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Recombination and Mutation of a New Chinese Strain of HP-PRRSV SCMY2023

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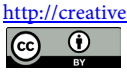


Abstract

In this study, RT-PCR was performed on lung tissue samples from sick pigs in a suspected outbreak of porcine reproductive and respiratory syndrome (PRRS) at a pig farm in Mianyang City, Sichuan Province, China. Positive samples were inoculated into Marc-145 cells to observe lesions. The Marc-145 cells with cytopathic lesions were identified by indirect immunofluorescence. The whole genome sequences of the isolated and purified strains were amplified by RT-PCR and analyzed for homology and genetic evolution. A strain of porcine reproductive and respiratory syndrome virus (PRRSV), named SCMY2023 (GenBank No. PQ179742), was successfully isolated. SCMY2023 has a genome length of 15,321 base pairs (without a poly A tail). Nucleotide and amino acid homology analyses suggest that this strain belongs to Lineage 8, a variant of the highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) prevalent in China. Recombination and genetic evolution analyses indicate that this isolate is a PRRSV variant that recombined with HuN-ZZ (Lineage 8, 98.79% homology) on the backbone of the SCSN2020 strain (Lineage 8, 99.35% homology) in the recombination region from 4407 to 13,107 nucleotides (ORF1a to ORF3). In-depth study of the genetic recombination of this isolate can provide a reference for the prevention and control of PRRS.

Keywords

PRRSV, Isolation and Identification, Recombinant Virus, Prevention and Control Strategy

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS), commonly known as porcine blue ear disease, was first identified in North America in 1987 [1]. The pathogen responsible is the porcine reproductive and respiratory syndrome virus (PRRSV), a single-stranded positive RNA virus belonging to the Arterivirus genus of the Arteriviridae family in the order Nidovirales. PRRSV causes reproductive disorders in sows and respiratory disease in pigs of all ages, posing significant threats and losses to the global pig industry [2] [3]. PRRSV has a total length of approximately 15 kb, and the genome contains multiple open reading frames (ORFs): ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF6, and ORF7 [4] [5]. Of these, ORF1a and ORF1b encode viral nonstructural proteins (NSP), whereas ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF6, and ORF7 encode viral structural proteins GP2, E, GP3, GP4, GP5, M, and N [6] [7]. The gene encoding the nonstructural protein 2 (Nsp2) is characterized by various insertions, deletions, and mutations. It has the highest degree of variation among non-structural protein-encoding genes and is often used as a molecular genetic marker for PRRSV [8] [9]. The GP5 protein encoded by ORF5 is the most varied among the structural proteins of PRRSV. It contains loci associated with viral neutralization and protection, making it a major candidate for developing a subunit vaccine [10] [11].

PRRSV can be categorized into two genotypes based on genetic and antigenic differences: PRRSV-1 and PRRSV-2. The nucleotide homology between them is about 55% to 70%, and the amino acid homology is about 50% to 80% [12]. Based on the PRRSV ORF5 gene sequence, PRRSV-2 types are categorized into nine lineages (Lineage 1 to 9), with Lineage 1, Lineage 3, Lineage 5, and Lineage 8 being the most prevalent in China [13] [14]. In the face of the risks and challenges posed by PRRSV, vaccination is the most effective and commonly used strategy to protect swine farms from infection. However, there is growing evidence that many live attenuated vaccines can recombine with field strains to form new strains. Additionally, point mutations and genomic recombination of wild-type strains have led to an increasingly complex PRRSV gene pool, making it difficult to control the virus [15]-[18].

In previous PRRSV studies, it has been found that the epidemiology of strains from different lineages in Chinese swine herds is characterized by distinct traits, with Lineage 8 playing a significant role. Since 2016, strains prevalent in Sichuan Province have mainly exhibited multiple recombinations, including JXA1-Like (Lineage 8) with NADC30-Like; VR2332-Like (closely related to Lineage 8) with JXA1-Like and NADC30-Like; and JXA1-Like with NADC30-Like and QYYZ-Like (Lineage 3) combinations [19]-[21]. The emergence of these recombination patterns suggests complex interactions between Lineage 8 strains and other lineage strains, significantly impacting the prevalence and variation of PRRSV in swine populations. Sichuan Province is a key pig-breeding region in China, where pig farming plays a crucial role in the local agricultural economy. The large number and dense distribution of pig herds provide a hotbed for PRRSV transmission

and mutation, conducive to studying its evolutionary dynamics in complex breeding environments. Since 2016, prevalent PRRSV strains in Sichuan Province have shown diverse and complex recombination patterns, indicating a rich viral gene pool in the swine herds and frequent interactions between strains of different lineages. This creates a natural laboratory for studying recombination mechanisms and evolutionary patterns. Additionally, the frequent movement of pigs between farms in this region increases the chance of virus transmission and spread, potentially accelerating mutation and the generation of new strains. Selecting Sichuan Province as the study area can help reveal the influence of local breeding patterns and pig movement on PRRSV evolution, providing a scientific basis for developing prevention and control strategies.

In this study, we isolated and purified a new strain of PRRSV from diseased and dead pig lung tissues on a farm in Mianyang City, Sichuan Province. This strain, named SCMY2023 (GenBank No. PQ179742), has a full genome length of 15,321 bp (excluding the poly A tail). Homology and genetic evolution analyses showed that this strain belongs to Lineage 8, a recombinant variant of the HP-PRRSV currently prevalent in China. Recombination and genetic evolution analyses indicated that this isolate is a recombinant from SCSN2020 (Lineage 8, 99.35% homology) as the primary virus parent and HuN-ZZ (Lineage 8, 98.79% homology) as the secondary virus parent. The recombination region is located between 4407 - 13,107 nt (ORF1a-ORF3). An in-depth study of the genetic recombination of this isolate may provide a reference for the prevention and control of PRRS.

2. Materials and Methods

2.1. Disease Material Collection, Virus Identification and Virus Isolation

Lung, liver, and spleen tissues were collected from diseased pigs during a suspected outbreak of porcine reproductive and respiratory syndrome at a farm in Mianyang City, Sichuan Province. These tissues were ground using a tissue homogenizer with saline, then labeled and frozen at −80˚C. RNA extraction from the tissue fluids and the HP-PRRSV JXA1 strain (used as a positive control and maintained in our laboratory) was performed according to the instructions of the Mini BEST Viral RNA/DNA Extraction Kit Ver.5.0 (Takara, Dalian, China). RNA extraction was also conducted following the instructions of the PrimeScript™ RT Reagent Kit (Takara, Dalian, China). RNA was extracted from the tissue fluid and the HP-PRRSV JXA1 strain (isolated and kept in our laboratory as a positive control) and then reverse transcribed according to the instructions of the PrimeScript™ RT reagent Kit (Takara, Dalian, China). The PRRSV nucleocapsid (N) protein was selected for monitoring because it is highly conserved [22]. The sequence of the CDS region of the PRRSV N protein was queried on the NCBI website (GenBank No. NP_047413.1), and identification primers were designed accordingly (**Table 1**). PCR reactions were performed on the reverse transcription products according to the instructions of the PrimeSTAR® Max DNA Polymerase

