



# The prevalence and genetic diversity of porcine circovirus types 2 and 3 in Northeast China from 2015 to 2018

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## Abstract

A total of 472 samples from domestic pigs collected in China from 2015 to 2018 were tested for the presence of porcine circovirus types 2 and 3 (PCV2 and PCV3, respectively) by conventional polymerase chain reaction analysis. The prevalence of PCV2, PCV3, and PCV2/3 co-infection was 50.0%, 13.3%, and 6.78%, respectively. The complete genomic sequences of 66 PCV2 isolates and four PCV3 isolates were determined. Based phylogenetic analysis, the PCV2 isolates were assigned to three genotypes, PCV2a, PCV2b, and PCV2d, representing 13.6% (9/66), 25.8% (17/66), and 60.6% (40/66) of the total, respectively. All four PCV3 isolates shared a high degree of similarity in their complete nucleotide sequences (98.8–99.8% identity) and ORF2 amino acid sequences (98.6–99.5% identity). These results indicate that all three PCV2 genotypes (PCV2a, PCV2b, and PCV2d) are present on pig farms and that PCV2d has become the predominant genotype. The predicted amino acid sequences of the four PCV3 isolates indicated that PCV3-CN-JL53/PCV3-CN-LN56, PCV3-CN-HLJ3, and PCV3-CN-0710, belonged to the genotypes PCV3a, PCV3b, and PCV3a-IM, respectively. In view of the great harm that PCV2 causes to the pig industry, the epidemic trend of PCV3 should continue to be closely monitored. This study provides information about the prevalence, genetic diversity, and molecular epidemiology of PCV2 and PCV3 in China from 2015 to 2018.

## Introduction

Porcine circovirus (PCV), a member of the genus *Circovirus*, family *Circoviridae*, has two genotypes: porcine circovirus type 1 (PCV1) and porcine circovirus type 2 (PCV2) [26]. PCV1 was first identified as a contaminant of PK-15 cell culture in 1974 but is generally recognized as

non-pathogenic to pigs [34]. PCV2 is the etiological agent of PCV-associated disease (PCVAD), is generally considered to be one of the primary causative agents threatening the swine industry worldwide, and has resulted in large economic losses [22]. Recently, a novel PCV strain, designated as PCV3, was identified by metagenomic sequencing as the cause of acute porcine dermatitis and nephropathy syndrome (PDNS)-like clinical signs and reproductive failure in sows [24]. Moreover, a PCV3 virus stock from the rescue of an infectious PCV3 DNA clone was shown to successfully reproduce PDNS-like disease by PCV3 infection [14].

The PCV genome consists of a closed circular single strand of DNA. Within the genus *Circovirus*, non-pathogenic PCV1 and pathogenic PCV2 have a similar genomic organization. In most strains, the PCV2 genome is nine nucleotides longer than that of PCV1 (1768 vs. 1759 nucleotides [nt], respectively). PCV2, which is one of the smallest animal viruses, has no envelope and a closed single-stranded DNA genome of 1766–1769 nt [20]. The genome contains four well-characterized open reading frames (ORFs). PCV2 ORF1 encodes two replication proteins (Rep and Rep') that are both essential for viral replication via the rolling-circle

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replication mechanism [3]. PCV2 ORF2 encodes the capsid (Cap) protein, which is the only structural component of the virion and the dominant immunogenic antigen [21]. PCV2 ORF3 encodes an apoptotic protein [19], while ORF4 is not essential for viral replication, but has been associated with modulating the host immune response via regulation of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes during infection [13]. The PCV3 genome has two main ORFs: ORF1, which encodes a protein involved viral replication, and ORF2, which encodes the major structural Cap protein.

Point mutations and genetic recombination contribute to the evolution of PCV2 resulting in the generation of genetic diversity and driving rapid and global spread of the virus. Genotypic shifts may be associated with differences in pathogenicity and vaccine immunity. Based on pairwise sequence comparisons and linearized phylogenetic trees, as proposed by the EU consortium on PCV disease in 2008, PCV2 is classified into three genotypes: PCV2a, PCV2b, and PCV2c [32]. The PCV2d genotype was first identified by Guo et al. in 2010 [9], and it became the predominant genotype worldwide around 2012 [36]. Meanwhile, a new genotype, designated as PCV2e, was identified in swine in midwestern U.S. states and Mexico from 2006 to 2015 [5, 11]. In addition, a new genotype, designated as PCV2f, was identified in a retrospective study of PCV2 infection in China [1]. With an increasing number of complete PCV2 sequences available, a phylogenetic method based on a p-distance of 0.0035 for ORF2 and 0.02 for the complete sequences was recently proposed for classification of new isolates, but further refinement is needed ([www.PCVD.eu](http://www.PCVD.eu)). Hence, new criteria have been proposed: a maximum intra-genotype p-distance of 13% (based on the ORF2 gene), bootstrap support at the corresponding internal node of > 70%, and the availability of at least 15 sequences [7]. PCV3 was first reported in the USA in 2016 and has since been detected in China [2], Brazil [35], Korea [15], Japan [10], and many European countries [4, 6, 33]. PCV3 infection has been implicated as the cause of PDNS and reproductive failure, as well as cardiac and multisystemic inflammation [25]. Moreover, increasing evidence has shown that PCV3 coinfection with other pathogens might be associated with increased pathogenicity in pigs [2].

Since 2016, coinfection of PCV3 with PCV2 has frequently been reported in clinical samples from diseased pigs. Zhang et al. reported that, according to the results of a nano-dPCR assay, 14.7% of the samples were positive for PCV2, 16.6% were positive for PCV3, and 6.8% were positive for both viruses [40]. Ku et al. demonstrated that the positive rates for PCV2 and PCV3 in these samples were 62.2% (138/222) and 34.7% (77/222), respectively. The coinfection rate of PCV3 with PCV2 was 15.8% (35/222) [16].

The aim of this study was to analyze the genetic diversity of PCV2 and PCV3 by phylogenetic analysis based on

complete genome sequences to provide information regarding the occurrence of PCV2 and PCV3 in pigs in Northeast China from 2015 to 2018.

## Materials and methods

### Cells and monoclonal antibodies (mAbs) against PCV2

PK-15 cells, free of PCV1 contamination, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA), 100 IU of penicillin per mL, and 100 µg of streptomycin per mL at 37 °C in a humidified 5% CO<sub>2</sub> incubator. Based on the PCV2a Cap sequence, 13 mAbs (1H10, 2C1, 3A5, 4D6, 4F5, 5B9, 6B10, 8E10, 8G12, 10D3, 8E4, 5B8, and 1D2) were generated and preserved in our laboratory.

### Sample information

A total of 472 samples (including serum, lungs, and lymph nodes) were collected from diseased and healthy pigs in China from 2015 to 2018. Of the 472 clinical samples, 286 were from the northeastern provinces of Liao Ning, Ji Lin, and Hei Longjiang, while the others were collected from the provinces of Shan Dong (30), He Nan (24), Inner Mongolia (32), He Bei (37), Jiang Xi (33), Shan Xi (12), and Guang Xi (18). All samples were stored at -20 °C and submitted to the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (Harbin, China) for detection of PCV2 and PCV3.

### Polymerase chain reaction (PCR) analysis of PCV2 and PCV3

Viral genomic DNA was extracted from 200 µL of serum or tissue homogenate using a TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions and stored at -80 °C. The primers used for detection of PCV2 and PCV3 are listed in Table 1. For detection of PCV2 and PCV3, each 25-µL reaction mixture contained 12.5 µL of 2× *Taq* polymerase premix (TaKaRa Biotech, Dalian, China), 0.25 µL of each primer at a concentration of 10 µM, 2 µL of DNA template, and 10 µL of sterile water. The PCR amplification conditions were as follows: 2 min at 94 °C, then 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C for PCV2 and 30 s at 72 °C for PCV3, followed by a final elongation step of 10 min at 72 °C. The amplified PCR products for PCV2 and PCV3 were 1057 and 357 bp in length, respectively, as determined by 1% agarose gel electrophoresis.

