A Thesis

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Genetic and Phylogenetic Analysis of Porcine Circovirus Type 2 on Jeju Island, South Korea, 2019-2020: evidence of a novel inter-genotypic recombinant

GRADUATE SCHOOL JEJU NATIONAL UNIVERSITY

Department of Veterinary Medicine

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Abstract

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Porcine circovirus type 2 (PCV2) is the most ubiquitous viral pathogen of pigs and has persistently affected the global swine industry. Since first being identified in South Korea in 1999, the virus has undergone considerable genetic diversity and genotype shifts during the past two decades. These events have contributed to the co-existence of genotypes PCV2a, PCV2b, and PCV2d in Korean pig populations, which may promote viral recombination. The genotypic and phylogenetic characteristics of PCV2 strains circulating in pig herds on Jeju Island from 2019 to 2020 were the focus of this study. Genotype-specific

PCR indicated that PCV2d is the dominant viral genotype and that co-infections with PCV2d and PCV2a (75%) or PCV2a and PCV2b (25%) are common in provincial pig herds. The whole-genome sequences of 11 PCV2 strains, including three PCV2a, two PCV2b, and six PCV2d, were determined. A genomic comparison showed that all viruses had the highest nucleotide identity with their corresponding genotypic reference strain. Notably, genetic and phylogenetic analyses revealed that one PCV2d strain, KNU-1931, exhibited nucleotide variation at the ORF1 gene when compared to other PCV2d strains but shared high similarity to that of PCV2b strains. Comprehensive recombination analysis demonstrated that KNU-1931 originated from natural recombination within ORF1 between PCV2b (the minor parent) and PCV2d (the major parent) strains. Our findings provide evidence regarding the frequency of genetic recombination between two different PCV2 genotypes circulating in the domestic field, illustrating the importance of continuous inter-genotypic recombination for viral fitness under the concomitance of multiple genotypes

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Keywords: PCV2, Multiple genotypes, Inter-genotypic recombination, Jeju.

CONTENTS

Ⅰ. Introduction

Porcine circovirus type 2 (PCV2) was first identified in the late 1990s and was initially associated with PCV2-systemic disease (PCV2-SD) [1]. A number of pathological conditions related to PCV2 infection have been described in the last two decades, including PCV2-reproductive disease (PCV2-RD), PCV2-lung disease (PCV2-LD), PCV2-enteric disease (PCV2-ED), and porcine dermatitis and nephropathy syndrome (PDNS). All PCV2-related manifestations are collectively referred to porcine circovirus-associated diseases (PCVADs)[35, 36](Table 1).

PCV2 is a small, nonenveloped virus with a circular, covalently closed single-stranded DNA genome and is a member of the genus Circovirus in the family Circoviridae [31]. The PCV2 genome is 1.76-1.77 kb in length and contains four open reading frames (ORFs; ORF1 - ORF4). ORF1 is located on the positive strand and encodes the nonstructural replicase proteins Rep and Rep′ that are responsible for viral replication, while ORF2 is placed on the complementary strand and codes for the exclusive structural protein Cap that is associated with immunogenicity [4,18,30]. ORF3 and ORF4 are embedded in the antisense direction of ORF1 and encode nonstructural proteins that regulate virus-induced apoptosis [14, 24](Fig. 1).

Table 1. Main clinical signs observed in different PCVDs (adapted from Segales, 2012)

PCVADs	Replaced terminology	Main clinical signs
PCV2 subclinical infection (PCV2-SI)	None	Decreased average daily gain without and evident clinical sign
PCV2 systemic disease (PCV2-SD)	Postweaning multisystemic wasting syndrome (PMWS) Porcine circovirosis PCV2-associated systemic Infection	Wasting, weight loss, decreased rate of weight gain clinically evident, ill thrift or poor-doer
PCV2 lung disease (PCV2-LD)	PCV2-associated respiratory disease Proliferative and necrotizing pneumonia (PNP)	Respiratory distress, dyspnea
PCV2 enteric disease (PCV2-ED)	PCV2-associated enteritis	Diarrhea
PCV2 reproductive disease (PCV2-RD)	PCV2-associated reproductive failure	Regular return-to-estrus Abortions or mummifications
Porcine dermatitis and nephropathy syndrom (PDNS)	None	Dark red papules and macules on skin, mainly in hind limbs and perineal area