kit (Takara, Dalian, China). Cell homogenates positive by RT-PCR were filtered through a 0.22 μm filter and inoculated with Marc-145 cells. These were cultured in 10% fetal bovine serum (FBS) in DMEM at 37˚C and 5% CO_2 for 2 - 4 days until cytopathic effect (CPE) appeared. After three generations of blind transmission, the cell lysates were collected by freeze-thawing three times, and the RT-PCR steps were repeated. The target virus was confirmed in the positive cell lysates, which were then used to infect Marc-145 cells again. The cell cultures were re-identified by indirect immunofluorescence assay (IFA) using a monoclonal murine antibody against PRRSV N protein (Guangzhou Chance Bio-technology Co. Ltd, China, dilution 1:1000) and a fluorescent secondary antibody, goat anti-mouse IgG Alexa Fluor® 488 (Proteintech, America, dilution 1:500). In the virus identification process, the negative control was made from the homogenate of healthy pig tissues not infected with PRRSV, collected, processed, and stored the same way as the diseased tissues. The electrophoresis results after PCR amplification of the negative control were observed. If the target fragment band (372 bp) did not appear, it indicated no contamination by exogenous PRRSV nucleic acid, and the results were reliable. If the target band appeared, it suggested possible contamination, and the experiment needed to be investigated and repeated for verification. During the collection process, tissues (lung, liver, and spleen) were collected from at least three pigs to ensure sample representativeness. In virus identification and isolation experiments, the PCR test was repeated three times for each sample. The results were considered valid only if all three tests were consistent. For cell culture and subsequent assays, three replicate wells were set up for each experiment to minimize experimental error.

Table 1. PRRSV N protein primer sequences.

Primer name	Primer sequence (5'-3')
N Protein-F	ATGCCAAATAACAACGGCAAG
N Protein-R	TCATGCTGAGGGTGATGCTGT

2.2. Virus Whole Genome Amplification and Sequencing

The NCBI website was accessed to design 10 pairs of overlapping whole genome amplification primers (**Table 2**) based on the full-length sequence of JXA1 (GenBank No. EF112445.1) using Primer 5 software, synthesized by Sangyo Bioengineering (Shanghai) Co. The PCR program was set up as follows: 98˚C for 5 min, 98˚C for 10 s, 55˚C for 10 s, 72˚C for 30 s, followed by 30 cycles of 72˚C extension for 10 min, and termination at 4˚C. The PCR products were purified using the Agarose Gel DNA Recovery Kit (Solarbio, Beijing, China) and ligated to the pTOPO-Blunt Vector according to the TOPO-Blunt Lightning Cloning Kit instructions (GeneBetter, Beijing, China). Then, the ligated product was transferred into DH5α receptor cells (Takara, Dalian, China) at a 1:10 ratio. The plate was spread and cultured according to the instructions, and bacterial culture and PCR

identification were performed as previously set. The positive bacterial solution was sent to Sangon Bioengineering (Shanghai) Co. For the whole genome amplification experiment, three replicate tubes were set up for each primer pair to ensure reliable results. After sequencing, each fragment was verified by bidirectional sequencing to ensure accuracy. Fragments with doubtful results were re-amplified and sequenced to eliminate possible errors.

Table 2. JXA1 genome-wide amplification primers.

Primer name	Primer sequence (5'-3')	Amplified region/bp	Segment length/bp
JXAI-1F	ATGACGTATAGGTGTTGGCTCTATGC	1 - 1641	1641
JXAI-1R	AGGGAGCCTGAGGATTTGGAT		
JXAI-2F	ATCGCCAACCGGATGGT	1522 - 3178	1657
JXAI-2R	CGATGATGGCTTGAGCTGAGTA		
JXAI-3F	TGTCATCAAGCAGCTCCCTGT	3110 - 4770	1661
JXAI-3R	AAGGACGAGGTTCGCGGT		
JXAI-4F	TTTCCCGCTGGAGTGAAAGTT	4666 - 6353	1688
JXAI-4R	GCTGTCAGAAGCCTGATCATCAG		
JXAI-5F	AATGAGATTCTCCCAGCTGTCCT	6241 - 7997	1757
JXAI-5R	GCGCCTAATATCACAAGCCTGTAT		
JXAI-6F	GGAAACACTGGGATTGATGGC	7893 - 9652	1760
JXAI-6R	CCACACCAGATTATAACAGGACAATG		
JXAI-7F	CGTACGCCACTGCCTGTG	9571 - 11,233	1663
JXAI-7R	GGGAGGGACTCAGCAACTTCT		
JXAI-8F	TGCTTCCGGAGACAGTCTTCA	11,140 - 12,887	1748
JXAI-8R	GAACCATGAACCCTAGTTCGTCAT		
JXAI-9F	TGAACTCATGGTGAATTACACGGT	12,740 - 14,358	1619
JXAI-9R	GTAATGGAAAACGCCAAAAGCA		
JXAI-10F	GAGTTGTGCTTGATGGTTCCG	14,230 - 15,321	1092
JXAI-10R	TAATTACGGCCGCATGGTTC		

2.3. Virus $TCID_{50}$ Experiment

Marc-145 cells were inoculated into 96-well plates. When cell density reached 80%, the virus was serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} with DMEM containing 2% FBS. The diluted virus was then used to infect Marc-145 cells, with 8 replicate wells for each concentration. The cells were incubated for 96 hours at 37˚C with 5% CO_2. The number of CPE wells was observed for each viral

concentration, and the viral titer was calculated using the Reed-Muench method. This experiment was repeated independently three times, each using freshly cultured Marc-145 cells and freshly diluted virus solution to ensure result stability. The viral titer was determined by calculating the mean and standard deviation of each experiment's results. The final viral titer was accepted only when the mean and standard deviation across the three experiments were within a reasonable range.

2.4. Virus Whole Genome Sequence Comparison Analysis

The 10 sequenced fragments were spliced using DNAMAN software. The nucleotide and amino acid homology between the spliced SCMY2023 virus strain and the representative strains of each lineage (Table 3) were compared using Megalign software to determine the lineage of the isolates. To ensure accuracy, each sequence comparison was repeated three times. For discrepant results, the sequence data's accuracy was further checked, and the analysis was repeated until stable and consistent results were obtained.

Table 3. Reference sequences for homology analysis and their sources.