**Table 1** Primers used in this study

Primer name	Primer sequence	Product size (bp)
PCV2-F56	CAGCAAGAAGAATGGAAGAAGCGGA	1057
PCV2-R1112	CCAGGACTACAATATCCGTGTAAC	
PCV2-CF	GGAAGCTTCAGTAATTTATTTTCATATGGAA	1767
PCV2-CR	GGAAGCTTTTTTATCACTTCGTAATGGTT	
PCV3-F28	GATCCACGGAGGTCTGTAGG	357
PCV3-R384	CACGTACCCTTGCAAGTGTG	
PCV3-F958	GGACTAGTAATGTTGTACCGGAGGAG	2000
PCV3-R964	GGACTAGTAATATATAATACCTTAGCCACAAAATTA AC	

### Amplification of the complete genomic sequences of PCV2 and PCV3

The complete genomic sequences of PCV2 and PCV3 were amplified by PCR using specific primer pairs (Table 1). The whole genomes of 66 PCV2 isolates and four PCV3 isolates were amplified, cloned, and sequenced. Each 50- $\mu$ L reaction mixture for PCR amplification contained 25  $\mu$ L of 2 $\times$  buffer, 10  $\mu$ L of 2 mM dNTP, 0.5  $\mu$ L of each primer at a concentration of 10  $\mu$ M, 2  $\mu$ L of DNA template, 1  $\mu$ L of KOD FX Neo polymerase solution (Toyobo, Shanghai, China), and 11  $\mu$ L of sterile water. The PCR amplification conditions were as follows: 2 min at 94  $^{\circ}$ C, then 35 cycles of 10 s at 98  $^{\circ}$ C, 30 s at 55  $^{\circ}$ C for PCV2 and 30 s at 52  $^{\circ}$ C for PCV3, and 1 min at 68  $^{\circ}$ C, followed by a final elongation step of 7 min at 68  $^{\circ}$ C. The PCR products were analyzed by 1% agarose gel electrophoresis. The PCR products of PCV2 (1766–1768 bp) and PCV3 (2000 bp) were purified using gel extraction kits (Axygen Biotech, Hangzhou, China), according to the manufacturer's instructions and cloned into the pMD18-T vector (TaKaRa Biotech, Dalian, China). The ligated products were introduced into competent *Escherichia coli* Top10 cells (Tiangen Biotech, Beijing, China) by transformation. The plasmids were confirmed by restriction enzyme digestion with *Hind*III for PCV2 and *Spe*I for PCV3, and then sequenced by Comate Bioscience (Jilin, China) using the primers M13 (-47) and RV-M.

### Alignment and phylogenetic analysis

Phylogenetic analysis is regarded as a powerful tool for investigating the evolution of viruses. In this study, multiple alignments were made using the Clustal W of the Molecular Evolutionary Genetics Analysis program, version 7.0 (MEGA7.0), and phylogenetic trees were constructed using the maximum-likelihood (ML) method based on 1000 bootstrap replicates. Alignment analysis was performed with DNASTar (Version 7.0) using the Clustal W method based on the amino acid sequences of

ORF1 and ORF2 of PCV2, and ORF2 of PCV3. Details of all sequences used in this study are summarized in Table 2.

### Reactivity of mAbs to different PCV2 isolates

In order to test the reactivity of mAbs to the different PCV2 genotypes, nine confirmed positive recombinant plasmids (pMD18-PCV2a-CY29052, PCV2a-YL2801, PCV2a-BX3109, PCV2b-YB2206, PCV2b-HC72302, PCV2b-HD625062, PCV2d-ZB2811, PCV2d-FX3116, and PCV2d-DP2209) were extracted using a TIANprep Midi Plasmid Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. After digestion with *Hind*III, genomic DNA was separated from plasmid DNA by electrophoresis on a 1% agarose gel. The method for constructing an infectious clone of PCV2 has been reported previously [8]. The rescued PCV2 clone was confirmed using an immunoperoxidase monolayer cell assay (IPMA) as described previously [18]. An immunofluorescence assay (IFA) was used to test the reactivity of different PCV2 mAbs to the rescued PCV2 isolates. Briefly, PK-15 cells in 96-well plates were infected with PCV2a-CY29052, PCV2a-YL2801, PCV2a-BX3109, PCV2b-YB2206, PCV2b-WK01015, PCV2b-HD625062, PCV2d-ZB2811, PCV2d-FX3116, or PCV2d-DP2209 or mock-infected for 48 h, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, and then permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. Afterward, the cells were incubated at 37 $^{\circ}$ C for 1 h with each of the 13 mAbs (diluted 1:500 in PBS) against PCV2 Cap. After washing three times, the cells were incubated at 37 $^{\circ}$ C for 1 h with goat anti-mouse IgG Abs conjugated with DyLight<sup>TM</sup> 488 fluorescent dye (diluted 1:500 in PBS; Pierce, Rockford, IL). The fluorescent signals were visualized using an EVOS inverted fluorescence microscope (Life Technologies, Carlsbad, CA, USA). The IFA assays were performed independently three times for each PCV2 isolate.

**Table 2** Information about PCV2 and PCV3 isolates obtained in this study

No.	Name	Area	Genotype	Genome size (nt)	Accession no.
1	BX3109	Hei Longjiang	PCV2a	1768	MK347388
2	WK040515	Liao Ning	PCV2a	1767	MK347366
3	CY29052	Hei Longjiang	PCV2a	1768	MK347405
4	YL2801	Hei Longjiang	PCV2-IM1	1767	MK347380
5	HLJ290315	Hei Longjiang	PCV2a	1768	MK347370
6	LN27F16	Liao Ning	PCV2a	1768	MK347374
7	HN32F16	He Nan	PCV2a	1768	MK347381
8	SD384F16	Shan Dong	PCV2-IM3	1768	MK347400
9	LY625123	Liao Ning	PCV2a	1768	MK347399
10	YB2206	Ji Lin	PCV2b	1767	MK347362
11	HC72302	Hei Longjiang	PCV2b	1767	MK347393
12	WK090315	Ji Lin	PCV2b	1767	MK347406
13	WK070215	Ji Lin	PCV2b	1766	MK347390
14	WK01015	Liao Ning	PCV2b	1767	MK347408
15	HLJ120215	Hei Longjiang	PCV2b	1767	MK347394
16	WK040615	Liao Ning	PCV2b	1766	MK347402
17	HD625062	He Bei	PCV2b	1767	MK347349
18	HLJ172415	Hei Longjiang	PCV2b	1767	MK347359
19	NMG3L16	Inner Mongolia	PCV2b	1767	MK347386
20	HB2015	He Bei	PCV2b	1767	MK347411
21	NMG6F16	Inner Mongolia	PCV2b	1767	MK347385
22	LN590516	Liao Ning	PCV2b	1767	MK347352
23	WK09015	Ji Lin	PCV2b	1767	MK347395
24	HLJ120515	Hei Longjiang	PCV2b	1767	MK347384
25	HB140615	He Bei	PCV2b	1767	MK347360
26	LN200615	Liao Ning	PCV2b	1767	MK347396
27	DL1802	Liao Ning	PCV2d	1767	MK347391
28	YK1805	Liao Ning	PCV2d	1767	MK347369
29	DP2209	Liao Ning	PCV2d	1767	MK347401
30	BD2210	He Bei	PCV2d	1767	MK347412
31	XT2807	He Bei	PCV2d	1767	MK347375
32	ZB2811	Shan Dong	PCV2d	1767	MK347389
33	FX3116	Hei Longjiang	PCV2d	1767	MK347398
34	PF29053	Hei Longjiang	PCV2d	1767	MK347379
35	TL3004	Hei Longjiang	PCV2d	1767	MK347410
36	XA07174	Hei Longjiang	PCV2d	1767	MK347358
37	LYD07215	Ji Lin	PCV2d	1767	MK347383
38	JMS07252	Hei Longjiang	PCV2d	1767	MK347373
39	LY625121	Liao Ning	PCV2d	1767	MK347409
40	PT625135	Liao Ning	PCV2d	1767	MK347355
41	HB12F16	He Bei	PCV2d	1767	MK347361
42	BJ19L16	He Bei	PCV2d	1767	MK347354
43	SX20F16	Shan Xi	PCV2d	1767	MK347377
44	SX21L16	Shan Xi	PCV2d	1767	MK347351
45	SD385F16	Shan Dong	PCV2d	1767	MK347387
46	SD446F16	Shan Dong	PCV2d	1767	MK347371
47	SD542L16	Shan Dong	PCV2d	1767	MK347353
48	HB560616	He Bei	PCV2d	1767	MK347364
49	JL1201415	Ji Lin	PCV2d	1767	MK347397
50	JL9201415	Ji Lin	PCV2d	1767	MK347404
51	GX2015	Guang Xi	PCV2d	1767	MK347413

