian, China). The PCR cycle parameters were as follows: 98 °C for 15 s, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s, and extension at 72 °C for 1-3 min, and finally, 1

Table 1 The prevalence of PRRSVs in Fujian province from 2009 to 2014

Year	Number of samples			Year	Number of samples		
	Total	Positive	% positive		Total	Positive	% positive
2009	380	25	6.58	2012	460	54	11.74
2010	420	39	9.29	2013	469	60	12.79
2011	447	51	11.41	2014	504	68	13.49

cycle of 5 min at 72 °C. Recombinant clones were sent to Ruibo Life Technologies Corporation (Beijing, China) for sequencing. Each fragment was independently sequenced at least three times.

Phylogenetic analysis

Twelve complete genomes, 53 ORF5 genes (Table S2 in the supplemental material), and 53 NSP2 genes (Table S3 in the supplemental material) were sequenced. Reference PRRSV sequences in GenBank were included in phylogenetic analyses as controls. Sequences were aligned in CLUSTAL X (version 1.83), and ORF5 sequences were genotyped according to the global PRRSV classification systems [16]. Sequences were also analyzed in BEAST v1.4.3 under the assumption of a relaxed, uncorrelated lognormal clock, and under an HKY85 model of nucleotide substitution with a gamma-distributed rate of variation. Independent runs of chain length 8 × 105 were combined to provide an effective sample size > 200 for all parameters. After removal of a visually conservative 10% burn-in period, 9,961 trees were aggregated in TreeAnnotator to maximize clade credibility, with posterior probabilities taken as a measure of branch robustness [17, 18]. A phylogenetic tree based on complete genome sequences was constructed by neighbor-joining in MEGA 6.0, using maximum composite likelihood and bootstrap confidence values from 1,000 replicates.

Potential N-glycosylation sites in GP5 was identified using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) at the default probability threshold of above 0.5 (http://www.cbs.dtu.dk/services/NetNGlyc/ output. Php).

Recombination analysis

Recombination events were detected using RDP v.4.22 [19] as described by Ramos et al. [20]. Potential recombination events were tested by seven different algorithms (RDP, GeneConv, BootScan, MaxChi, Chimera, SiScan, and 3Seq) with Bonferroni correction and a highest acceptable p-value of 0.01.

Virus cross-neutralization assay

The live attenuated virus vaccine strain JXA1-R, which was derived from HP-PRRSV strain JXA-1, has been most widely used throughout Fujian Province since 2009. Therefore, it is important to evaluate whether antibody induced by JXA1-R could neutralize representative PRRSV strains isolated in Fujian. Four serum samples (#1-4) were collected from four individual piglets on day 35 after vaccination with the JXA1-R vaccine. Cross-neutralization was assayed as described previously [21]. Briefly, sera from infected pigs were serially diluted two-fold in DMEM, and 100 μL of each dilution was mixed with an equal volume of virus at 10^3 $TCID_{50}$/mL. After incubation at 37 °C for 1 h, mixtures were added to MARC-145 monolayers in 96-well plates. Infection in each well was assessed by indirect immunofluorescence assay (IFA) five days thereafter. Neutralizing antibody (NA) titers against other PRRSV isolates were calculated using the Reed-Muench method [22]. Data were analyzed by Student's t-test, and $p < 0.05$ was considered statistically significant.

Viral growth properties and kinetics

Growth properties and kinetics of the viruses FJ3.2 and FJ7-2 were assessed following previously described procedures [23]. Briefly, MARC-145 cells or PAM cells were cultured in 6-well plates and infected at an MOI of 0.01. After incubation at 37 °C for 1 h, cells were washed and incubated in RPMI-1640 with 3% FBS at 37 °C for 96 h. The virus-infected supernatants were collected every 12 h, and viral titers were determined and expressed as $TCID_{50}$/mL.

Results

PRRSV detection

Clinical samples from 2009 to 2014were tested in the present study by RT-PCR. Of the 2,680 samples collected from different swine herds located in Fujian, 297 samples (11.1%) were positive (Table 1) and 53 positive strains were selected for further analysis.

Phylogenetic analysis of ORF5

Based on global PRRSV classification systems [16], Fujian viruses belonged to lineages 1 (NADC30-like, 10 strains), 3 (QYYZ-like, 1 strain), 5.1 (VR2332-like, 5 strains), and 8.7 (JXA1-like, 37 strains) (Fig. 1). Strains in lineage 8.7 from different farms in Fujian were further classifiable into four subgroups, with 30 of 53 strains belonging to subgroup I and being closely related to JXA1. Interestingly, this subgroup consisted mainly of strains isolated in 2009-2013. On the other hand, strains FJE1 and FJLY08 were classified as subgroup II and were closely related to HB-1(sh)/2002, a strain with an intermediate subgenotype. Subgroup III consisted of two strains isolated in 2014, while subgroup IV consisted of strains FJI6, FJ11, and FJ5I (Fig. 1).