Sequence name	GenBank
SCMY2023	PQ179742.1
SCSN2020	OQ883907.1
JXA1	EF112445.1
HuN-ZZ	OR250810.1
CH-1a	AY032626.1
VR2332	U87392.3
QYYZ	JQ308798.1
GM2	JN662424.1
NADC34	MF326985.1
ISU10	KT257966.1
NADC30	JN654459.1
CHsx1401	KP861625.1
Lelystad virus	M96262.2
fs 2108	OR800929.1
CH-YY	MK450365.1
qy 2008	OR800928.1
sg 2108	OR800927.1
sg 2104	OR800926.1
WUH3	HM853673.2
P-GX-080630	GQ466009.1
SD 2020	MW408254.1

2.5. Phylogenetic and Recombination Analysis

The reference virus sequence was obtained by logging into the official NCBI website and uploading the full gene sequence of the SCMY2023 virus strain to BLASTn for comparison (Table 3). Recombination events were analyzed using Simplot software (Version 3.5.1, 200 bp sliding window, 20 bp step) and RDP4 software (Version 4.100, 200 bp sliding window, 20 bp step). A phylogenetic tree was constructed using MEGA 6.0 software to analyze the homology and genetic evolution. This tree compared the whole genome of the SCMY2023 isolate with the reference strain, the ORF5 gene, and the Nsp2 amino acid. Mutations, insertions, and deletions in the ORF5 and Nsp2 regions of the isolate were compared with those of the closest homologous reference strain. The recombination analyses using Simplot and RDP4 software were repeated three times to ensure the reliability of the detected recombination events. When constructing the phylogenetic tree, different methods (e.g., neighbor-joining, maximum likelihood) were used to construct the tree three times. Comparative analyses were then conducted to determine the final phylogenetic relationships only when the topology and branching relationships were consistent. For any abnormal results or uncertainties, relevant data and parameter settings were further examined to ensure the accuracy and stability of the analysis results.

3. Results

3.1. Pathogenetic Identification of Diseased Material Tissues

The JXA1 strain kept in our laboratory was used as a positive control. The RNA of the diseased tissue was extracted and reverse transcribed. The reverse transcription product was then used as a template for a PCR experiment, and the PCR amplification product was identified by 1% agarose gel electrophoresis. The results showed that a fragment of 372 bp was amplified from the diseased tissue, as expected (Figure 1), suggesting the presence of PRRSV in the diseased tissue.

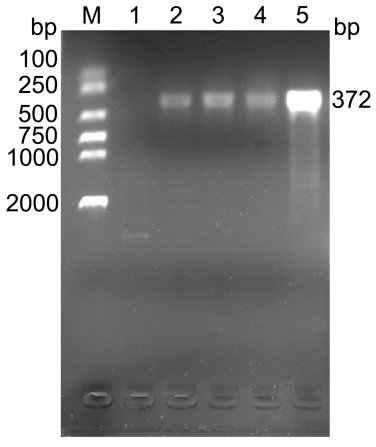


Figure 1. Pathogenicity PCR identification electropherograms: the individual lanes are, from left to right: M. DL 2000 DNA Marker; 1. Negative control; 2 - 4. Diseased tissue; 5. JXA1 positive control.

3.2. Virus Isolation and Characterization

The PCR-positive samples were inoculated into Marc-145 cells for successive passages, and the CPE phenomenon still appeared after three generations, with infected cells showing contraction, rounding, aggregation, and massive shedding (**Figure 2(b)**). The positive samples were infected with Marc-145 cells for 72 hours for IFA identification, and the infected group showed green fluorescence of PRRSV N protein antigen positivity compared with the normal control group (**Figure 3(e)** and **Figure 3(f)**). These results further confirmed the presence of PRRSV in the diseased tissues.

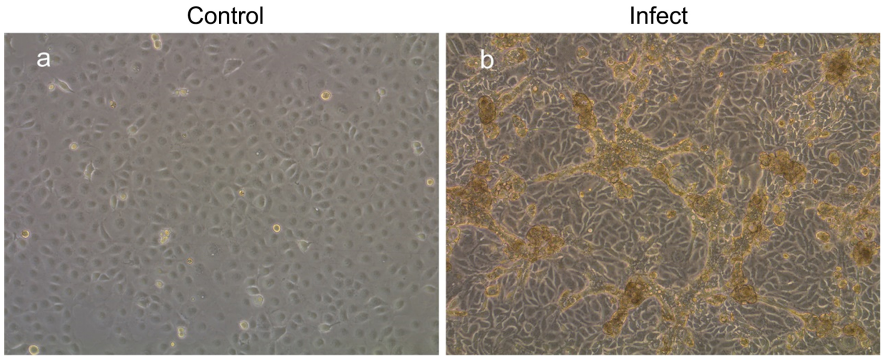


Figure 2. (a) Normal Marc-145 cells were cultured for 96 hours without any cytopathic effect (CPE). (b) CPE was observed in SCMY2023-infected Marc-145 cells after three generations of blind passaging.

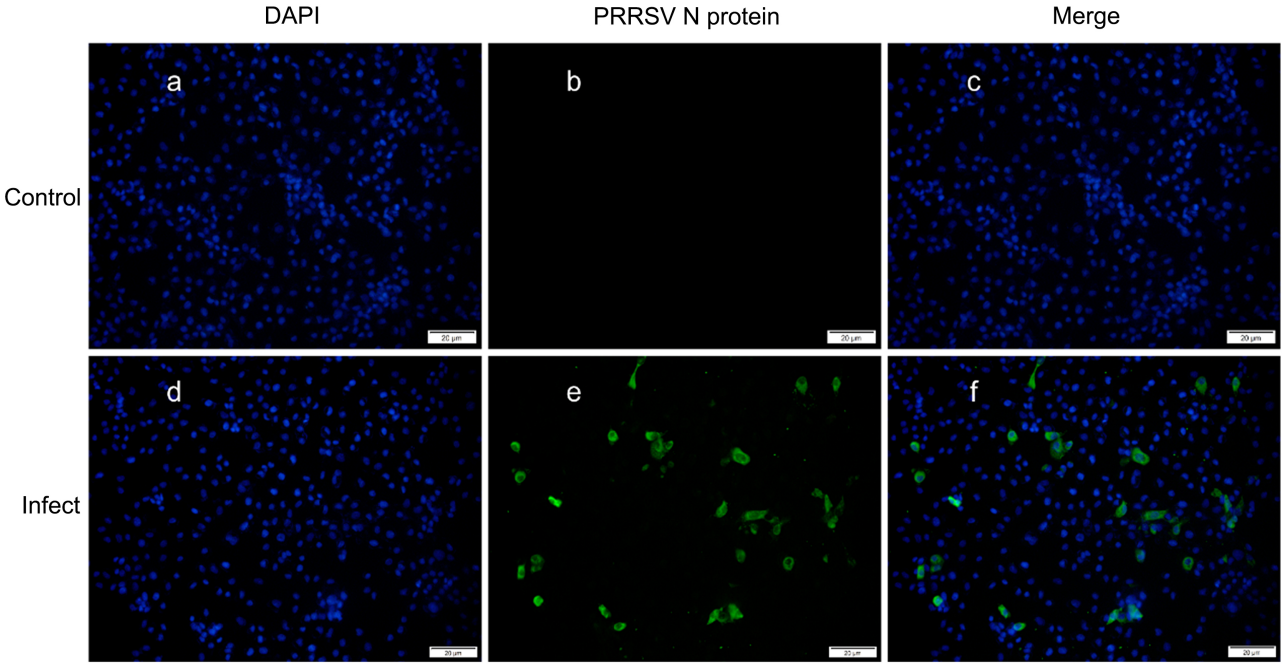


Figure 3. (a) Normal Marc-145 cells were cultured for 72 hours and then stained with DAPI. (b) Normal Marc-145 cells were cultured for 72 hours and identified using IFA with the PRRSV N protein. (c) Merge of (a) and (b). (d) SCMY2023-infected Marc-145 cells were cultured for 72 hours and then stained with DAPI. (e) SCMY2023-infected Marc-145 cells were cultured for 72 hours and identified using IFA with the PRRSV N protein. (f) Merge of (d) and (e). All pictures are scaled to 20 μm.