**Table 2** (continued)

No.	Name	Area	Genotype	Genome size (nt)	Accession no.
52	HLJ2015	Hei Longjiang	PCV2d	1767	MK347407
53	JLA022915	Ji Lin	PCV2d	1767	MK347357
54	HLJ09215	Hei Longjiang	PCV2d	1766	MK347365
55	HLJ0915	Hei Longjiang	PCV2d	1767	MK347368
56	WK05015	Hei Longjiang	PCV2d	1767	MK347382
57	WK030415	Ji Lin	PCV2d	1767	MK347350
58	HLJ090215	Hei Longjiang	PCV2d	1767	MK347403
59	HLJ09115	Hei Longjiang	PCV2d	1767	MK347376
60	LN210615	Liao Ning	PCV2d	1767	MK347367
61	WK02015	Inner Mongolia	PCV2d	1767	MK347372
62	YJ070315	Hei Longjiang	PCV2d	1767	MK347378
63	GX130315	Guang Xi	PCV2d	1767	MK347348
64	WK080315	Ji Lin	PCV2d	1767	MK347356
65	GX130415	Guang Xi	PCV2d	1767	MK347392
66	JS250315	Jiang Su	PCV2d	1767	MK347363
67	CN-LN56	Liao Ning	PCV3a	2000	MK347416
68	CN-JL53	Ji Lin	PCV3a	2000	MK347414
69	CN-HLJ3	Hei Longjiang	PCV3b	2000	MK347415
70	CN-0710	Hei Longjiang	PCV3-IM	2000	MK347417

### Restriction fragment length polymorphism (RFLP) analysis of PCV2

In this study, a linearized genome of PCV2 containing a *Hind*III restriction enzyme site inserted into the viral genome was constructed. Each primer was modified by two nucleotides to create a *Hind*III restriction enzyme site as a traceable marker (G to A and C to G at positions 996 and 997, respectively). Using this method to linearize the complete PCV2 genomic sequences, the locations of restriction endonuclease sites were identified using Bioedit software (version 7.1.3.0; Biosoft, Cambridge, UK). PCV2a has no *Ase*I site, while PCV2b and PCV2d have only one. The purified PCR-PCV2 products were first digested with *Ase*I to distinguish PCV2a from PCV2b and PCV2d. Based on the analysis of PCV2b and PCV2d, PCV2b has only one *Sac*I site, while PCV2d has one or two. In this way, PCV2d was positively identified when three fragments appeared, but the genotype could not be determined when two fragments appeared after digestion by *Sac*I because PCV2b has only one *Sac*I site. The rescued virus could be differentiated from wild-type PCV2 by PCR and RFLP analysis.

### GenBank accession numbers

The complete genomic sequences of the PCV2 and PCV3 isolates analyzed in this study have been submitted to the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>)

using the Sequin DNA sequence submission tool (<https://www.ncbi.nlm.nih.gov/Sequin/>).