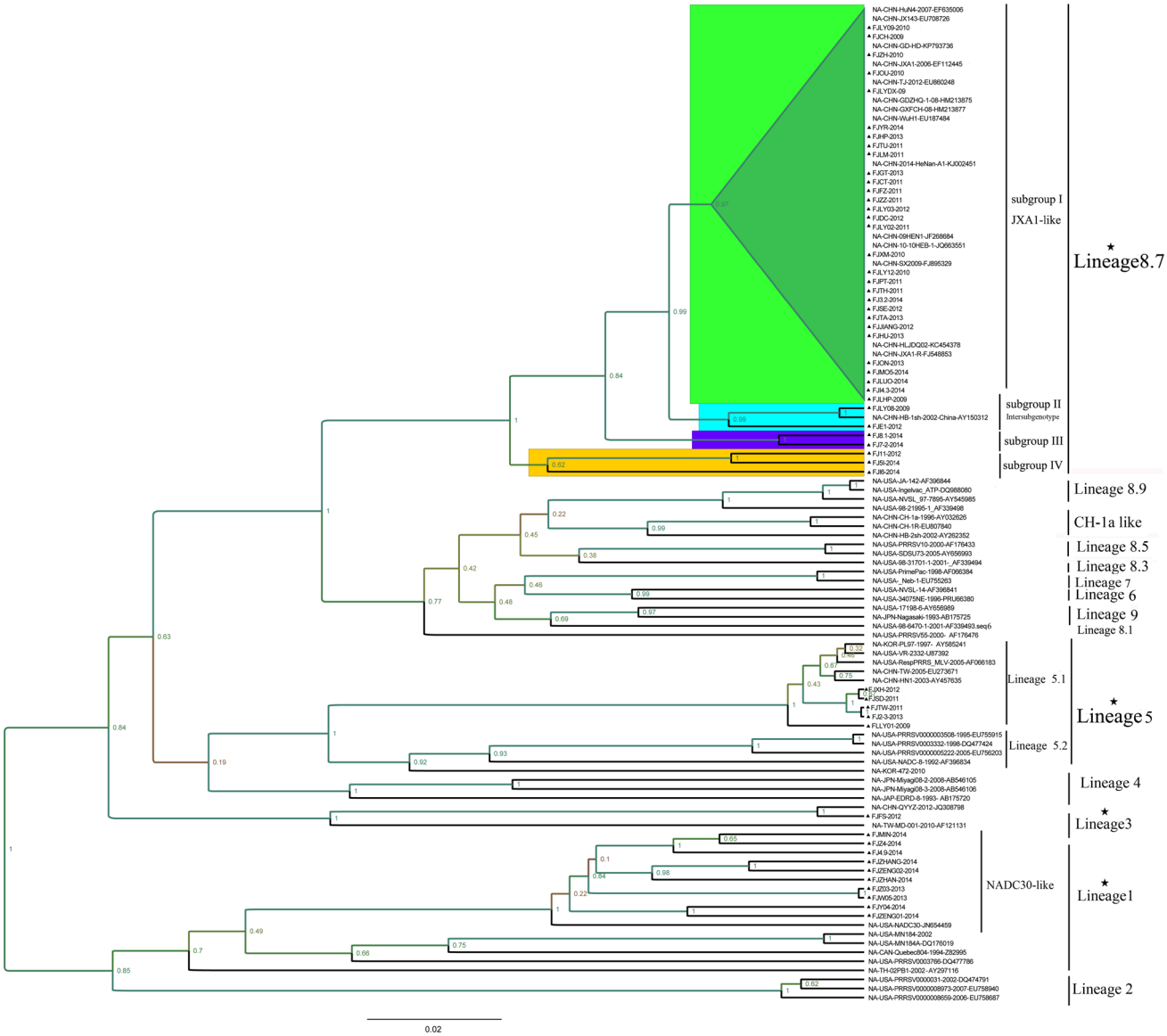


Fig. 1 Phylogenetic tree based on ORF5 genes from 53 Fujian isolates and reference viruses. Reliability was assessed by Bayesian posterior probability analysis. Representative Fujian isolates are indicated by black triangles (▲)

Analysis of the GP5 amino acid sequence

GP5 is the most variable PRRSV protein and has thus been used as a marker of genetic diversity. The Fujian viruses were found to have acquired extensive mutations in the primary neutralizing epitope (^{37}SHF/LQLIYNL^{45}), decoy epitope, and potential glycosylation sites (^{27}V/ALVN^{30}) in GP5 [24, 25] (Table 2). In particular, the isolates were found to be highly variable at position 39 (L/F^{39}→I/S^{39}), with two additional mutations (N^{44}→K^{44} and L^{41}→S^{41}) observed in sublineage 8.7. Moreover, the substitutions V^{29}→A^{29} and V^{27}→A^{27} were present in decoy epitopes in lineages 1 and 3 and sublineage 8.7, while N^{30}→S^{30} was observed in lineages 1 and 3 (see Fig. S1 in the supplemental material). N44 is the most critical amino acid residue for PRRSV infectivity [26]. However, we found an N44K mutation in strains FJ3.2 and FJ7-2, which were isolated in 2014. These two viruses produced high titers in both MARC-145 cells and PAM cells by 96 h post-infection and were most similar to HP-PRRSV FJLYDX (Fig. 2).

Analysis of the NSP2 amino acid sequence

Of the various nonstructural proteins in PRRSV, NSP2 is the most genetically variable and is regarded as an ideal marker of virus evolution and epidemiology [16, 27].

The 53 NSP2 proteins examined varied from 849 to 984 amino acids in length. Surprisingly, comparison with the

Table 2 Amino acid substitutions in the GP5 envelope protein of 53 isolates compared to VR2332

Position	Amino acid substitutions in GP5																		
	3	4	9	10	11	13	14	15	16	18	19	23	24	25	26	27	29	30	32
Substitutions	3	1	18	11	2	16	1	9	7	4	7	14	16	16	6	10	15	3	11
Position	33	34	35	36	61	62	72	81	88	91	92	93	94	98	101	102	103	104	106
Substitutions	16	23	24	1	6	1	1	4	1	2	10	1	15	5	11	14	1	8	4
Position	117	121	124	136	137	151	152	153	158	159	161	163	164	168	170	178	185	187	189
Substitutions	3	17	13	1	7	21	1	2	2	1	15	3	17	17	5	2	7	7	16
Position	191	192	194	196	198	199	200	192	194	196	198	199	200						
Substitutions	1	10	2	17	1	2	21	10	2	17	1	2	21						