3.3. SCMY2023 Whole Genome Segmentation Amplification

The RNA of the SCMY2023 isolate was extracted for reverse transcription and verified by PCR amplification using 10 pairs of primers mentioned in Table 2. The results of agarose gel electrophoresis, shown in Figure 4, indicated a total of 10 fragments, all matching the expected size.

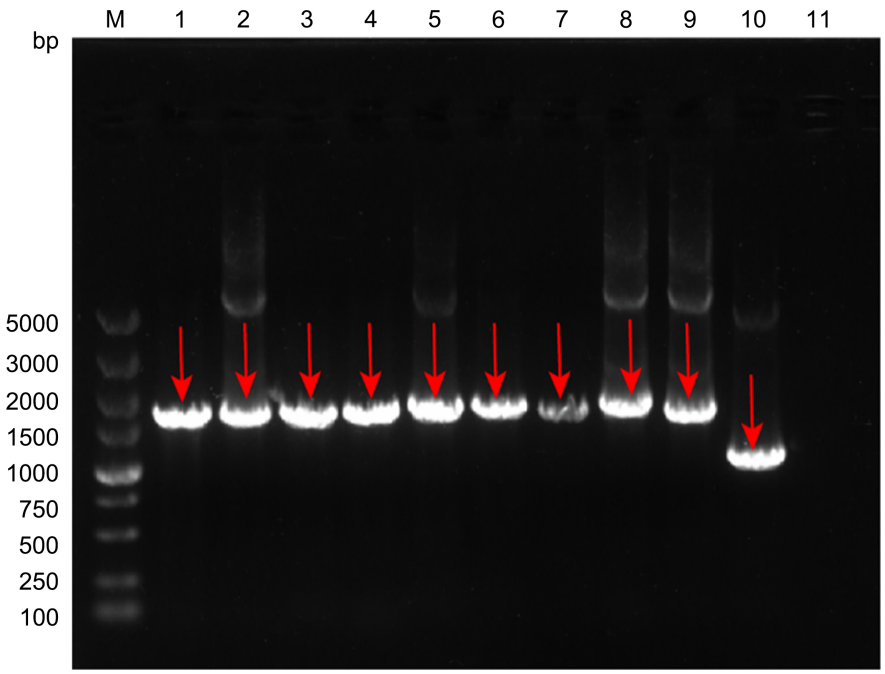


Figure 4. SCMY2023 whole genome segmented amplification electrophoresis results: The individual lanes are, from left to right, M. DL 5000 DNA Marker; 1 - 5. 5'UTR-ORF1a; 6 - 7. ORF1a-ORF1b; 8. ORF1b-ORF2; 9. ORF3-ORF5; 10. ORF5-5'UTR; 11. Negative control.

3.4. Virus $TCID_{50}$ Results

Marc-145 cells were inoculated into 96-well plates. Once the cells reached a suitable density, the third generation of the SCMY2023 isolate was infected with Marc-145 cells using successive dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} with DMEM containing 2% FBS. Eight wells were set up for each viral concentration and incubated for 96 hours at 37˚C with 5% CO_2. The number of CPE wells for each viral concentration was observed. The viral titer of the SCMY2023 isolate was calculated to be $10^{-3.75}$ $TCID_{50}$/mL according to the Reed-Muench method (MOI = 0.01).

3.5 Genome-Wide Characterization of SCMY2023

The whole genome sequence of SCMY2023 was assembled using DNAMAN software, with a length of 15,321 bp (excluding the poly A tail). The non-coding regions (UTR) were 189 nt (5'UTR) and 151 nt (3'UTR), respectively. Using DNAMAN's sequence comparison function, the nucleotide and amino acid homology of SCMY2023 were compared with representative strains of different

PRRSV spectrums (all strains were compared without the poly A tail). The results showed that the nucleotide homology of SCMY2023's whole genome with the representative strains ranged from 58.18% to 99.51%. The homology with Lelystad (PRRSV-1) was the lowest at 58.18%, while the homology with JXA1 (PRRSV-2, Sublineage 8.3) was the highest at 99.51%, followed by CH-1a (PRRSV-2, Sublineage 8.1) at 94.56%. Amino acid homology analysis showed that SCMY2023 had the lowest homology with Lelystad at 57.35%, and the highest with JXA1 and CH-1a at 99.51% and 94.54%, respectively (Table 4). Comparison with the reference strain showed that the SCMY2023 isolate had the highest nucleotide homology (98.70% - 100.00%) with the JXA1 strain across various regions of the genome (Table 5). This analysis indicates that the SCMY2023 isolate does not belong to PRRSV-1 but rather to Lineage 8 of PRRSV-2.