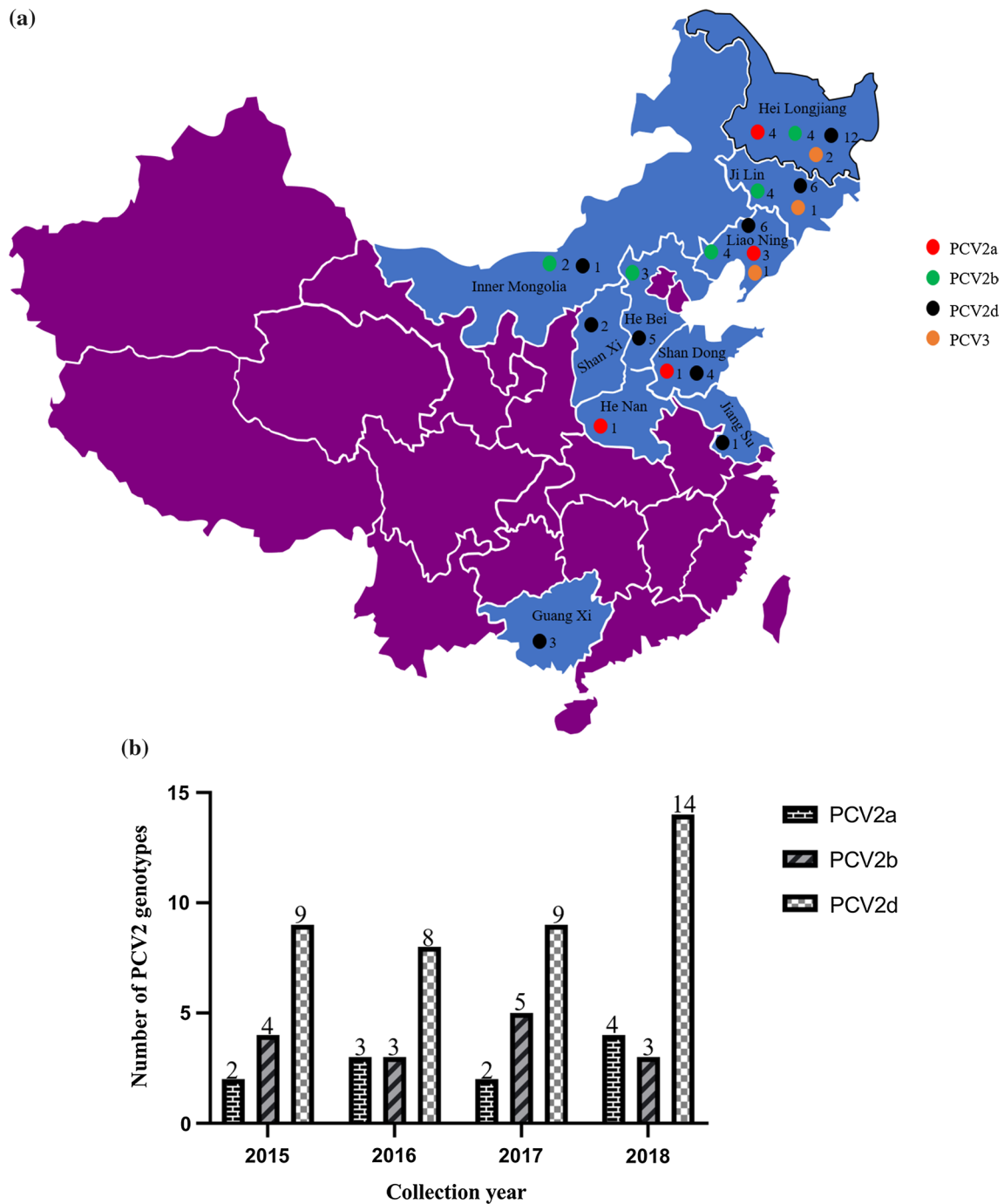
## Results

### Prevalence and complete sequences of PCV2 and PCV3

A total of 472 samples collected from diseased and healthy pigs in different geographical areas of China were tested for PCV2 and PCV3 by conventional PCR with specific primers. The prevalence of PCV2, PCV3, and PCV2/3 coinfection was 50.0% (236/472), 13.3% (63/472), and 6.78% (32/472), respectively. The number of PCV2a, b, and d isolates and PCV3 isolates in each province for which complete genome sequences were determined is shown in Fig. 1a and the prevalence of the different PCV2 genotypes by year is shown in Fig. 1b. All of these sequences were submitted to the GenBank database.

### Phylogenetic analysis based on the complete genome sequences of PCV2

The complete genomic sequences of 66 PCV2 isolates were assigned to three genotypes: PCV2a, PCV2b, and PCV2d. Based on phylogenetic analysis, PCV2a, PCV2b, and PCV2d represented 13.6% (9/66), 25.8% (17/66), and 60.6% (40/66) of the total, respectively (Fig. 2). The genotypes PCV2c, PCV2e, and PCV2f were not found



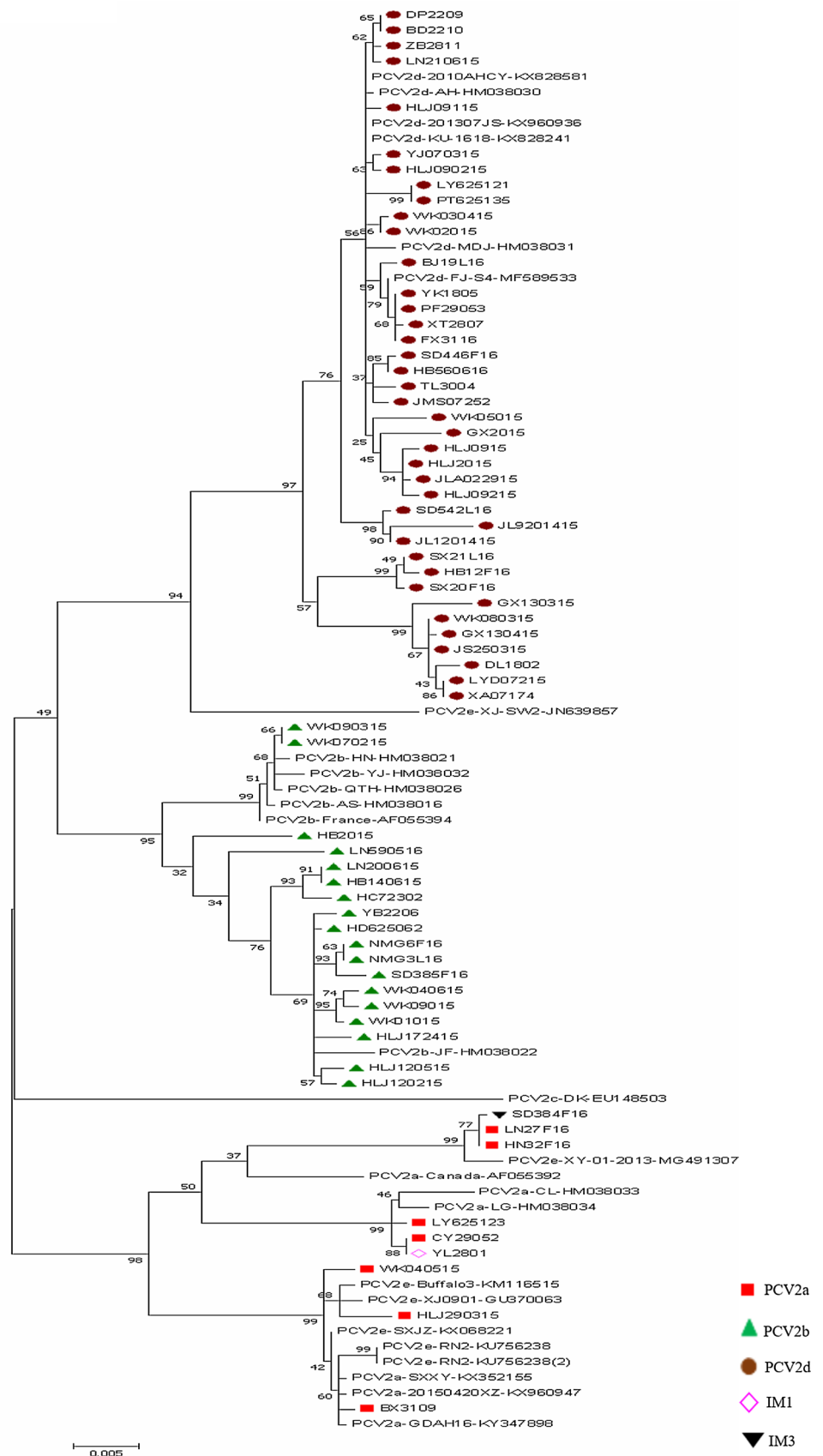
**Fig. 1 a.** A total of 66 PCV2 and four PCV3 samples were collected from 10 provinces in China (blue). The colored dots represent the genotypes found in each province. The number of isolates of each

genotype is shown with the corresponding colored dot. **b.** The relationship between the PCV2 subtype and collection year

in this study. The within-group sequence identity range for PCV2a, PCV2b, and PCV2d was 96.4%–99.9%, 98.1%–99.9%, and 97.3%–100%, respectively. Moreover, PCV2d, the predominant genotype, shared 94.9%–95.6%

and 96.0%–97.3% sequence identity with PCV2a and PCV2b, respectively, while PCV2a and PCV2b shared 95.6%–96.0% sequence identity.

**Fig. 2** Phylogenetic analysis of the 66 PCV2 and 25 reference sequences based on the complete sequence of PCV2. The phylogenetic tree was constructed using the ClustalW alignment algorithm of MEGA7.0 by the ML method based on 1000 bootstraps replicates



## Comparison of PCV2 ORF1 sequences

PCV2 ORF1 encodes the rep and rep' proteins, which are essential for viral replication and have conserved amino acid sequences. The within-group sequence identity range for PCV2a ORF1, PCV2b ORF1, and PCV2d ORF1 was 99.4%–100%, 98.4%–100%, and 98.1%–100%, respectively. In addition, PCV2d ORF1 shared 98.1%–100% and 98.4%–100% sequence identity with ORF1 of PCV2a and PCV2b, respectively, while PCV2a ORF1 and PCV2b ORF1 shared 98.4%–100% sequence identity. The sixth amino acid of the rep proteins encoded by PCV2a and PCV2b is an asparagine residue, while in PCV2d, it is an asparagine or serine.