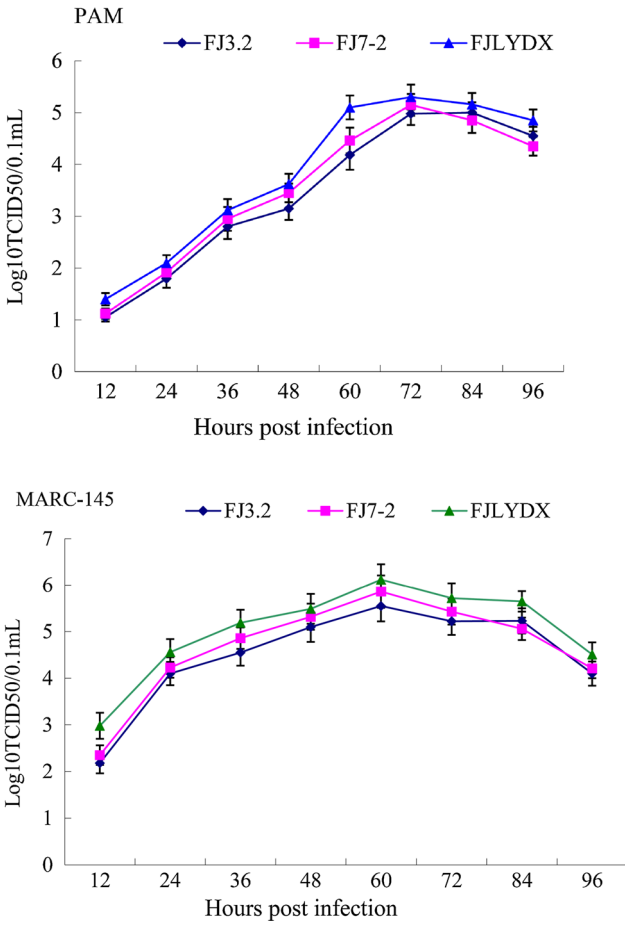


Fig. 2 Growth kinetics of FJ3.2, FJ7-2, and HP-PRRSV FJLYDX isolates in PAM and MARC-145 cells

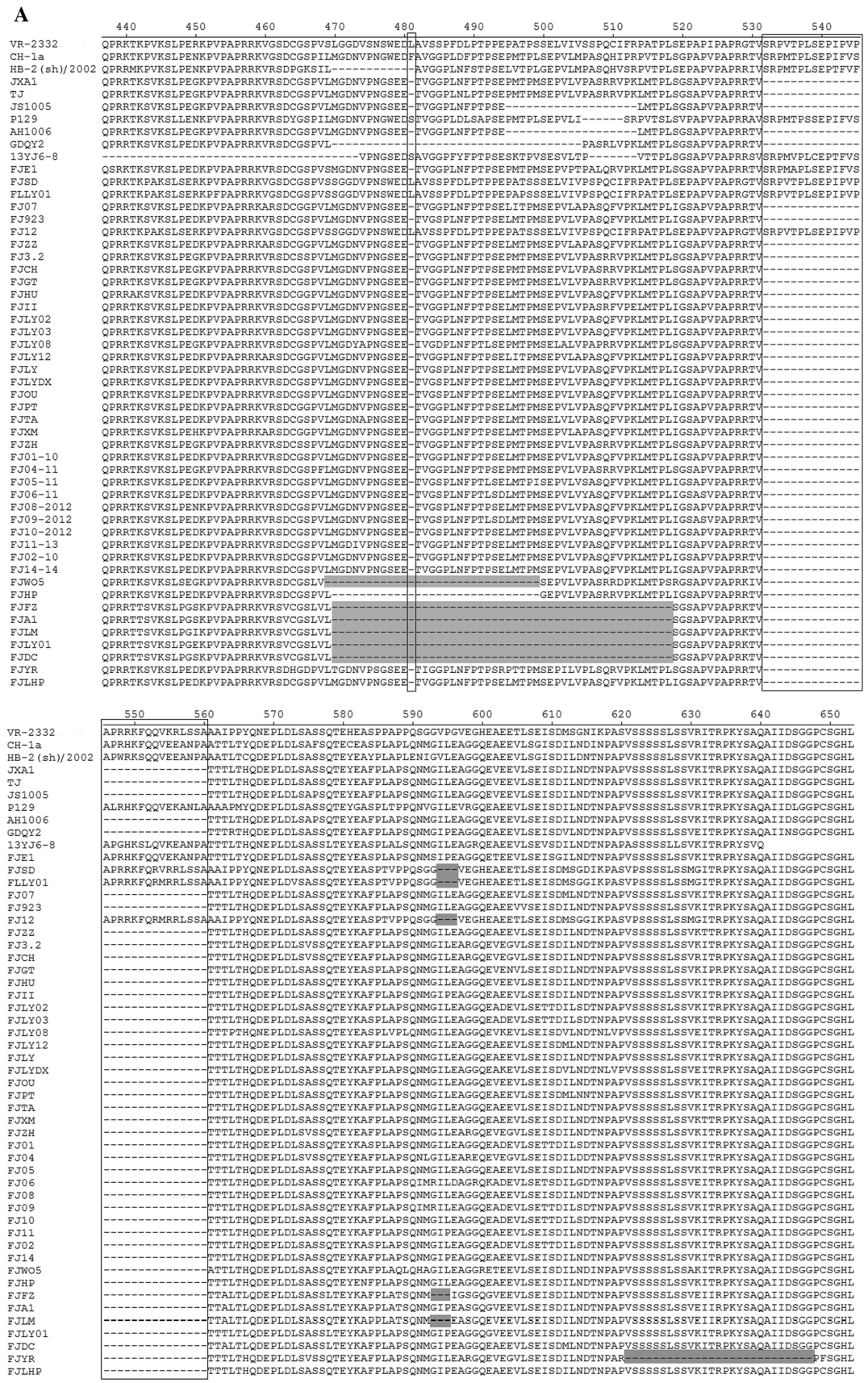
reference strains VR2332 and CH-1a indicated that the Fujian viruses had acquired three new discontinuous deletions at amino acids 470-518, 469-499, and 621-647. Furthermore, a 30-aa deletion at positions 532-560 was also observed in highly pathogenic strains (Fig. 3A). On the other hand, nine strains harbored discontinuous deletions similar to those in MN184 and NADC30 [28], including at amino acids 321-431 (111 aa), 481 (1 aa), and 502-520 (19 aa). Of note, a strain with these deletions has become increasingly prevalent since 2014 (Fig. 3B). Alignment of the FJFS isolate to VR2332 indicated that a 36-residue insertion at aa 813-848 was present along with a 30-residue deletion at positions 489-518 (Fig. 3C).