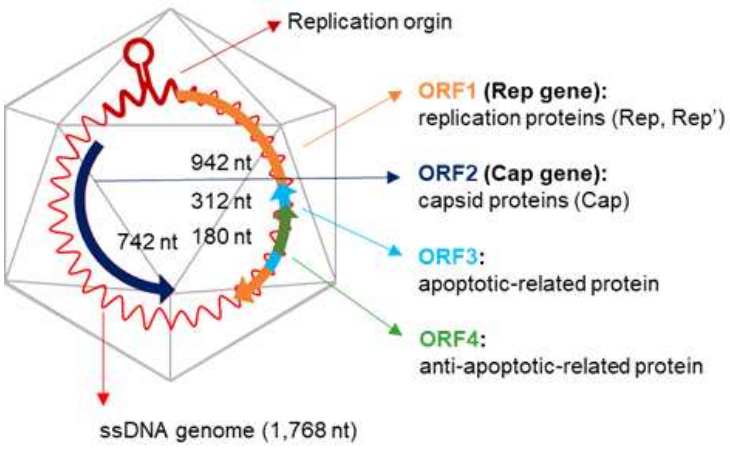

Fig. 1. Schematic representation of the PCV2 genome and the four ORFs producing proteins.

PCV2 is divided into five genotypes (PCV2a, PCV2b, PCV2c, PCV2d, and PCV2e) based on the ORF2 sequence [5, 14, 36, 41]. PCV2a, PCV2b, and PCV2d are the prevalent genotypes in the global pig population [11, 30, 41], while PCV2c has only been isolated from archived serum samples in Denmark [6] and feral pigs in Brazil [9]. A retrospective investigation revealed a new phylogenetic cluster that has been proposed as PCV2e [5](Fig. 2).

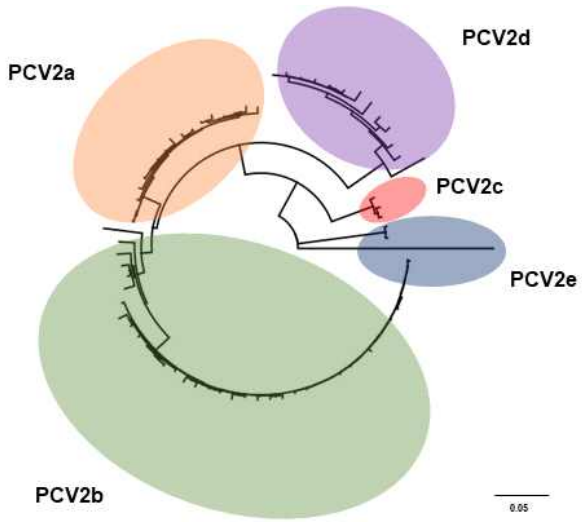

Fig. 2. Phylogenetic tree representing the five PCV2 genotypes descrived to date. Scale bars indicate nucleotide substitutions per site.

In South Korea, PCV2 was first identified in 1999 in pigs with PCV2-SD [27]. PCV2a was the initial predominant genotype until the early 2000s, but a genotype shift from PCV2a to PCV2b occurred around 2002 [2]. Furthermore, the continuous countrywide circulation of classic PCV2a and PCV2b strains resulted in the appearance of recombinant PCV2 strains via inter-genotypic recombination within ORF1 [20]. A recent molecular epidemiology