## Phylogenetic analysis based on PCV2 ORF2

In general, the ML tree based on complete genome sequences of PCV2 isolates was similar to that obtained using ORF2 sequences (Fig. 3). Based on the complete genome sequence, the ORF2 sequence of PCV2-IM1 (intermediate clade, IM1) was close to that of PCV2a and PCV2d [36]. When the phylogenetic tree was constructed based on PCV2 ORF2, the LY2801-IM1 isolate belonged to PCV2d, while it belonged to PCV2a in the tree based on the complete PCV2 sequence. Interestingly, the SD384F16 isolate belonged to PCV2b when the phylogenetic tree was constructed based on PCV2 ORF2, while it belonged to PCV2a in the tree based on the complete sequences and was designated as SD384F16-IM3. The within-group amino acid sequence identity ranges for PCV2a-ORF2, PCV2b-ORF2, and PCV2d-ORF2 were 93.6%–100%, 98.3%–100%, and 97.3%–100%, respectively. In addition, PCV2d-ORF2 shared 88.0%–92.2% and 92.8%–95.2% sequence identity with ORF2 of PCV2a and PCV2b, respectively, while PCV2a-ORF2 and PCV2b-ORF2 shared 91.0%–93.0% sequence identity. The individual amino acid sequence variations in ORF2 of the different genotypes are listed in Table 3.

## Phylogenetic analysis based on the complete genomic sequence of PCV3

Four PCV3 isolates were sequenced and compared to reference isolates of PCV3 and other circoviruses, using MEGA7.0 (Fig. 4). The four PCV3 isolates had a within-group nucleotide sequence identity range of 98.9%–99.7% and 98.8%–99.6% nucleotide sequence identity to isolates five isolates from the USA (PCV3-2164, PCV3-29160, PCV3-US/MO2015, PCV3-US/MN2016, and PCV3-US/SD2016), 99.0%–99.3% nucleotide sequence identity to isolates from the EU, and 98.8%–99.8% nucleotide sequence identity to reference PCV3 isolates from China.

## Comparison of PCV3 ORF2 sequences

PCV3 ORF2 contains 645 nucleotides encoding 214 amino acids. Four PCV3 ORF2 sequences shared 98.3%–99.4% nucleotide sequence identity and 98.1%–99.5% amino acid sequence identity. Amino acid 24 and 27 of Cap were crucial for distinguishing genotype PCV3a from PCV3b. When compared to PCV3a, PCV3b contained the amino acid changes A<sup>24</sup>V and R<sup>27</sup>K, while PCV3a-IM contained A<sup>24</sup> and K<sup>27</sup> [17], indicating that PCV3-CN-JL53/PCV3-CN-LN56, PCV3-CN-HLJ3, and PCV3-CN-0710 belong to genotype PCV3a, PCV3b, and PCV3a-IM, respectively. The four PCV3 Cap sequences identified in this study shared 98.1%–99.5% amino acid and 98.4%–99.4% nucleotide sequence identity, respectively.

## Reactivity of mAbs with the rescued PCV2 clones

Nine PCV2 infectious clones were successfully rescued and identified by IPMA (Fig. 5) as described previously [18]. The reactivity of mAbs with the nine PCV2 strains is shown in Table 4. Four of the 13 mAbs (3A5, 8G12, 8E10, and 10D3) reacted with all nine rescued PCV2 isolates, while 1H10, 2C1, 4F5, 4D6, 5B9, 6B10, 5B8, 8E4, and 1D2 reacted with PCV2a-CY29052 and PCV2a-BX3109, but not PCV2-YL2801-IM1, PCV2b-WK01015, PCV2b-YB2206, PCV2d-ZB2811, PCV2d-FX3116, and PCV2d-DP2209. Based on the ORF2 sequence, the reactivity characteristics of PCV2a-YL2801 were predicted to be similar to those of PCV2b and PCV2d isolates.

## RFLP analysis results

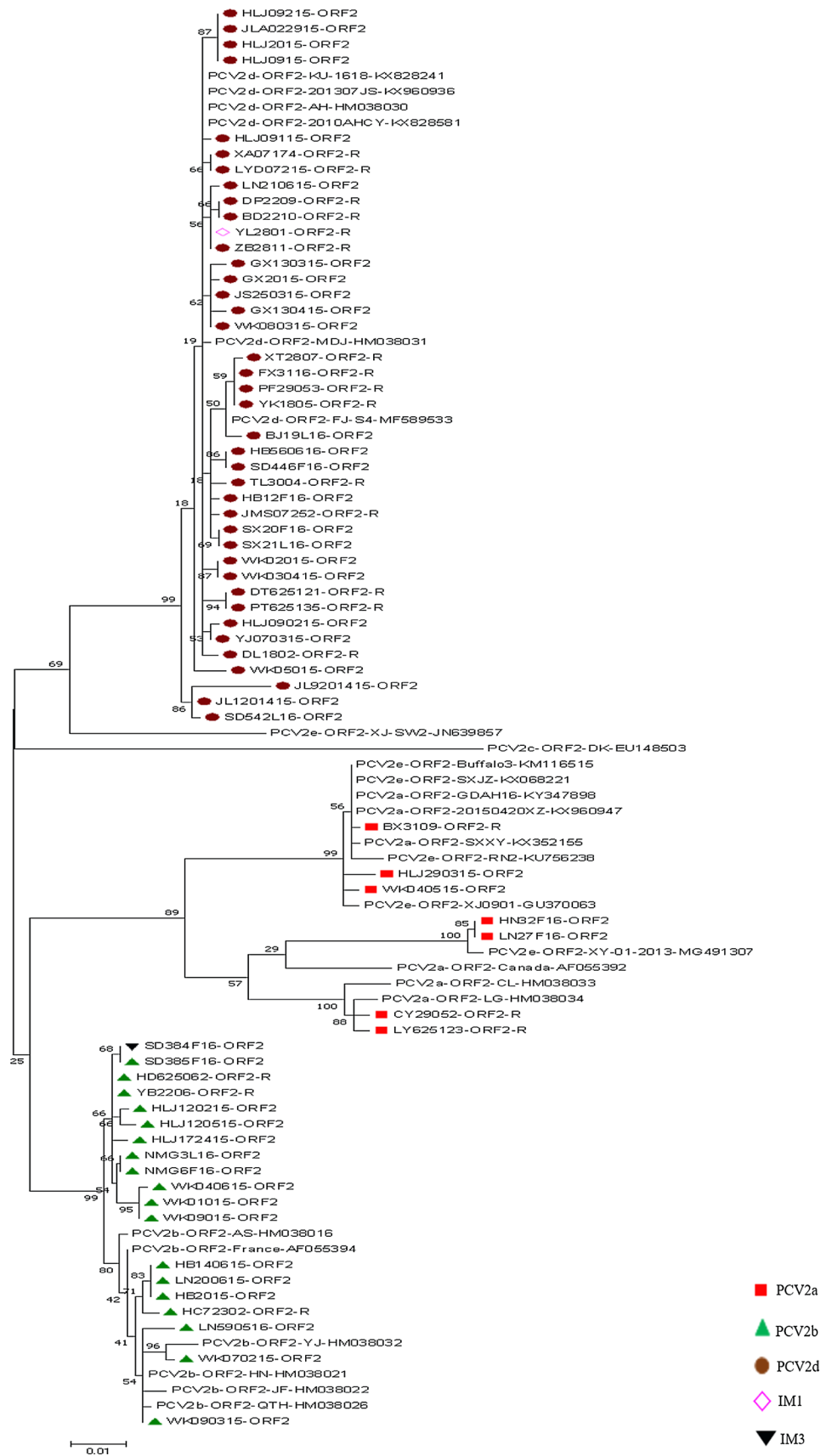
An analysis of the PCV2 genome sequence using Bioedit software revealed that the PCV2a isolates had no *AseI* site, while the PCV2b and PCV2d isolates had only one each. When digested with *AseI*, PCV2a was represented by one band of 1767–1768 bp, while PCV2b and PCV2d were represented by bands of 511–512 and 1255–1256 bp, respectively (Fig. 6). PCV2a was differentiated from PCV2b/2d by RFLP analysis. PCV2b and PCV2d could be digested by *SacI*, and PCV2d was distinguished when three fragments of 223, 616, and 928 bp appeared, but not when two fragments of 616 and 1151 bp appeared (Fig. 7). The information of RFLP analysis is summarized in Table 5.