Recombination analysis

FJW05 clustered with highly pathogenic JXA1-like strains in phylogenetic trees based on ORF1b and ORF2-4 but clustered with NADC30-like strains in trees based on ORF5-7. Accordingly, FJW05 formed a minor branch between JXA1 and NADC30 clusters in a phylogenetic tree based on the full-length genome sequence (Fig. 4). Collectively, these results indicate that the FJW05 has a mosaic structure. To identify possible recombination events, we evaluated potential recombinants using seven algorithms (RDP, GeneConv, BootScan, MaxChi, Chimera, SiScan, and 3Seq) implemented in RDP 4.22. Two inter-lineage recombination events between lineages 1 (NADC30-like FJZ03) and 8.7 (JXA1-R) were identified, with recombination breakpoints at positions 1-1,090 and 13,770-15,563 (with reference to the VR-2332 strain, Fig. 4A-B). In addition, FJW05 was isolated from herds vaccinated with JXA1-R, suggesting that the vaccine may have spontaneously recombined with NADC30-like PRRSV strains.

Analysis of full-length genomic sequences

A total of 12 complete genome sequences were obtained from different herds (Table 3). Excluding poly(A) tails, these genomes were 15,016-15,407 nucleotides in length. Genome sequence alignments revealed 88.2-99.4% identity with VR2332, 83.4-99.2% identity with JXA1, 88.2-97.1% identity with CH-1a, and 82.9-97.1% identity with NADC30. However, the identity was 82.1% to 99.3%

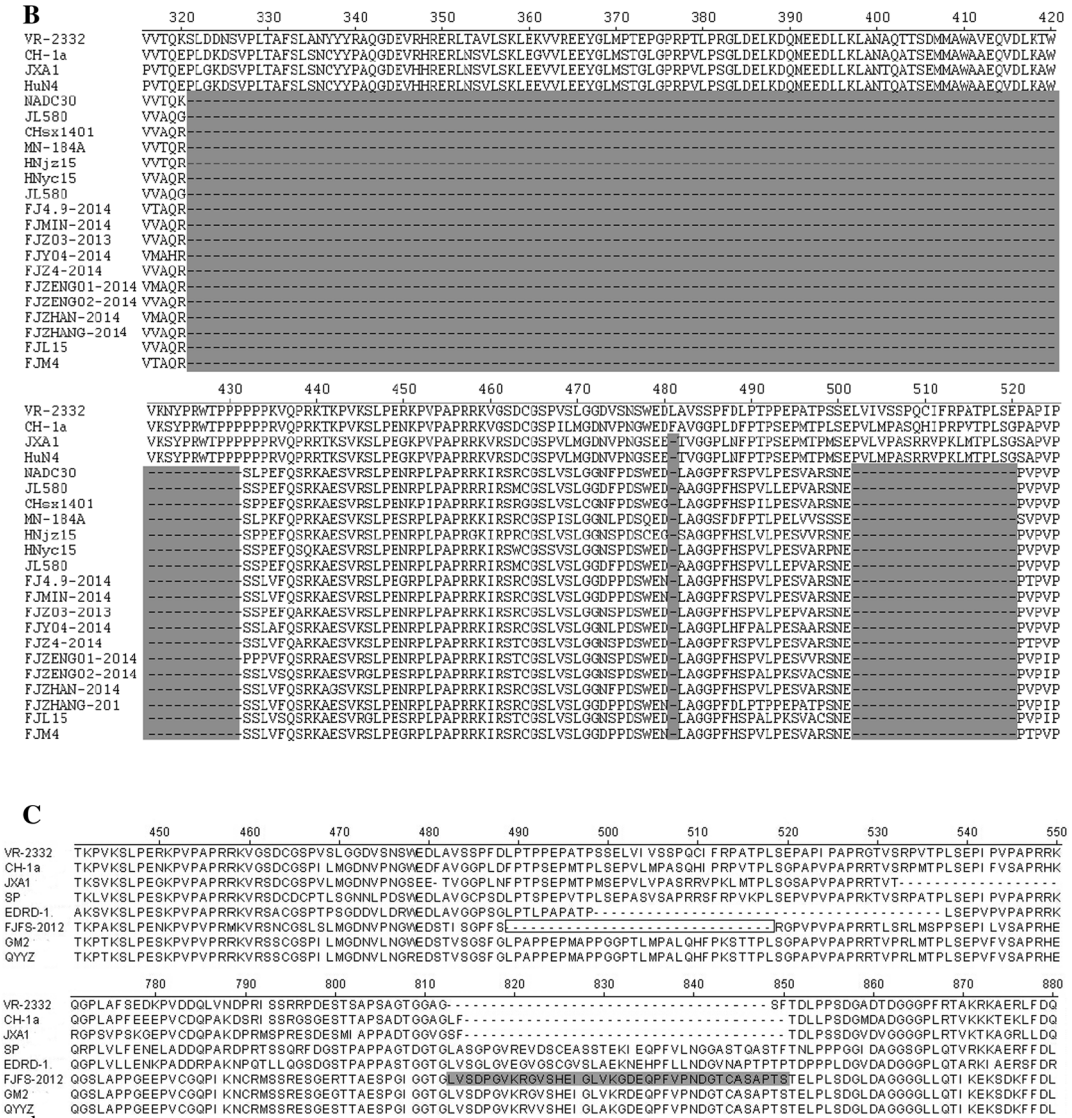

A
VR-2332
CH-1a
HB-2(sh)/2002
JXA1
TJ
JS1005
P129
AH1006
GDQY2
13YJ6-8
FJE1
FJSD
FLLY01
FJ07
FJ923
FJ12
FJZZ
FJ3.2
FJCH
FJGT
FJHU
FJII
FJLY02
FJLY03
FJLY08
FJLY12
FJLY
FJLYDX
FJOU
FJPT
FJTA
FJXM
FJZH
FJ01
FJ04
FJ05
FJ06
FJ08
FJ09
FJ10
FJ11
FJ02
FJ14
FJWO5
FJHP
FJFZ
FJA1
FJLM
FJLY01
FJDC
FJYR
FJLHP

among strains. Of note, FJZH and FJCH were most similar (99.5%-99.6%) to JXA1-R, a modified live virus vaccine from highly pathogenic JXA1, although the identity to JXA1 was also 99.1-99.2%.