study indicated that a second genotype shift to PCV2d occurred nationwide before 2012 and that the co-existence of multiple genotypes (PCV2a, PCV2b, and PCV2d) is common in Korean swine herds [23]. Interestingly, this study also reported the occurrence of a genotype shift to PCV2d in Jeju Province, the largest island of South Korea, where the trade of live pigs from the mainland is not permitted [23]. In addition, infection pattern analysis at the farm level confirmed that single infections with PCV2d (57.1%) or dual infections with PCV2d (28.6%) and PCV2a or PCV2b have been frequent in this province [23]. However, despite the genotype shift, there is limited information regarding the incidence of new inter-genotypic recombination of PCV2 in South Korea. Therefore, we aimed to expand our knowledge of the genetic diversity of PCV2 isolates in pig herds on Jeju Province from 2019 to 2020 based on complete genome sequences. This study provides direct evidence of an emerging novel recombinant strain that originated from a natural inter-genotypic recombination event, and the genome of this strain is composed of a PCV2d backbone with a partial ORF1 sequence from of PCV2b circulating in South Korea.

Ⅱ. Materials and methods

Clinical sample collection

Clinical samples (blood, oral fluid, or feces) were obtained from pigs of different ages from eight commercial farrow-to-finish farms located in the Hallim district of Jeju Province from October 2019 to March 2020. Due to the ubiquitous nature of PCV2 in the pig population, there are no seasonal variations in the prevalence of PCV2 infection.

The farms have been clinically affected by PCVAD-like symptoms, including respiratory disorders and wasting and are distributed within a 5-km radius (Fig. 3). Details about the sample collection are provided in Table 2.

Table 2. Number of clinical samples collected from pigs of different ages in 8 commercial farms from October 2019 to March 2020

Farms	Age of pigs	No. of sample			Total
		Serum	Oral fluid	Feces	
Farm A	Weaning	5	5	-	10
	Growing	5	5	-	10
Farm B	Weaning	-	5	5	10
	Growing	-	5	5	10
	Finishing	-	5	5	10
Farm C	Weaning	-	5	5	10
	Growing	-	5	5	10
	Finishing	-	5	5	10
Farm D	Weaning	5	5	5	15
	Growing	5	5	5	15
	Finishing	5	5	5	15
Farm E	Weaning	-	5	5	10
	Growing	-	5	5	10
	Finishing	-	5	5	10
Farm F	Suckling	10	-	-	10
	Weaning	10	-	-	10
	Growing	10	-	-	10
Farm G	Suckling	10	-	-	10
	Weaning	10	-	-	10
	Growing	10	-	-	10
Farm H	Suckling	5	5	5	10
	Weaning	5	5	5	10
Total		95	80	70	

Ages of pigs: Weaning pigs (40 days), Growing pigs(70~100 days), Suckling pigs(10 days), Finishing pigs (130 days)

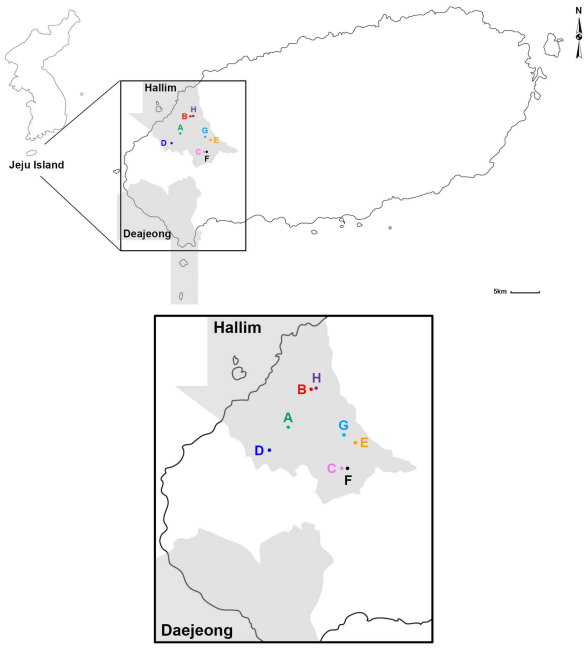

Fig. 3. Geographic location of the farms on Jeju Island.