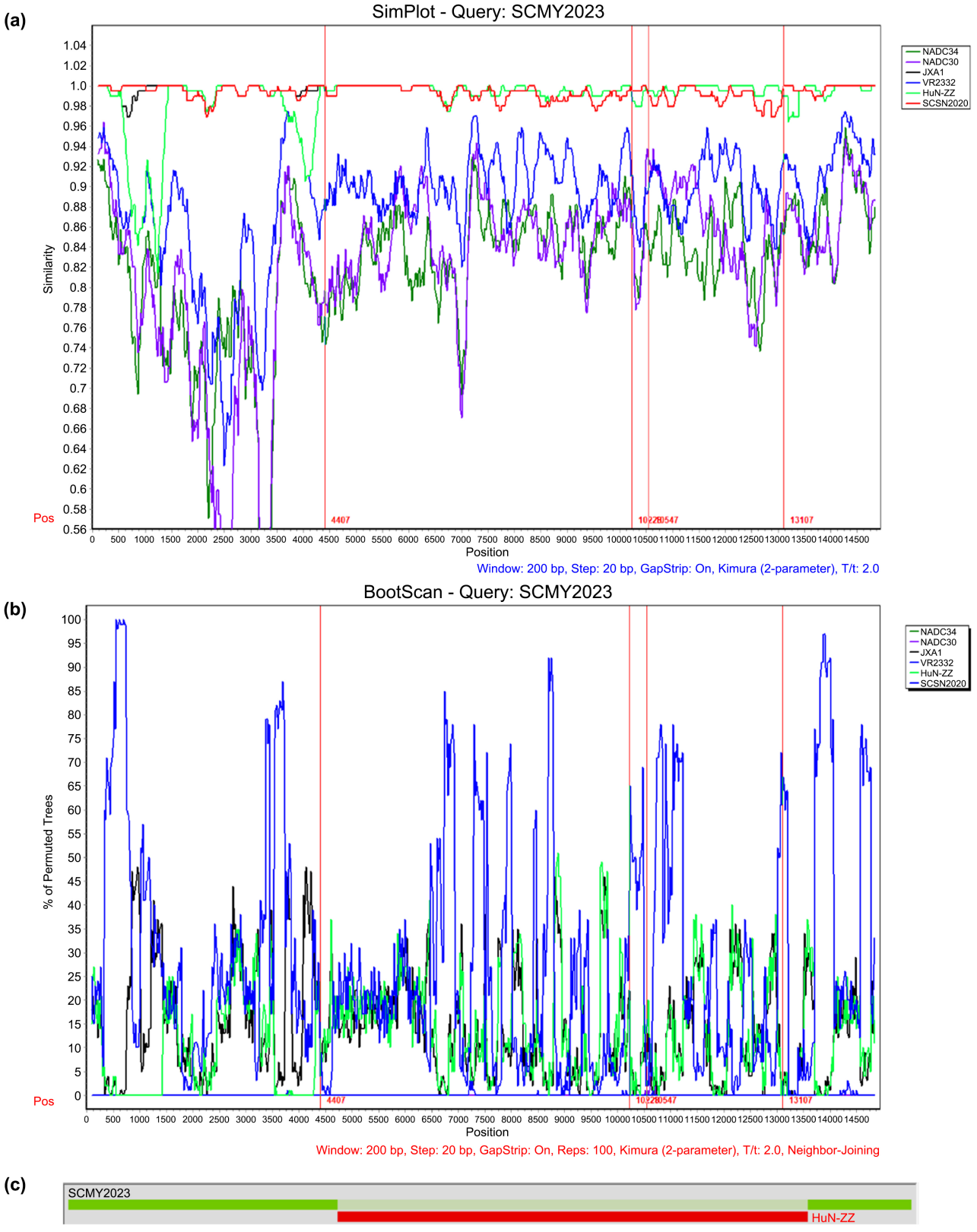
Table 4. Nucleotide and amino acid homology between the complete genome of SCMY2023 and representative strains of different lineages.

Homology (%)	Sublineage 1.5		Sublineage 1.8		Lineage 3		Sublineage 5.1	Sublineage 8.1	Sublineage 8.3	PRRSV-1
	NADC34	ISU-10	NADC30	CHsx1401	QYYZ	GM2	VR2332	CH-1a	JXA1	Lelystad
Nucleotide	82.33	81.88	82.71	81.93	86.66	86.49	89.03	94.56	99.51	58.18
Amino acids	82.22	81.94	82.71	81.92	86.69	86.48	89.02	94.54	99.51	57.35

Table 5. Comparison of nucleotide homology between each open reading frame of SCMY2023 and representative strains of each spectrum.

Homology (%)	Lineage 1	Lineage 3	Lineage 5	Lineage 8		PRRSV-1
	NADC30	QYYZ	VR2332	JXA1	CH-1a	Lelystad
5'UTR	91.62	95.26	92.06	100.00	93.37	51.80
ORF-1a	77.62	83.04	86.51	99.54	92.94	53.31
ORF-1b	71.39	90.17	90.90	99.41	95.82	62.45
ORF-2	86.25	90.14	93.13	99.87	96.24	63.55
ORF-3	83.66	90.59	89.15	100.00	95.82	62.05
ORF-4	87.52	94.79	90.32	98.70	96.83	64.86
ORF-5	86.07	84.08	89.55	99.50	95.36	61.33
ORF-6	88.76	90.86	94.86	99.81	97.33	69.14
ORF-7	90.86	89.52	93.82	99.73	95.97	60.35
3'UTR	89.40	88.74	93.38	100.00	96.03	54.30