A phylogenetic tree based on the full-length genome sequence, constructed from 36 representative PRRSVs and Fujian isolates (Table S4 in the supplemental material) indicated that the Fujian viruses cluster into five groups (Fig. 5). Isolates FJZH, FJCH, FJOU, FJYR, and FJW05 belonged to subgroup I, of which the reference strain is JXA1, while isolate FJE1 belonged to subgroup II, of which the reference strain is HB-1(sh)/2002. FJFS clustered with QYYZ as subgroup III, while VR2332 clustered with FJSD as subgroup IV. Finally, group V consisted of FJZ03, FJY04, FJM4, FJL15, and NADC30 (Fig. 5). The phylogenetic tree suggests that type 2 PRRSV has acquired considerable genetic variation and diversity.

Virus cross-neutralization

To evaluate whether antibody induced by the JXA1-R vaccine could neutralize representative PRRSV strains



◄**Fig. 3** Alignment of partial NSP2 amino acid sequences from representative Fujian isolates. NSP2 sequences from VR2332 (EF536003), CH-1a (AY032626), JXA1 (EF112445), NADC30 (JN654459), MN184A (DQ176019), SP (AF184212), QYYZ (JQ308798), and EDRD-1 (AB288356) were also used for comparison. (A) Known deletions in highly pathogenic strains (1+29aa) are indicated by a solid box, and previously unreported deletions are highlighted in dark gray. (B) In comparison to VR2332, a 131-aa in NSP2 was observed in some strains and is highlighted in dark gray. (C) Deletions in strain FJFS are indicated by a solid gray box, and insertions are highlighted in dark gray

◄**Fig. 3** continued

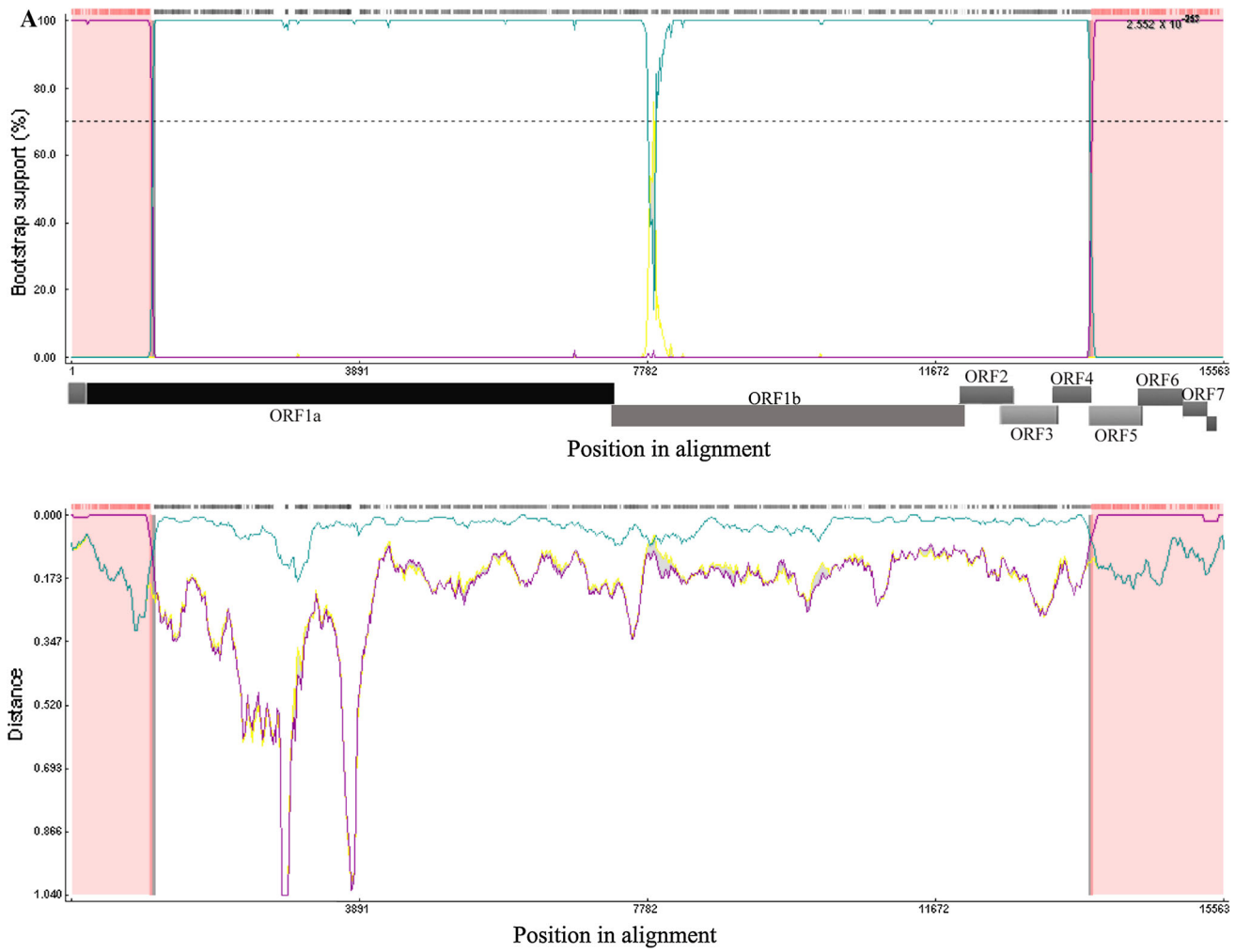


Fig. 4 Recombination between modified live vaccine JXA1-R and lineage 1 strain FJZ03 generates FJW05. (A) Two recombination break points were detected by RDP and bootscanning at the position 1-1090 and 13,770-15,563 of the sequence alignment (with reference to the VR-2332 strain). (B) Phylogenies of the parental regions in JXA1-R and FJZ03 are shown in a similarity plot