Real-time quantitative PCR (qPCR) analysis

The collected fecal samples were diluted 1:10 (w/v) with phosphate-buffered saline. The fecal suspensions, as well as the blood and oral fluids, were centrifuged for 10 min at 4,500 × g (Hanil Centrifuge FLETA5, Incheon, South Korea). The clarified supernatants and serum samples were initially subjected to real-time quantitative PCR (qPCR) analysis for the detection of PCV2, as previously described [21]. The reaction was performed using a One Step SYBR PrimeScript RT-PCR Kit (TaKaRa, Otsu, Japan) and a Thermal Cycler Dice Real Time System (TaKaRa) according to the manufacturer's protocol under the following conditions: 1 cycle of 95℃ for 10 min, and 40 cycles of 95℃ for 5

sec and 60℃ for 34 sec. Samples with mean cycle threshold [Ct] values of <40 were considered to be positive for PCV2. The PCV2-positive DNA samples were subjected to additional PCR to determine PCV2 genotypes using three genotype-specific primer sets (Table 3 and 4). The complete genomes of representative PCV2 isolates with mean Ct values of <25 were also sequenced. The PCR was performed using genotype-specific or PCV2 sequencing primers and TaKaRa Ex Taq DNA polymerase (TaKaRa, Otsu, Japan) according to the manufacturer's protocol under the following conditions: an initial PCR activation step at 94℃ for 5 min, 30 cycles of denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec and extension at 72°C for 40 sec or 1 min 50 sec, followed by a final extension step at 72°C for 10 min.

Table 3. List of primers used in this study

Primers	Nucleotide sequence (5′-3′)	Purpose	Location (nt)
PCV2C-F	GCTGGCTGAACTTTTGAAAGT	PCV2 sequencing	497-517
PCV2C-R	AAATTTCTGACAAACGTTACA		470-490
PCV2-132-F	GAGCGCAAGAAAATACGGGAG	PCV2 sequencing	132-152
PCV2-795-R	GCTGGTAATCAGAATACTGCG		795-815
PCV2-Realtime-F	CCAGGAGGGCGTTCTGACT	PCV2 detection	1534-1552
PCV2-Realtime-R	CGTTACCGCTGGAAAAGGAA		1613-1632
PCV2ab 2NF	GGTTGGAAGTAATCAATAGTGGA	PCV2a-specific	1209-1231
PCV2a 2NR	GGGGAACCAACAAAATCTC		1467-1485
PCV2ab 2NF	GGTTGGAAGTAATCAATAGTGGA	PCV2b-specific	1209-1231
PCV2b 2NR	GGGGCTCAAACCCCCGCTC		1466-1484
PCV2d 2NF	GGTTGGAAGTAATCGATTGTCCT	PCV2d-specific	1208-1230
PCV2d 2NR	TCAGAACGCCCTCCTGGAAT		1531-1550

Nucleotide sequence analysis

The full-length genomes of 11 PCV2 strains were PCR amplified with specific primer sets (Table 3). The PCR amplicons were gel-purified, cloned using the pGEM-T Easy Vector System (Promega, Madison, WI), and sequenced in both directions using two commercial vector-specific T7 and SP6 primers. The complete genomic sequences of the PCV2 strains were deposited in GenBank under respective accession numbers (Table 5). We selected ten colonies for sequencing analysis from each pGEM-T cloning to rule out the possibility of a mixed infection in a single sample, as well as to exclude any contamination that may have occurred during sample preparation.

Multiple alignments and phylogenetic analyses

The sequences of ORF2 genes and the complete genomes of 74 global PCV2 strains from the GenBank database were used to produce sequence alignments and phylogenetic analyses. ClustalX 2.0 [38] was used to generate multiple sequence alignments and determine the percent nucleotide divergences. Phylogenetic trees were constructed from the aligned nucleotide or amino acid sequences using the neighbor-joining method and were subjected to bootstrap analysis with 1000 replicates to determine the percent reliability values for each internal node of the tree [33]. All phylogenetic trees were generated using MEGA X software [22].

Recombination analysis

Recombination events were detected using three methods. First, whole-genome sequences were aligned and analyzed with the Recombination Detection Program (RDP 4 version 4.95) to simultaneously detect potential recombination events using eight algorithms (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq, and LARD) [28]. A PCV2 sequence was considered recombinant when the recombination signal was supported by at least four of these methods with p-values of less than 0.01 to ensure reliability. Recombination breakpoint detection by at least four methods was considered as confirmation of a putative recombination event. Second, the potential recombination events and breakpoints were further verified by similarity plot analysis using SimPlot version 3.5.1 [26]. Finally, the putative recombination data were supported by phylogenies of the parental genome regions, analyzed as described above.