3.6. SCMY2023 Gene Recombination and Genetic Evolution Analysis



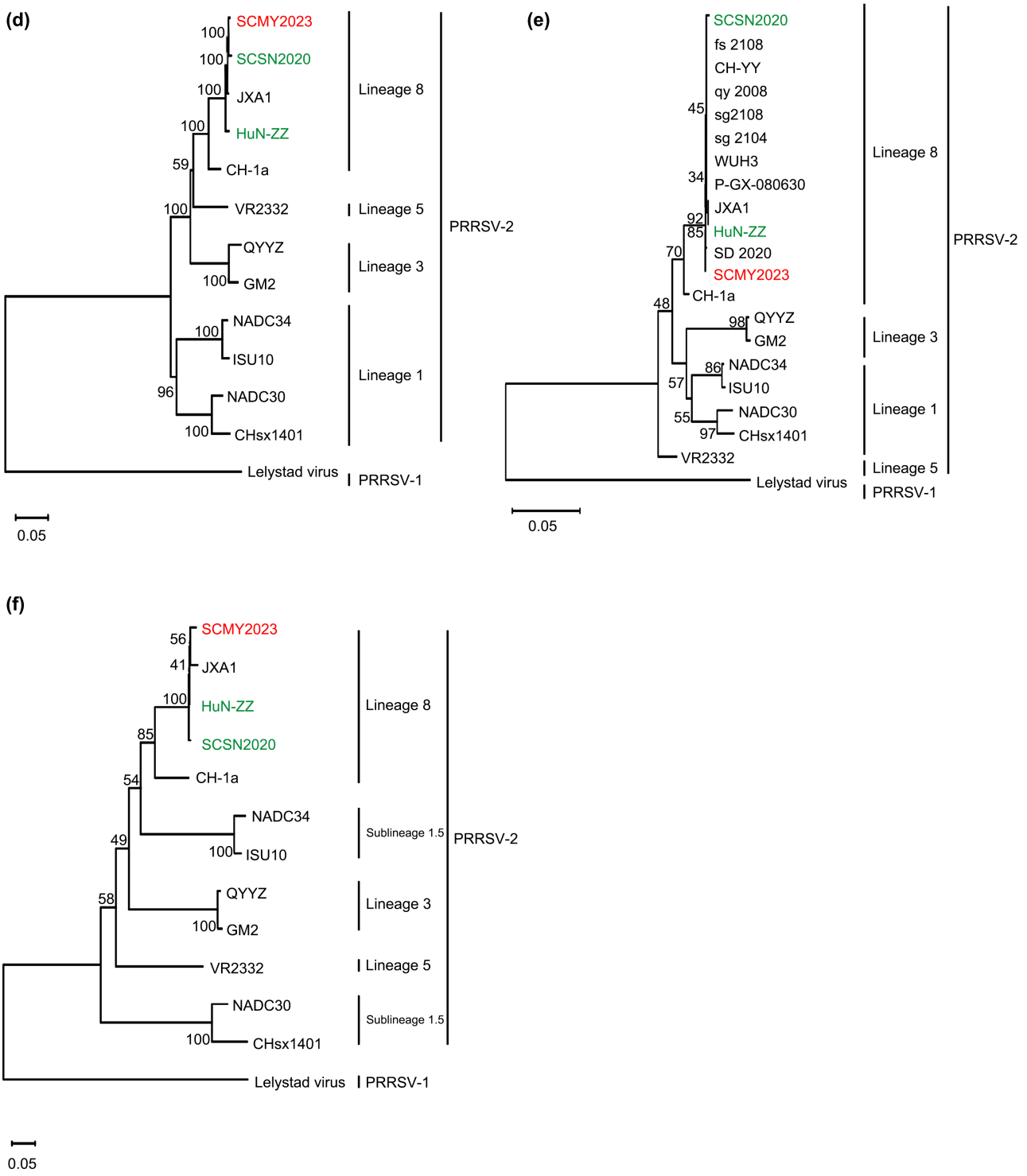


Figure 5. (a) Recombination analysis was conducted, and a similarity map was generated using Simplot 3.5.1 software with a window size of 200 bp and a step size of 20 bp. SCMY2023 was used as the query sequence in the similarity plot analysis. (b) BootScan analysis was performed and plotted using Simplot 3.5.1 software with the same window and step sizes, using SCMY2023 as the query sequence. (c) Recombination regions were shown with segments of different colors, and the two recombination breakpoints were detected by RDP v4.1016. Green segments represent the sequence of the SCSN2020 strain. The red segment represents the sequence of the HuN-ZZ strain. (d) Genome-wide evolutionary tree, neighbor-joining method, 1000 bootstrap. (e) ORF5 evolutionary tree, neighbor-joining method, 1000 bootstrap. (f) Nsp2 evolutionary tree, neighbor-joining method, 1000 bootstrap.

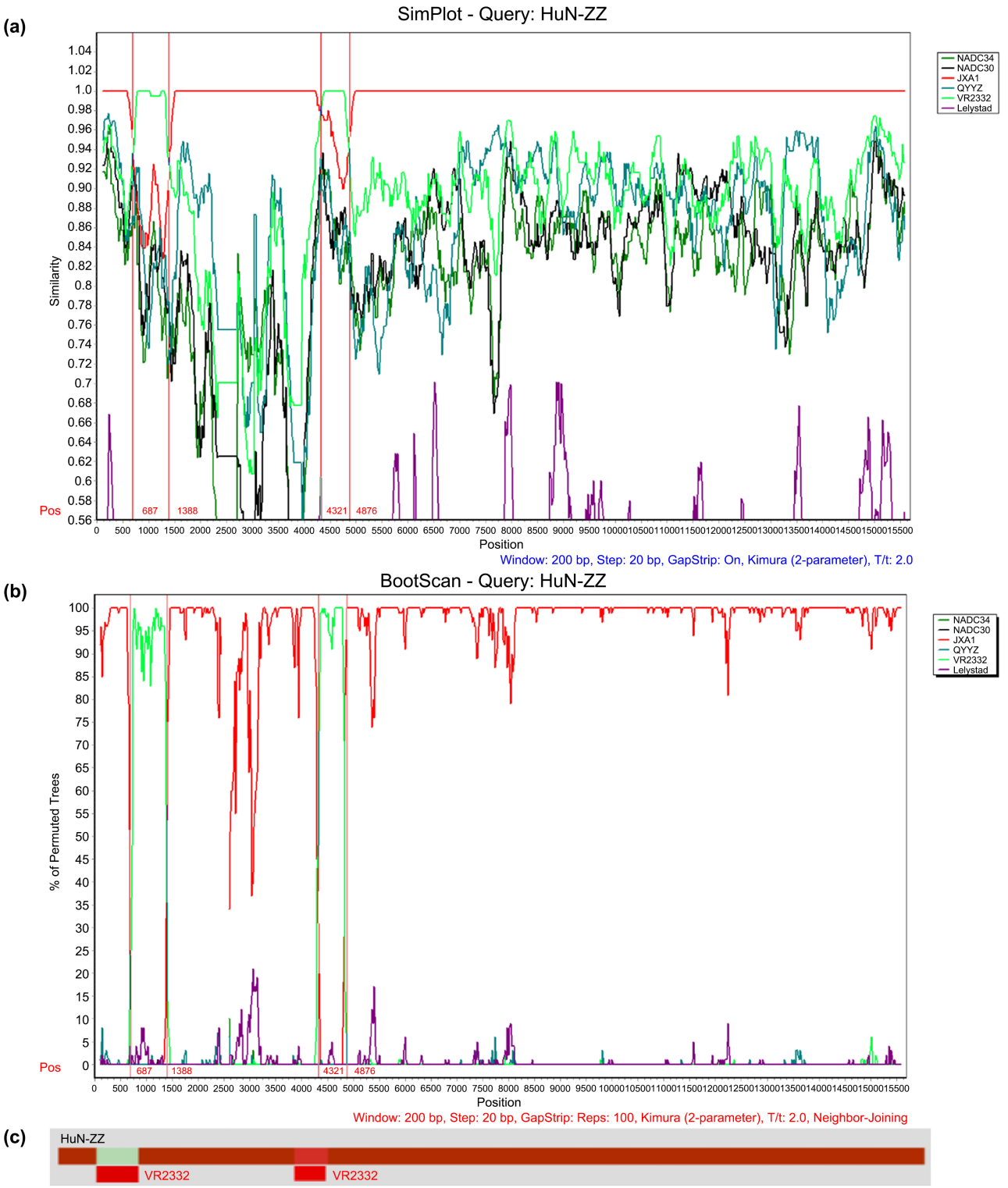


Figure 6. (a) Recombination analysis was conducted, and a similarity map was generated using Simplot 3.5.1 software with a window size of 200 bp and a step size of 20 bp. HuN-ZZ was used as the query sequence in the similarity plot analysis. (b) BootScan analysis was performed and plotted using Simplot 3.5.1 software with a window size of 200 bp and a step size of 20 bp, with HuN-ZZ as the query sequence. (c) Recombination regions were shown with segments of different colors, and the two recombination breakpoints were detected by RDP v4.1016. Red segments represent the sequence of the JXA1 strain, while two other color segments represent the sequence of the VR2332 strain.