FJZH (JXA1-like, lineage 8.7), FJFS (QYYZ-like, lineage 3), FJSD (VR-2332-like, lineage 5.1), FJZ03 (NADC30-like, lineage 1), and FJW05 (mosaic) isolated in Fujian, sera (#1-4) were collected from four individual piglets vaccinated with the JXA1-R vaccine. Neutralizing antibody titers against FJZH, FJFS, FJSD, FJZ03, and FJW05 were calculated and were found to be significantly higher against FJZH and FJSD than against FJFS, FJW05, and FJZ03 ($P < 0.05$, Fig. 6).

Discussion

Since emerging in the 1990s, PRRSV has become widespread and continues to evolve rapidly, with virulent variants rapidly appearing and spreading. Despite global efforts to control and eradicate this virus, it continues to cause major economic losses throughout the world. Thus, we sought to investigate the genetic diversity and epidemiology of PRRSV in southeastern China in 2009-2014.

Due to the rapid growth in the number of PRRSV sequences deposited in the databases, it would be technically difficult for researchers to genotype the PRRSV by analyzing all of the sequences in the databases. A systematic classification of type 2 PRRSV has been conducted based on analysis of all available PRRSV ORF5 sequences in the databases including field isolates around the world and vaccine strains [16]. In this system, Type 2 PRRSV was divided into nine monophyletic lineages (1-9), HP-PRRSVs were clustered in sublineage 8.7. We can estimate the frequency of PRRSV transmission between different regions, identify new virus types, determine the major source of PRRSV and shed light on the direction of future vaccine development based on this system. The type 2 PRRSV isolates collected in this survey were found to belong to lineages 1, 3, 5 (5.1), and 8 (8.7) based on global

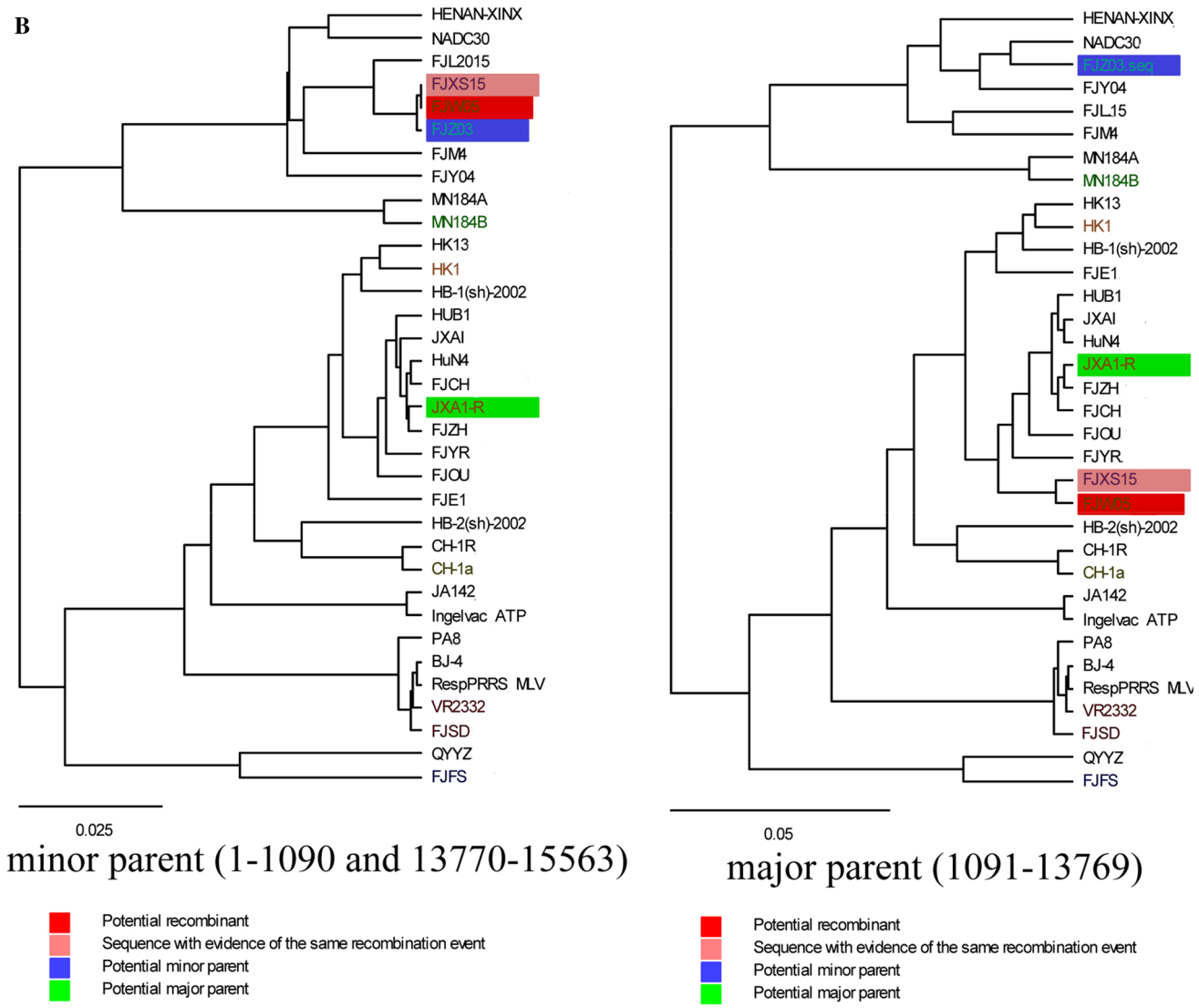


Fig. 4 continued

genotyping [16]. Of these, sublineage 8.7 was predominant in Fujian from 2009 to 2013. The FJFS isolate (lineage 3) is closely related to the isolate QYYZ and is thus likely to have originated in southern China before spreading into Fujian [29]. The six viruses in sublineage 5.1 are closely related to the Ingelvac PRRS modified live vaccine, while lineage 1 isolates are closely related to NADC30 and hence may have been introduced from North America in 2013. Previous studies have shown that mutation of N44 results in progeny that are noninfectious [26]. In the present study, FJ3.2 and FJ7-2 were found to have an N44K mutation, but they exhibited high replication rates and high titers in MARC-145 and PAM cells. Further study is warranted to determine the biological characteristics of N44 deletion isolates.