Ⅲ. Results

Real-time qPCR for PCV2 detection was performed in all samples obtained from the eight farms. The PCV2-positive samples with low Ct values representing each farm were selected and further used to determine the genotypes of the PCV2 strains circulating on Jeju Island using genotype-specific conventional PCR (Table 4). As expected, PCV2d was detected in all samples from PCV2-positive farms, confirming its high prevalence in the provincial herds (Fig. 4). Interestingly, no singly occurring PCV2d infection was discovered at the farm level. Two or more PCV2 genotypes commonly co-circulated in all eight farms; dual infections with PCV2a and PCV2d were identified on six farms and triple infections with PCV2a, PCV2b, and PCV2d were found on two farms (Table 5).

Table 4. Results of genetic analyses in clinical samples collected from pigs of different ages in eight commercial farms from October 2019 to March 2020

Farms	Age of pigs	Sample type	qPCR (Ct-value)	Genotyping	Sequencing (genotype)
Farm A	Weaning	Serum-1	23.34	PCV2a/d	KNU-1930 (PCV2a)
		Serum-2	28.28		
		Serum-3	–[a]		
		Serum-4	30.23		
		Serum-5	32.51		
		Oral fluid-1	33.54		
		Oral fluid-2	35.87		
		Oral fluid-3	31.79		
		Oral fluid-4	25.35	PCV2a/d	
		Oral fluid-5	21.93	PCV2a/d	KNU-1929 (PCV2d)

	Growing	Serum-1	32.53		
		Serum-2	−		
		Serum-3	−		
		Serum-4	−		
		Serum-5	−		
		Oral fluid-1	31.19		
		Oral fluid-2	30.60		
		Oral fluid-3	−		
		Oral fluid-4	−		
		Oral fluid-5	−		
Farm B	Weaning	Oral fluid-1	−		
		Oral fluid-2	−		
		Oral fluid-3	−		
		Oral fluid-4	−		
		Oral fluid-5	−		
		Feces-1	−		
		Feces-2	−		
		Feces-3	−		
		Feces-4	−		
		Feces-5	−		
	Growing	Oral fluid-1	21.63		
		Oral fluid-2	20.65	PCV2a/d	
		Oral fluid-3	24.97		
		Oral fluid-4	27.49		
		Oral fluid-5	31.59		
		Feces-1	21.45		
		Feces-2	19.23	PCV2a/d	KNU-1931 (PCV2d)
		Feces-3	−		
		Feces-4	−		
		Feces-5	−		
	Finishing	Oral fluid-1	22.44		
		Oral fluid-2	29.18		
		Oral fluid-3	23.71		
		Oral fluid-4	−		
		Oral fluid-5	−		
		Feces-1	31.98		
		Feces-2	31.93		
		Feces-3	−		
		Feces-4	−		
		Feces-5	−		
Farm C	Weaning	Oral fluid-1	28.89		
		Oral fluid-2	−		
		Oral fluid-3	−		
		Oral fluid-4	−		
		Oral fluid-5	−		
		Feces-1	30.81		
		Feces-2	−		
		Feces-3	−		

		Feces-4	–		
		Feces-5	–		
	Growing	Oral fluid-1	32.41		
		Oral fluid-2	26.01	PCV2a/d	KNU-2013/ KNU-2014 (PCV2d/PCV2a)
		Oral fluid-3	–		
		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	27.33		
		Feces-2	–		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
	Finishing	Oral fluid-1	33.71		
		Oral fluid-2	–		
		Oral fluid-3	–		
		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	–		
		Feces-2	–		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
Farm D	Weaning	Serum-1	–		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	–		
		Oral fluid-1	–		
		Oral fluid-2	–		
		Oral fluid-3	–		
		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	29.85		
		Feces-2	–		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
	Growing	Serum-1	33.63		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	–		
		Oral fluid-1	24.67	PCV2a/d	KNU-2015 (PCV2d)
		Oral fluid-2	23.18	PCV2a/d	KNU-2016 (PCV2a)
		Oral fluid-3	–		