## GenBank accession numbers

The complete sequences of PCV2 and PCV3 identified in this study have been submitted to the GenBank database under the accession numbers MK347348 to MK347417 (Table 2).



**Fig. 3** Phylogenetic analysis of the 66 PCV2 and 25 reference sequences based on the ORF2 of PCV2. The phylogenetic tree was constructed using the ClustalW alignment algorithm of MEGA7.0 by the ML method based on 1000 bootstraps replicates



**Table 3** Information about amino acid (AA) mutations in PCV2 ORF2

AA mutation site	Genotype		
	PCV2a	PCV2b	PCV2d
8	Y/F	Y	F
47	A/S/T	T	T
53	F	F	I
57	V	I	V
59	A	R/K	K
63	T/S/R	K/R	R
68	A/S	A	N
72	L/M	M	M
77	D/N	N	N
80	V/L	L	L
86	T	S	S
88	K	P	P
89	I	R	L
90	S	S	T
91	I	V	V
121	S/T	S	T
130	F/V	V	V
131	M/P/T	T	T
133	A/V/S	A	A
134	T/P	T/P	N
151	P	T	T
169	S	S	R/G
185	M/L	L	L
187	I/L	L	L
190	S	A/T	T
191	K/A/R	G	G
206	K/T	I	I
210	D	E	D
215	V	V	I
232	K	N/T	N/K
234	*	*	K

\*no amino acid at this position

## Discussion

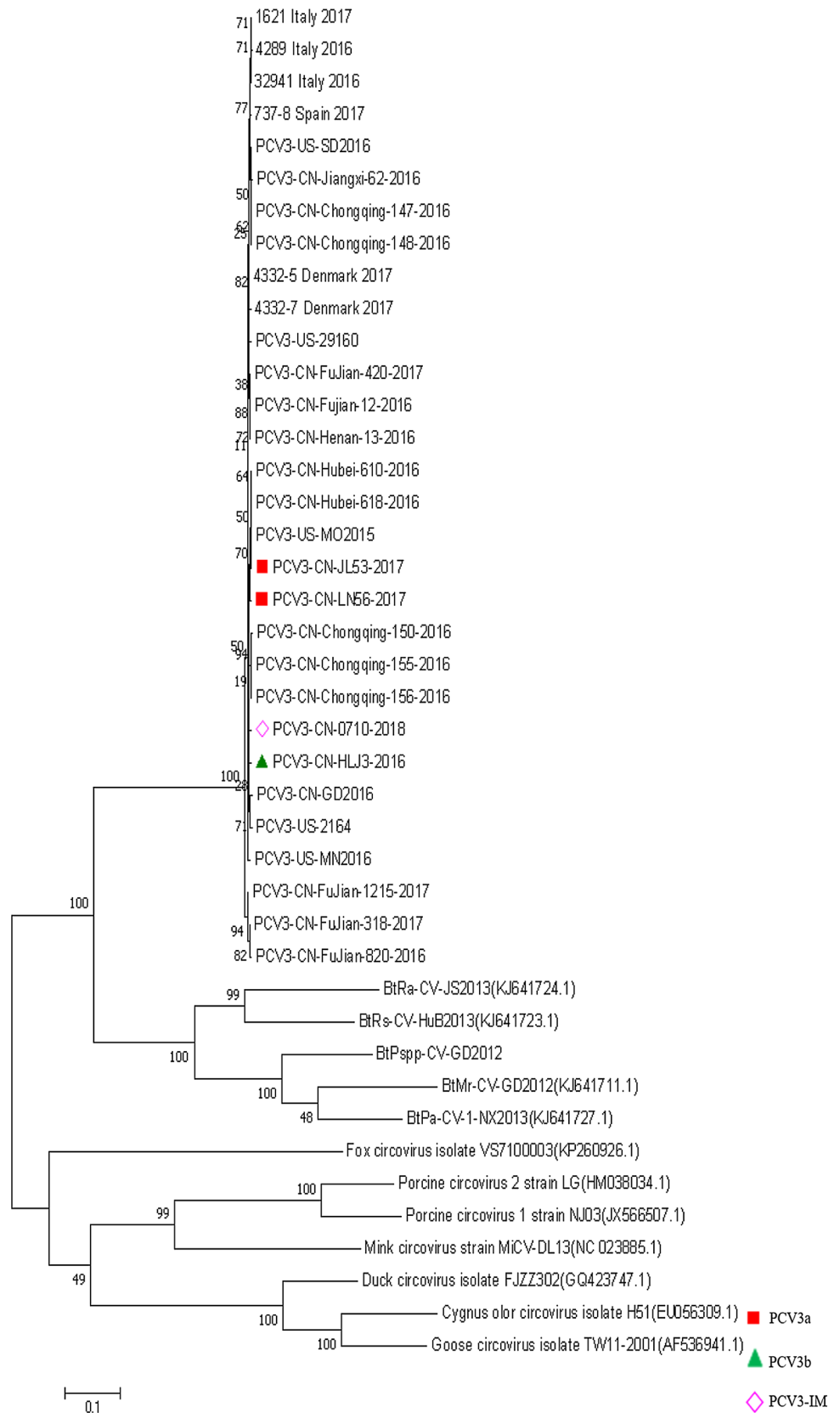
PCV2 is associated with several porcine disease syndromes, collectively known as PCVAD [22]. PCV2 seriously endangers the health of swine and causes tremendous economic losses to the swine industry in China [31, 38]. A high nucleotide substitution rate allows for the continuous evolution of PCV2 and the emergence of novel PCV2 strains. The genotype of PCV2 appears to have shifted twice: PCV2a to PCV2b around 2003 and PCV2b to PCV2d after 2013 [15, 39]. Recently, PCV2d was recognized as the predominant genotype worldwide. Although vaccines based on PCV2a could provide protection against infection with PCV2b and PCV2d [23], substantial genetic divergence among PCV2d