The entire gene sequence of the SCMY2023 isolate was analyzed using SimPlot and RDP4 software. The results showed that the SCMY2023 isolate is a PRRSV variant formed by recombination of the SCSN2020 strain (GenBank No. OQ883907.1) from PRRSV-2 Lineage 8 with HuN-ZZ (GenBank No. OR250810.1) as the parental virus. The recombination region was located at 4407 - 13,107 nt (ORF1a-ORF3) (**Figure 5(a)**, **Figure 5(b)**, and **Figure 5(c)**). These two recombination breakpoints divided the genome into three regions. Regions 1 - 4406 and 13,108 - 15,321 had high homology with SCSN2020, while the region 4407 - 13,107, where recombination occurred, was closer in affinity to the HuN-ZZ strain. Specific analysis revealed that the large recombination region of 4407 - 13,107 could be divided into three parts. The region 10,241 - 10,547 remained highly homologous to the major parental virus SCSN2020, showing no obvious recombination. However, the regions 4407 - 10,240 and 10,548 - 17,107 were more homologous to the secondary parental virus HuN-ZZ, with a clear recombination phenomenon. Phylogenetic evolutionary trees were constructed for the whole gene sequences, ORF5 gene sequences, and Nsp2 amino acid sequences of the SCMY2023 isolate and the reference strain. The results showed that, although all three evolutionary trees indicated SCMY2023 belonged to the PRRSV-2 lineage 8, the whole genome tree suggested SCMY2023 was genetically closer to SCSN2020 than to HuN-ZZ (**Figure 5(d)**); similarly, the phylogenetic trees of ORF5 and Nsp2 sequences showed that SCMY2023 was more homologous to HuN-ZZ (**Figure 5(e)** and **Figure 5(f)**). These results indicated that the SCMY2023 isolate was recombinant, with SCSN2020 as the primary parental virus and HuN-ZZ as the secondary parental virus. Further investigation revealed that the primary parental virus, SCSN2020, is a JXA1-like strain that has not undergone recombination [5]. In contrast, the secondary parental virus, HuN-ZZ, was recombined with the JXA1-like strain as the primary parental virus and VR2332-like as the secondary parental virus. The recombination positions are shown below (**Figure 6(a)**, **Figure 6(b)**, and **Figure 6(c)**).

3.7. Amino Acid Sequence Analysis of ORF5 and Nsp2 in SCMY2023 Isolates

During the genetic evolution of PRRSV, the gene sequences of its ORF5 and Nsp2 coding regions often undergo insertions, deletions, and mutations. These changes can affect the virus's heritability and pathogenicity, making outcomes difficult to predict accurately. By analyzing the amino acid sequences of the ORF5 and Nsp2 coding regions of SCMY2023 against reference strains using DNAMAN software, we identified several mutations and deletions. Specifically, the ORF5 coding region of SCMY2023 exhibited a leucine-to-phenylalanine mutation at position 24, and the amino acid at position 195 was consistent with the primary parental virus SCSN2020, which was arginine. However, a leucine-to-arginine mutation occurred compared to JXA1 and the secondary parental virus HuN-ZZ (**Figure 7(a)**). In the Nsp2 coding region of SCMY2023, the amino acid at position 9 remained

consistent with JXA1 and HuN-ZZ but mutated from glycine to glutamate compared to the major parental strain SCSN2020. At position 134, SCMY2023 was consistent with JXA1, differing from the two parental strains with a mutation from glutamate to glycine. At positions 178 and 191, the amino acids of the other three strains were consistent; however, SCMY2023 differed from the reference strain with mutations from cysteine to phenylalanine at position 178 and from leucine to phenylalanine at position 191, respectively. Notably, at positions 25, 105, and 128, SCMY2023 remained consistent with the amino acids of the two parental viruses but differed from the JXA1 strain with mutations of serine to glycine, valine to alanine, and arginine to glycine, respectively (**Figure 7(b)**).

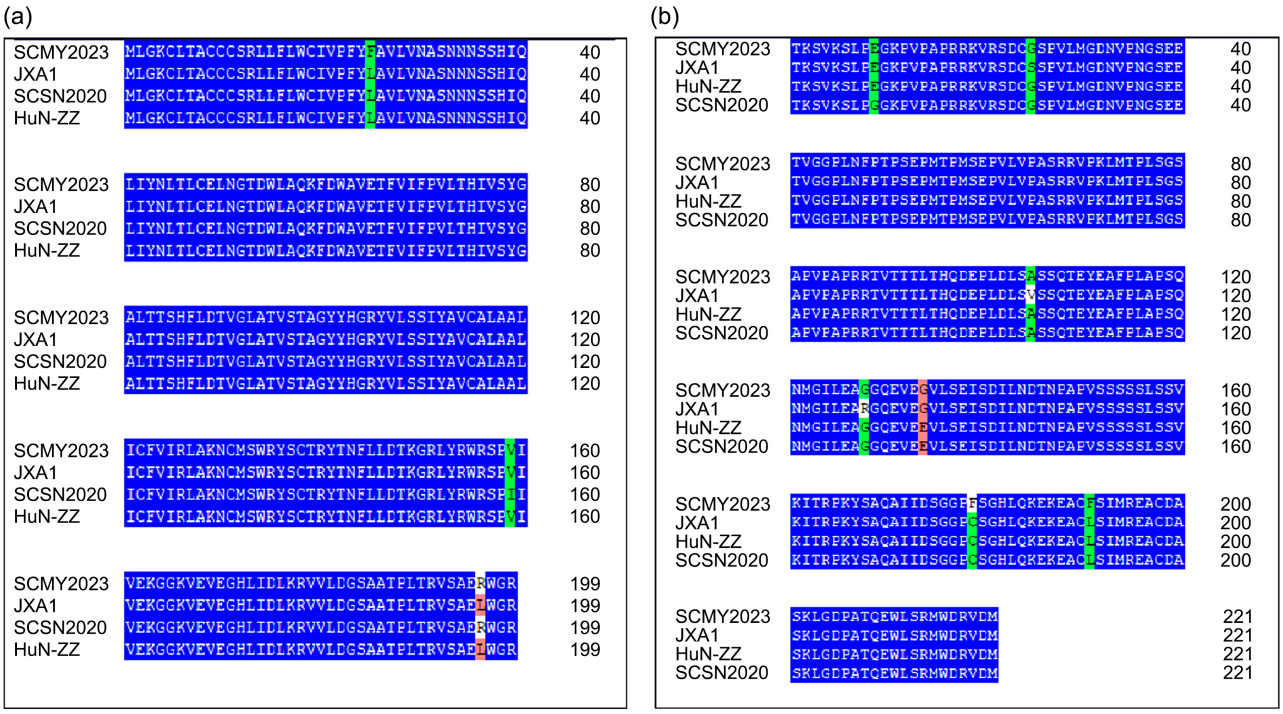


Figure 7. (a) ORF5 amino acid sequence comparison analysis. (b) Nsp2 amino acid sequence alignment analysis.