		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	29.39		
		Feces-2	–		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
	Finishing	Serum-1	–		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	–		
		Oral fluid-1	32.83		
		Oral fluid-2	–		
		Oral fluid-3	–		
		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	–		
		Feces-2	–		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
Farm E	Weaning	Oral fluid-1	26.93		
		Oral fluid-2	–		
		Oral fluid-3	–		
		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	22.18	PCV2a/d	
		Feces-2	25.14		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
	Growing	Oral fluid-1	21.29	PCV2a/d	
		Oral fluid-2	20.83	PCV2a/d	KNU-2017 (PCV2d)
		Oral fluid-3	–		
		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	23.27	PCV2a/d	
		Feces-2	29.41		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
	Finishing	Oral fluid-1	32.45		
		Oral fluid-2	–		
		Oral fluid-3	–		
		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	31.37		

		Feces-2	34.09		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
Farm F	Suckling	Serum-1	–		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	–		
		Serum-6	–		
		Serum-7	–		
		Serum-8	–		
		Serum-9	–		
		Serum-10	–		
	Weaning	Serum-1	–		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	–		
		Serum-6	–		
		Serum-7	–		
		Serum-8	–		
		Serum-9	–		
		Serum-10	–		
	Growing	Serum-1	–		
		Serum-2	–		
		Serum-3	21.18	PCV2a/b/d	KNU-2018 (PCV2b)
		Serum-4	23.43	PCV2a/b/d	
		Serum-5	–		
		Serum-6	–		
		Serum-7	–		
		Serum-8	–		
		Serum-9	–		
		Serum-10	–		
Farm G	Suckling	Serum-1	–		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	25.63		
		Serum-6	–		
		Serum-7	–		
		Serum-8	–		
		Serum-9	–		
		Serum-10	–		
	Weaning	Serum-1	–		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		

		Serum-5	–		
		Serum-6	–		
		Serum-7	–		
		Serum-8	–		
		Serum-9	–		
		Serum-10	–		
	Growing	Serum-1	–		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	–		
		Serum-6	–		
		Serum-7	27.43		
		Serum-8	–		
		Serum-9	26.12		
		Serum-10	24.63	PCV2a/b/d	KNU-2019 (PCV2b)
Farm H	Suckling	Serum-1	–		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	–		
		Oral fluid-1	–		
		Oral fluid-2	–		
		Oral fluid-3	–		
		Oral fluid-4	–		
		Oral fluid-5	–		
		Feces-1	29.39		
		Feces-2	–		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		
	Weaning	Serum-1	21.43		
		Serum-2	–		
		Serum-3	–		
		Serum-4	–		
		Serum-5	–		
		Oral fluid-1	17.05	PCV2a/d	KNU-2020 (PCV2d)
		Oral fluid-2	27.98		
		Oral fluid-3	27.57		
		Oral fluid-4	28.73		
		Oral fluid-5	32.14		
		Feces-1	25.51		
		Feces-2	–		
		Feces-3	–		
		Feces-4	–		
		Feces-5	–		

[a]Indicates negative qPCR signals, Ages of pigs: Suckling pigs(10 days), Weaning

pigs (40 days), Growing pigs(70~100 days), Finishing pigs (130 days)

Table 5. Summary of PCV2 isolates and origin/source

Farms	Sample source	Year of collection	Infection pattern	Name of isolate	Genotype	Accession number
Farm A	Oral fluid Serum	2019	PCV2a/d	KNU-1929	PCV2d	MT814841
				KNU-1930	PCV2a	MT814842
Farm B	Feces	2019	PCV2a/d	KNU-1931	PCV2d	MT814843
Farm C	Oral fluid	2020	PCV2a/d	KNU-2013	PCV2d	MT814844
				KNU-2014	PCV2a	MT814845
Farm D	Oral fluid	2020	PCV2a/d	KNU-2015	PCV2d	MT814846
				KNU-2016	PCV2a	MT814847
Farm E	Oral fluid	2020	PCV2a/d	KNU-2017	PCV2d	MT814848
Farm F	Serum	2020	PCV2a/b/d	KNU-2018	PCV2b	MT814849
Farm G	Serum	2020	PCV2a/b/d	KNU-2019	PCV2b	MT814850
Farm H	Oral fluid	2020	PCV2a/d	KNU-2020	PCV2d	MT814851