NSP2 is regarded as an ideal marker for molecular epidemiology and PRRSV evolution [16, 27, 30, 31]. In comparison to the reference strains VR2332 and CH-1a, seven discontinuous deletions within NSP2 were widespread in Fujian isolates. Genetic analysis of NSP2 genes showed that almost all strains from 2009 to 2013 contained 30-aa discontinuous deletions, and this has been recognized as the gene marker for HP-PRRSV [1, 10, 14, 15], suggesting that the HP-PRRSV strains were dominant in Fujian Province during 2009-2013. Strikingly, three of these deletions, at positions 470-518, 469-499, and 621-647, have never been reported previously. Whether these new deletions are associated with changes in virulence or pathogenicity requires further study, as most isolates retained genetic markers characteristic of highly pathogenic strains. Interestingly, 11 Fujian isolates harbored the same 131-aa NSP2 deletion as NADC30, although these isolates were quite divergent from each other (>12.0% dissimilarity), as well as from NADC30,

Table 3 Overview of the 10 complete genome sequences of Fujian Type 2 PRRSV

No.	Virus	GenBank accession no.	Collection year	Clinical signs	Genome length[a]	Nucleotide sequence identity (%)			
						VR2332	JXA1	NADC30	CH-1a
1	FJZ03	KP860909	2013	Respiratory	15016	85.9	84.0	97.1	84.9
2	FJY04	KP860910	2014	Respiratory	15016	85.0	83.4	96.0	84.2
3	FJW05	KP860911	2013	Reproductive failure	15226	88.2	95.4	85.7	92.5
4	FJSD	KP998474	2011	High fever	15402	99.4	89.4	86.3	91.3
5	FJE1	KP998475	2012	Reproductive failure	15407	88.8	96.0	84.0	94.2
6	FJFS	KP998476	2012	High fever	15424	87.1	87.9	82.9	88.2
7	FJCH	KP998477	2009	Respiratory/reproductive failure	15320	89.5	99.1	84.5	95.0
8	FJZH	KP998478	2010	Stillborn piglet	15320	89.5	99.2	84.4	95.0
9	FJOU	KP998479	2010	Respiratory/reproductive failure	15320	89.2	98.4	84.3	94.5
10	FJYR	KT804696	2014	Reproductive failure	15239	88.9	98.0	84.1	94.1
11	FJM4	KY412888	2014	Reproductive failure	15016	85.1	83.9	95.0	84.7
12	FJL15	KY412887	2014	High fever	15016	85.8	85.3	94.1	85.5

[a] Excluding the poly(A) tail

CH-1a, and VR-2332 (>15% dissimilarity). Of note, the FJFS strain harbored a 36-aa insertion in addition to the previously unreported 30-residue deletion at 469-499. Although the vaccine strain SP [32] and several Chinese strains [29] contain the same insertion, FJFS is only distantly related to SP (86.1% identity) and to the Chinese strain QYYZ (94.9% identity). In any case, the discontinuous deletions in NSP2 suggest that the virus may be evolving a more compact genome by eliminating dispensable segments [33].

All 12 strains except FJW05 clustered into the same five groups in separate phylogenetic trees based on the sequences of the complete genome and ORF5. Interestingly, FJW05 clustered into lineage 1 in trees based on ORF5, but into sublineage 8.7 in trees based on the complete genome. Analysis of the genomes also indicated that most glycosylation sites were highly conserved in the minor glycoproteins, although seven isolates had an N-glycosylation site at position 27 in GP3 that is absent in VR2332 and CH-1a. These sites might affect the host's ability to produce neutralizing antibodies.

Highly pathogenic PRRSV is widespread in Chinese swine herds [8–13, 15, 16], especially in Fujian, one of the largest livestock trading areas in China. Unfortunately, farm practices in the province are not stringently regulated, and Fujian is thus one of the most vulnerable to disease epidemics. Herd movements across provinces and national borders are also common. Hence, vaccination is one of the major measures applied to control swine PRRSV, and commercial live vaccines derived from highly pathogenic strains, including JXA1-R, HuN4-F112, TJM-F92, and GDr180, are the most widely used. Of note, strains FJZH and FJCH, which were isolated from two dying piglets on two separate farms, were found to have a high level of sequence identity (99.5-99.6%) to JXA1-R and are thus likely to be revertants, as reported previously [23]. In any case, overuse and cross-use of live vaccines is quite pervasive and appears to have resulted in rapid virus evolution, increased genetic diversity, and a wide range of virulence.