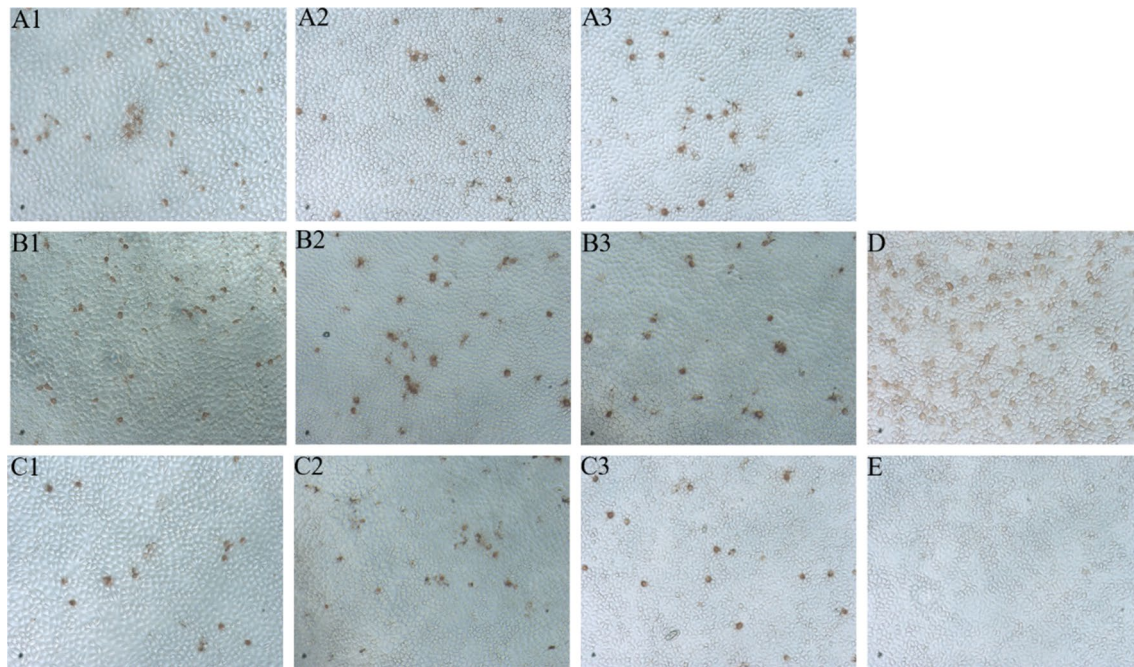
strains over time, which might have changed the pathogenicity of the virus, has been observed in PCV2 isolates collected from farms where the vaccine program had failed.

Since the first report of PCV3 in the USA in 2016, many studies have explored the epidemiology and source of the virus. Recently, PCV3 was successfully rescued and propagated in PK-15 cells for 15 passages, and this virus was capable of reproducing severe PDNS-like disease [14]. A high prevalence of PCV3 (44.2% [159/360]) has been reported in South Korea [15]. In northeast China, the total PCV3-positive rate was 33.3% (35/105), with rates of 17.8% (8/45), 66.7% (10/15), and 37.8% (17/45) in Hei Longjiang, Ji Lin, and Liao Ning provinces, respectively [12]. However, Qi et al. reported that the PCV3 positive rate in China was 12.2% (75/616) at the sample level and 24.1% (42/174) at the farm level [27]. In the present study, the PCV3-positive rate was lower 13.3% (63/472) at the sample level than in earlier studies in China, but is consistent with the results reported by Qi et al. in 2018 [27]. Moreover, Zhai et al. reported that the detection rate of PCV3 in the respiratory tract was much higher than that in the digestive tract [39]. The detection rate of PCV3 is thus closely related to the tissue of origin. A pair of PCV3-specific primers was used for PCR amplification of the complete PCV3 genome sequence, and a one-step PCR technique was established in our laboratory for amplification of PCV3. In the present study, we referred to the methods described by Li et al. to divide the PCV3 strains into different clades [17]. Based on the amino acid sequence of PCV3 Cap, four complete PCV3 sequences were obtained: PCV3-CN-JL53/PCV3-CN-LN56, PCV3-CN-HLJ3, and PCV3-CN-0710, which belonged to PCV3a, PCV3b, and PCV3a-IM, respectively. However, at present, there are no universally recognized rules for the classification of PCV3, as more complete sequences are needed to establish the criteria to be used. In this study, 50.0% (236/472) of the samples collected from 2015 to 2018 were positive for PCV2. In a previous retrospective study conducted in China, using samples collected from 1999 to 2017, the PCV2-positive rate was 54.31% (227/418) [37]. Qu et al. reported that, out of 674 samples collected from 2006 to 2016 in Hunan province, 62% were positive for that PCV2 and PCV2d had become the predominant genotype beginning in 2008 [28]. In this study, the complete genome sequences of 66 PCV2 isolates were determined, with PCV2a, PCV2b, and PCV2d representing 13.6% (9/66), 25.8% (17/66), and 60.6% (40/66) of the total respectively. The three main genotypes of PCV2 were widespread on pig farms, while PCV2d was the predominant PCV2 genotype in China. PCV2d has been reported to be the predominant genotype by several research groups [15, 28, 38].

The use of mAbs to identify the antigenic subtype of PCV2 has been reported previously [29, 30]. In this study, nine PCV2 isolates was rescued by the reverse genetics

**Fig. 4** Phylogenetic analysis based on the four complete PCV3 genome sequences obtained in this study and other reference sequences. The phylogenetic tree was constructed using the ClustalW alignment algorithm of MEGA7.0 by the ML method based on 1000 bootstraps replicates





**Fig. 5** Nine PCV2 infectious clones were rescued and identified by IPMA. A1, PCV2a-CY29052; A2, PCV2-YL2801-IM1; A3, PCV2a-BX3109; B1, PCV2b-YB2206; B2, PCV2b-WK01015; B3, PCV2b-

HD625062; C1, PCV2d-ZB2811; C2, PCV2d-FX3116; C3, PCV2d-DP2209; D, PCV2a-LG (positive control); and E, PK-15 cells (negative control)

**Table 4** Reactivity of rescued viruses with mAbs against PCV2 Cap

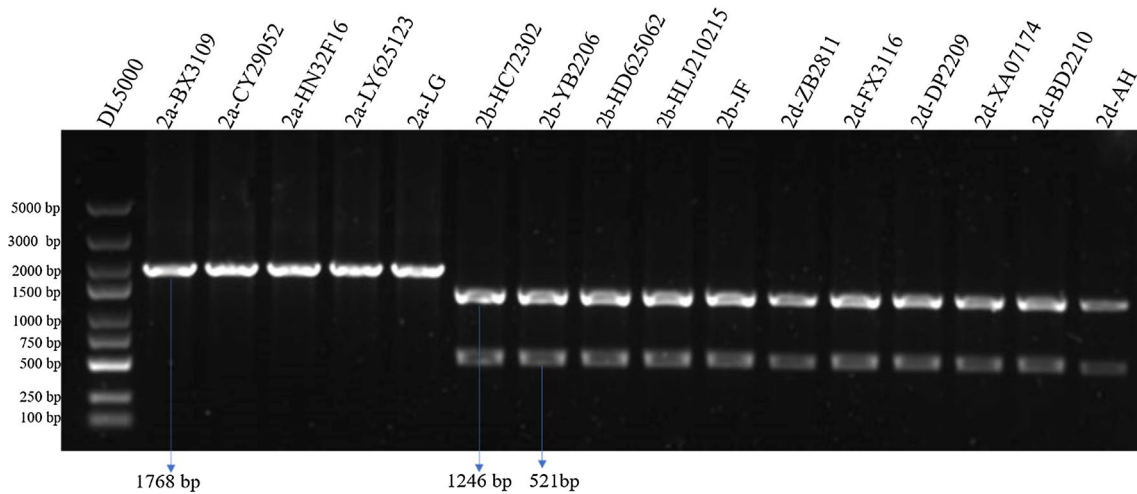
mAb	PCV2a			PCV2b			PCV2d		
	CY29052	YL2801	BX3109	YB2206	WK01015	HD625062	ZB2811	FX3116	DP2209
1H10	+	-	+	-	-	-	-	-	-
2C1	+	-	+	-	-	-	-	-	-
3A5	+	+	+	+	+	+	+	+	+
4F5	+	-	+	-	-	-	-	-	-
4D6	+	-	+	-	-	-	-	-	-
5B9	+	-	+	-	-	-	-	-	-
6B10	+	-	+	-	-	-	-	-	-
8G12	+	+	+	+	+	+	+	+	+
8E10	+	+	+	+	+	+	+	+	+
10D3	+	+	+	+	+	+	+	+	+
5B8	+	-	+	-	-	-	-	-	-
8E4	+	-	+	-	-	-	-	-	-
1D2	+	-	+	-	-	-	-	-	-