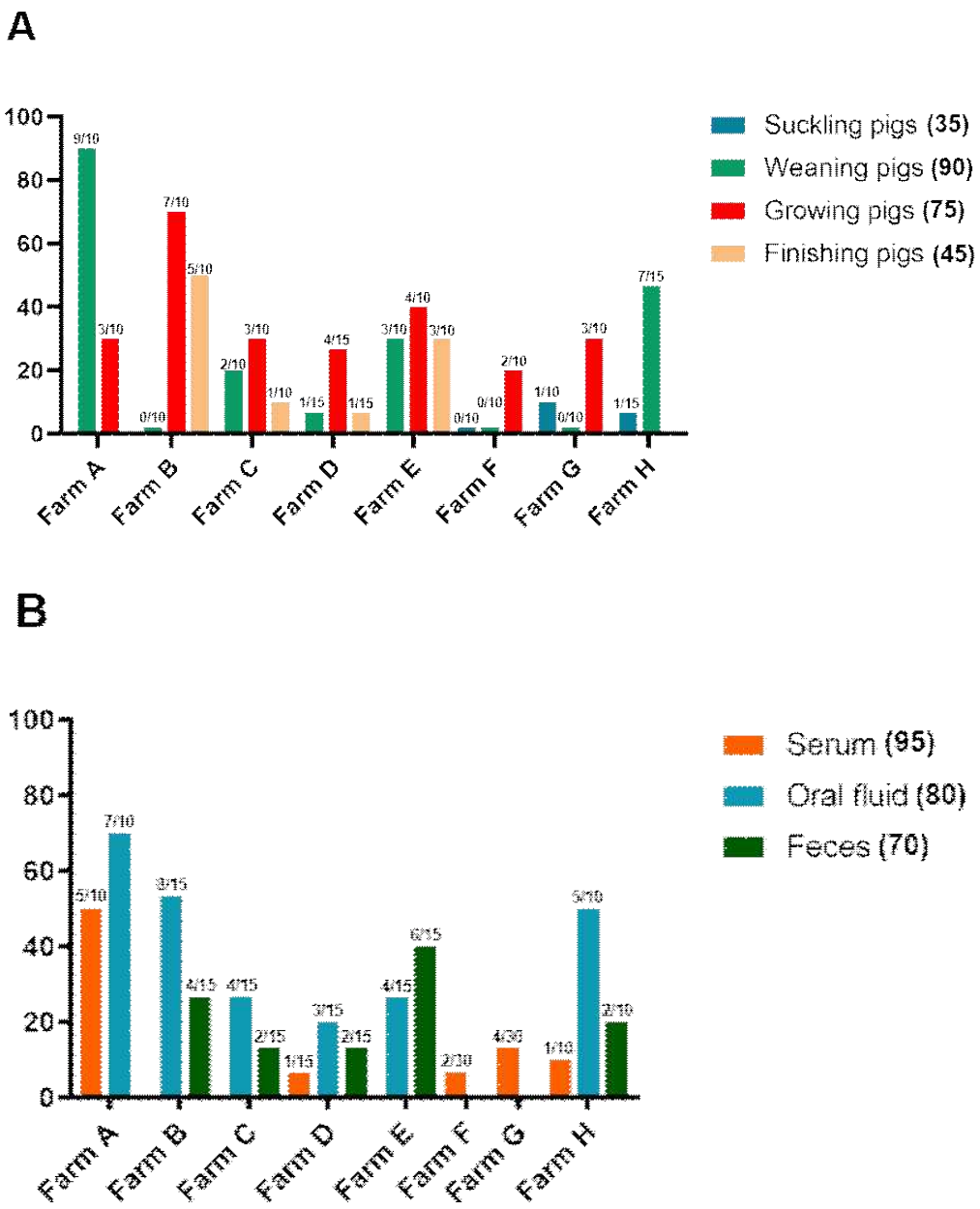

Fig. 4. Positive rate by age group (A) and sample type (B) of PCV2 at the farm level on Jeju Island. Positive rate was determined based on real-time qPCR analysis.