In this light, it is not surprising that highly pathogenic strains became the dominant circulating viruses in 2009. However, NADC30-like viruses might have been introduced in 2013, possibly from North America. As the genetic divergence of NADC30-like PRRSV from circulating strains would have significantly lowered the effectiveness of vaccines, the former rapidly became the predominant strain in 2014, especially in the absence of an effective strategy to control the disease or to monitor herd movements. Consequently, multiple PRRSV types now co-exist in swine herds, and recombination among strains has become a cause for concern. Live attenuated PRRSV vaccines have been a valuable tool in PRRS disease control [34]. In recent years, recombination between Chinese field strains and vaccine strains has been reported; these recombinant viruses showed markedly higher virulence than vaccine strains [35, 36]. Recombination is one of the important mechanisms of PRRSV evolution, playing a potential role in PRRSV replication, conditioning virulence, and immunization resistance [34, 37, 38]. In the present study, strain FJW05 appears to be a recombinant between the JXA1-

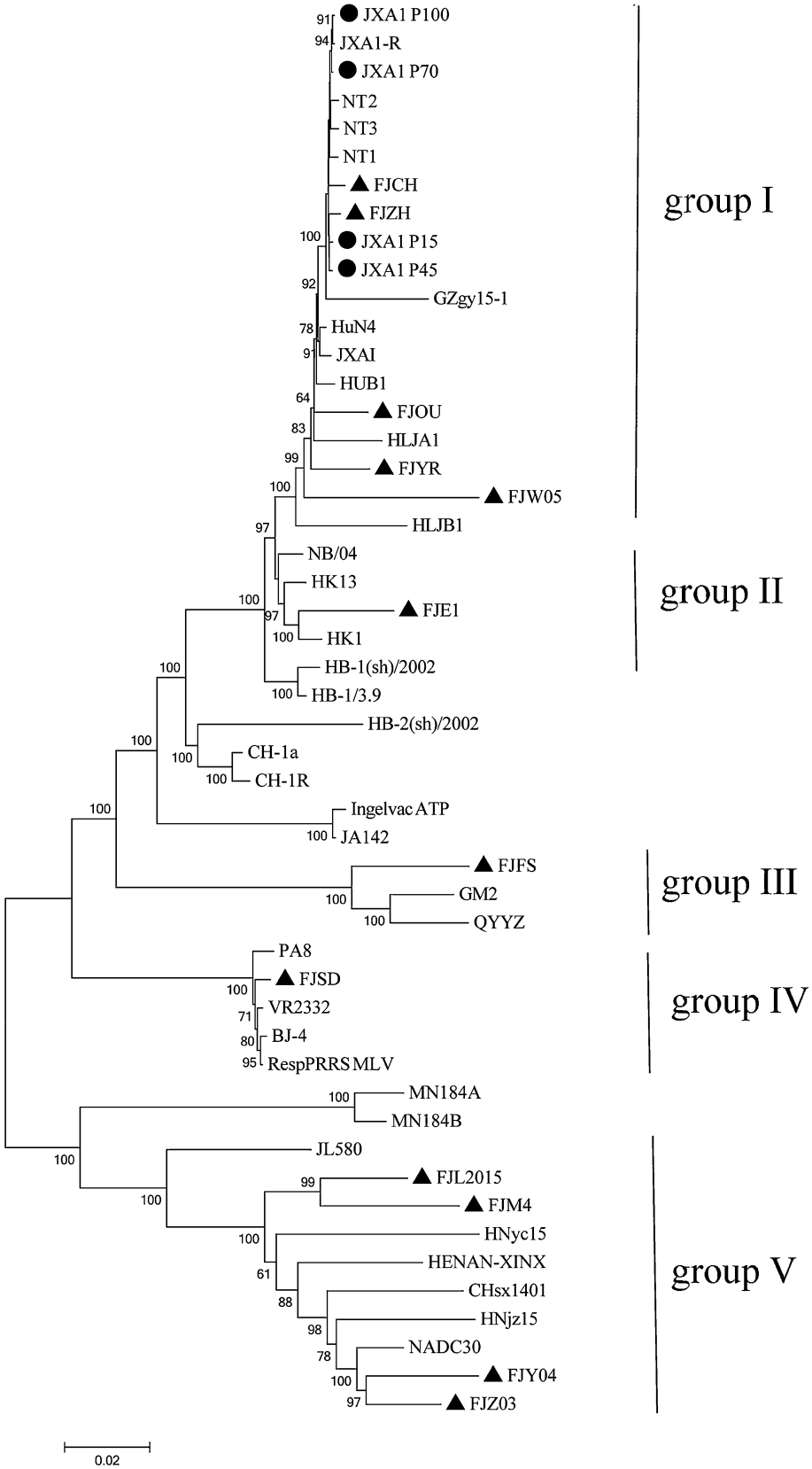


Fig. 5 Phylogenetic tree constructed from complete genome sequences of 12 isolates from 2009 to 2014 and of reference virus strains. Reliability was assessed by bootstrap analysis of 1,000 replications. Representative Fujian isolates are indicated by black triangles (▲). Derivatives of *in vitro*-passaged HP-PRRSV JXA1 in MARC-145 cells are indicated by a black circle (●)

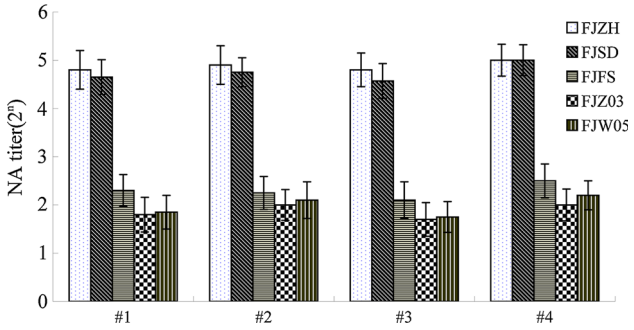


Fig. 6 Neutralizing antibody (NA) titers of pig immune sera against FJZH, FJSD, FJFS, FJZ03 and FJW05. Sera (#1, #2, #3 and #4) were collected from four individual piglets vaccinated with JXA1-R. Asterisks (**) indicate that the NA titer of the pig sera was significantly higher against FJZH and FJSD than against FJZ03 and FJW05 ($P < 0.01$). The error bars represent standard deviations of the three experiments

R vaccine and a NADC30-like lineage 1 virus. Importantly, the results of cross-neutralization assays suggested that the neutralizing antigens in NADC30-like FJZ03 and FJW05 are different from those in highly pathogenic PRRSV, indicating that the pathogenicity and biological characteristics of FJW05 warrant further study.

In summary, JXA1-like and NADC30-like viruses were the two predominant strains circulating in Fujian in 2009-2014. Our data enhance our understanding of the PRRSV epidemic in Fujian and may help veterinary workers establish suitable prevention and control policies for PRRS.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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