4. Discussion

As an RNA virus, PRRSV is prone to mutation during genetic evolution, a key feature of PRRSV. This is evident in frequent mutations within individual PRRSV strains and frequent recombination among multiple strains, resulting in a more complex PRRSV gene pool. Consequently, these viruses become increasingly difficult to control [10] [23]. According to published reports on PRRSV recombination, the number of reports of PRRSV-2 recombination is higher than that of PRRSV-1 [24]. This phenomenon appears to be related to the density of pig farming. In areas with high swine densities, such as Henan, Shandong, and Sichuan in China, or Iowa and Minnesota in the United States, the predominance of reported recombination cases in PRRSV may be due to the multispectral nature of PRRSV epidemics. This environment promotes co-infection within swine farms, creating

opportunities for recombinant mutations of PRRSV [15]. Vaccination remains the most effective and commonly used strategy to address PRRSV-induced infections in swine farms [25]. In light of the current status of vaccines and the characteristics of the SCMY2023 strain, more targeted vaccine strategies should be developed. Since SCMY2023 belongs to Lineage 8 and has a unique recombination pattern, research on the antigenic properties of strains in this lineage should be strengthened. A multivalent vaccine containing antigens specific to SCMY2023 should be developed to improve protective efficacy against these strains and their recombinant variants.

The genetic evolution of PRRSV mainly relies on recombination and mutation [26] [27], with the Nsp2 and ORF5 regions being highly susceptible to mutation in the whole genome sequence of PRRSV. The Nsp2 region contains immunogenic epitopes, indicating it is the immunogenic protein that triggers the production of specific antibodies during PRRSV infection [28]. The deletion and mutation of the Nsp2 region sequence tend to produce an amplification effect, potentially leading to altered PRRSV virulence or increased viral adaptations in the host, which can facilitate immune escape [6] [29]. For the ORF5-encoded GP5 protein, mutations in the ORF5 coding region may alter viral antigenic properties and host-virus interactions due to its role in mediating receptor binding and neutralizing antibodies [30]. These two regions are frequently the focus of attention in the study of mutations in PRRSV genes [5]. In this study, SCMY2023 isolates were compared with the Nsp2 and ORF5 regions of the reference strains JXA1, SCSN2020, and HuN-ZZ and were found to have the above mutation patterns (**Figure 7(a)** and **Figure 7(b)**). These mutation sites have not been reported in other references when comparing all previous studies [6] [19] [28] [31]-[35] based on the same region (Sichuan Province) within a contemporary time frame, which is a highlight of this study.

Based on the findings of this paper and practical applications, we propose the following meaningful conjectures: 1) The unique mutations in the Nsp2 and ORF5 regions of the SCMY2023 strain can guide the optimization of diagnostic methods. More specific diagnostic reagents can be developed based on these mutation sites, allowing for more accurate detection of pigs infected with this strain. This facilitates early diagnosis, timely isolation, and prevents further spread of the virus in the herd. 2) Given the high mutation rate of PRRSV, the virus needs continuous monitoring for both new strains and further mutations of known strains. For example, it is important to study the evolutionary dynamics of the SCMY2023 strain and its related parental strains in different environments and pig populations, as well as their potential new recombination events with other strains. This study has identified some effects of mutations in the Nsp2 and ORF5 regions of SCMY2023 strains on viral characterization, but many aspects still need further investigation. Specifically, how these mutations affect the viral replication mechanism, immune escape ability, and interaction with host cells. An in-depth understanding of these mechanisms can provide a theoretical basis for developing more effective

prevention and control strategies and vaccines. It is crucial to study vaccine immunity mechanisms, especially the effects of mutations in the Nsp2 and ORF5 regions on immune escape. This will help develop vaccines that induce a broader immune response, such as by optimizing the vaccine adjuvant or antigen delivery, to enhance the body's ability to recognize and clear the mutated strains. Since traditional prevention and control methods may have limitations against mutating PRRSV, future research should explore novel prevention and control techniques and strategies. For example, reverse genetic technology can be used to design and construct vaccine candidates in advance based on the mutation trends of prevalent strains. This approach can shorten the vaccine development cycle and enable a more timely response to emerging mutated strains.

In the swine farms covered in this paper, the farms where the SCMY2023 strain was isolated were characterized by overcrowded pig rearing environments and the misuse and abuse of vaccines by farm managers. Sick pigs exhibited clinical signs such as persistent high temperatures, convulsions, and diarrhea from onset to death. Both adult pigs and piglets suffered high mortality rates, especially piglets, highlighting the dangers of the persistent presence of HP-PRRSV. Notably, the SCMY2023 isolate was found in different pig farms in the same province as its main parental strain, SCSN2020, suggesting complexity in the regional spread of the virus.

It is recommended that the following measures be taken to reduce the risk of PRRSV infection and recombination during pig production. 1) Swine farm managers should select vaccines carefully based on local prevalent strains and vaccine efficacy data. Avoid using multiple types of vaccines indiscriminately to prevent recombination of vaccine strains with wild strains and the creation of new mutant strains. Specifically, in areas at risk of SCMY2023 strain epidemics, consider vaccines with better protection against this strain and its parent strains. Currently, the most common vaccine strains in Chinese pig farms include live vaccines such as R98, VR2332, JXA1-R, and TJM-F92. However, the current Lineage 1 virus is a recombinant dominant virus, and existing commercial PRRSV vaccines do not provide complete protection against this type of viral infection. Therefore, developing vaccines against Lineage 1 viruses is particularly necessary. 2) Strictly follow the vaccine instructions to ensure accurate dosing and timing. Additionally, regularly evaluate the vaccine's immunization effect and adjust the vaccination strategy based on the evaluation results. 3) Swine farms should determine the appropriate density of pigs based on their facilities and environmental conditions, avoid overcrowding, and provide enough living space to reduce virus transmission. A multi-point rearing mode can effectively reduce virus spread by dispersing pigs across different locations, preventing rapid transmission throughout the farm. Additionally, a certain distance should be maintained between feeding points, and hygiene management and disinfection measures should be strengthened to prevent cross-infection. 4) Pig farms should enhance disinfection protocols for personnel and vehicles. Personnel should change their clothes and shoes and undergo

full-body disinfection before entering the farms. Vehicles should be thoroughly cleaned and disinfected, especially the tires and undercarriage, before entering. Foreign animals are strictly prohibited to prevent virus introduction. If new pigs are introduced, they must be strictly isolated and quarantined to ensure they are not infected with PRRSV or other pathogens before joining the herd.

5. Conclusion

We comprehensively analyzed the new Chinese strain HP-PRRSV SCMY2023 and found that it has unique recombination and mutation patterns in NSP2 and ORF5. These patterns have not been reported before and are significant for predicting key regions of viral recombination. Meanwhile, since PRRSV shares a similar pattern of genetic evolution with other viruses in the Nidovirales, an in-depth study of PRRSV can help reveal the recombination mechanism and genetic evolution of the Nidovirales. Additionally, based on the findings of this paper, we propose scientific and practical conjectures, as well as preventive and control measures, to reduce PRRSV infections. These measures are crucial for minimizing the emergence of recombinant viruses and preventing PRRS.

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Authors' Contributions

Jida Li conceived and designed the study. Xianchang Zhu and Qian Lei performed the experiments. Wenhui Liu, Yang Xia, Zhaoxia Yang, Yi Zhang, and Bo Zhang analyzed the data. Xianchang Zhu drafted the manuscript. Jida Li critically reviewed and finalized the manuscript. All authors reviewed the manuscript before submission.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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