+, positive; -, negative

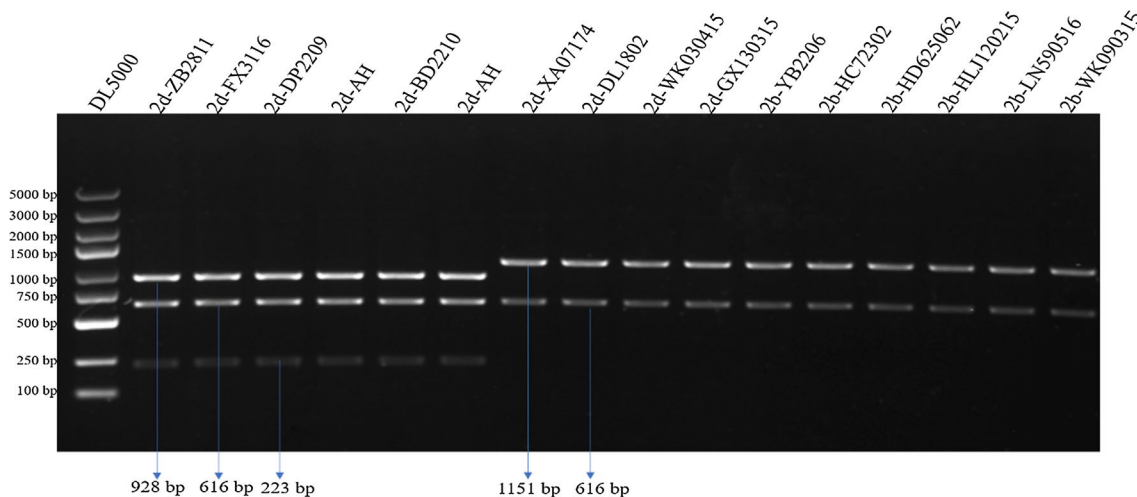
method described by Liu et al. [18]. Because thirteen mAbs were generated against PCV2a Cap, they reacted more strongly to the PCV2a subtype than to PCV2b and PCV2d. The mAbs 2C1, 3A5, 4F5, 4D6, 5B8, and 8E4 reacted with 2a-CY29052, BX3109, and PCV2a-LG (positive control). Based on phylogenetic analysis of complete sequences, the PCV2-YL2801-IM1 isolate belonged to the PCV2a genotype. However, the sequence of ORF2 was most similar to that of PCV2d. Based on this characteristic, the reaction

characteristics of PCV2-YL2801-IM1 with thirteen mAbs were similar to those of PCV2b and PCV2d. In comparison to the amino acid sequences of PCV2a with PCV2b and PCV2d, the mutations A(59)R/K, T(86)S, K(88)P, I(91)V, P(151)T, S(190)A/T, K/A/R(191)G, and K/T(206)I may be the key amino acids for the change of the reaction characteristics of mAbs.

PCR-RFLP is a relatively simple and rapid method that is expensive than sequencing for genotyping, allowing PCV2



**Fig. 6** Identification of PCV2 isolates by digestion with the restriction enzyme *AseI*. When digested with *AseI*, PCV2a was represented by one band of 1767–1768 bp, while PCV2b and PCV2d were represented by bands of 511–512 and 1255–1256 bp, respectively



**Fig. 7** Identification of PCV2 isolates by digestion with the restriction enzyme *SacI*. PCV2b and PCV2d could be digested by *SacI*, and PCV2d was positive when three fragments of 223, 616, and 928 bp

appeared, but the genotype could not be determined when two fragments of 616 and 1151 bp appeared

isolates from a large number of pigs on farms to be analyzed regularly. A genotypic change to PCV2 was revealed by RFLP analysis based on PCV2 ORF2, as reported previously by Takahagi et al. [36]. In this study, genotyping of PCV2 isolates based on RFLP analysis was performed using complete PCV2 sequences. We developed an RFLP method requiring only two restriction endonucleases (*AseI* and *SacI*). First, *AseI* was employed to discriminate PCV2a from PCV2b and PCV2d. Then, *SacI* was used to discriminate PCV2b from PCV2d, based on whether three fragments were produced, as mentioned above. When two fragments were observed, the use of *SacI* was insufficient to discriminate PCV2b from PCV2d. The PCV2 genome should be

further analyzed to identify more suitable restriction sites to distinguish different PCV2 genotypes.

In view of the great harm that PCV2 and PCV3 cause to the pig industry, it is necessary to monitor changes in the viral genome sequence and explore the impact of such changes on viral virulence. In order to investigate the prevalence of PCV2 and PCV3 in China from 2015 to 2018, a total of 472 samples collected from different regions of China were tested for PCV2 and PCV3. The complete genome sequences of 66 PCV2 isolates were determined, with PCV2a, PCV2b, and PCV2d representing 13.6% (9/66), 25.8% (17/66), and 60.6% (40/66) of the total, respectively. The three main genotypes of PCV2 were widespread on pig farms, while PCV2d was the predominant PCV2 genotype

**Table 5** RFLP analysis

Isolate	No. of <i>AseI</i> sites (position)	No. of <i>SacI</i> sites (positions)
2a-BX3109	0	2 (929, 1152)
2a-CY29052	0	2 (929, 1152)
2a-Canada	0	2 (929, 1152)
2a-CL	0	2 (929, 1152)
2a-HN32F16	0	1 (1152)
2a-LY625123	0	2 (929, 1152)
2a-LG	0	2 (929, 1152)
2a-LN27F16	0	1 (1152)
2a-SD384f16	0	1 (1152)
2a-WK040515	0	2 (929, 1152)
2a-GDAH16	0	2 (929, 1152)
2a-SXXY	0	2 (929, 1152)
2a-YL2801	0	2 (928, 1151)
2b-YB2206	1 (512)	1 (1151)
2b-HC72302	1 (512)	1 (1151)
2b-AS	1 (512)	1 (1151)
2b-HD625062	1 (512)	1 (1151)
2b-HLJ120215	1 (512)	1 (1152)
2b-HN	1 (512)	1 (1151)
2b-JF	1 (512)	1 (1151)
2b-LN590516	1 (512)	1 (1151)
2b-QTH	1 (512)	1 (1151)
2b-WK090315	1 (512)	1 (1151)
2b-YJ	1 (511)	1 (1150)
2b-France	1 (512)	1 (1151)
2d-XA07174	1 (512)	1 (1151)
2d-DL1802	1 (512)	1 (1151)
2d-GX130315	1 (512)	1 (1151)
2d-WK030415	1 (512)	1 (1151)
2d-2010AHCY	1 (512)	2 (928, 1151)
2d-BD2210	1 (512)	2 (929, 1152)
2d-TL3004	1 (512)	2 (928, 1151)
2d-FX3116	1 (512)	2 (928, 1151)
2d-201307JS	1 (512)	2 (928, 1151)
2d-AH	1 (512)	2 (927, 1150)
2d-BJ19L16	1 (512)	2 (928, 1151)
2d-MDJ	1 (512)	2 (927, 1150)
2d-XT2807	1 (512)	2 (928, 1151)
2d-ZB2811	1 (512)	2 (928, 1151)
2d-DP2209	1 (512)	2 (928, 1151)

in China. The epidemic trend and pathogenicity of PCV3 should be continuously monitored. Overall, the results of the present study provide detailed information regarding the prevalence, genetic diversity, and molecular epidemiology of PCV2 and PCV3, which should prove useful for future efforts to develop effective preventive and control measures.

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## Compliance with ethical standards

**Conflict of interest** The authors have no conflicts of interest to declare.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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