Subsequently, we were able to determine the full-length genomic sequences of 11 PCV2 isolates: three PCV2a, two PCV2b, and six PCV2d (Table 4). The complete genome length of PCV2 in this study was 1768 bp (PCV2a) or 1767 bp (PCV2b and PCV2d). The 11 PCV2 strains from Jeju were genotypically cognate, having 99.2-99.7%, 99.4%, and 98.9-99.9% sequence identity within a corresponding genotype and shared 98.5 - 98.7%, 99.5-99.6%, and 99.0-99.9% identity with PCV2a, PCV2b, and PCV2d reference strains, respectively (Fig. 5 and Table 6). The percent identities of ORF1 and ORF2 of the Jeju isolates between one another and to the reference strains are summarized in Fig. 6 and Table 7.

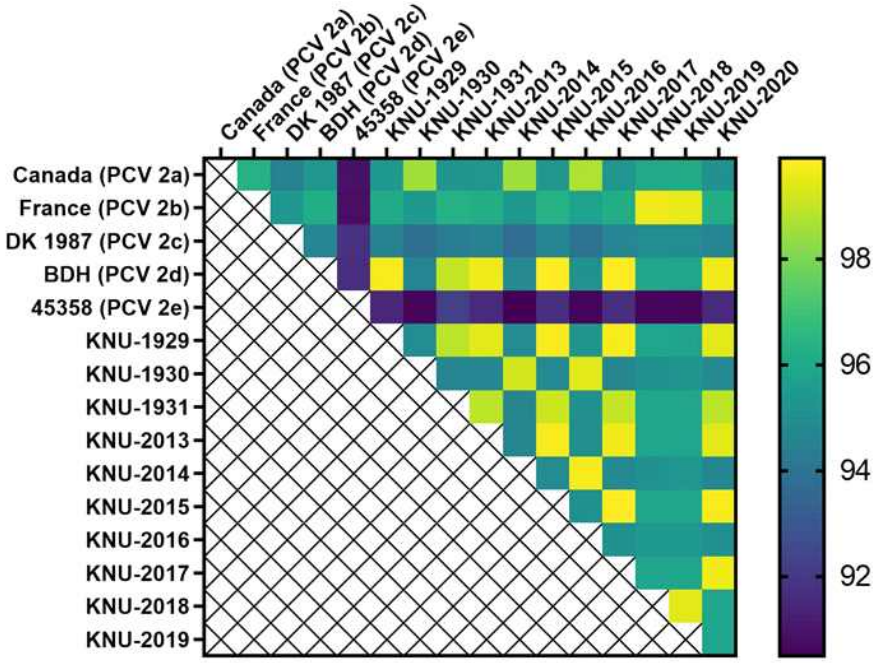

Fig. 5. Heatmap of full-length genomes identity of the PCV2 isolates and genotype-representative PCV2 strains. Yellow gradient bar represents the scale of similarity percentage.

Table 6. Comparison of the full-length genomes of the PCV2 isolates and genotype-representative PCV2 strains

Strain name (Genotype)	Nucleotide identity (%) (No. of nucleotide difference)															
	Canada	France	DK1987	BDH	45358	KNU-1929	KNU-1930	KNU-1931	KNU-2013	KNU-2014	KNU-2015	KNU-2016	KNU-2017	KNU-2018	KNU-2019	KNU-2020
Canada (PCV2a)		96.3 (65)	94.5 (97)	95.3 (83)	90.9 (161)	95.4 (81)	98.5 (26)	95.2 (84)	95.3 (83)	98.5 (25)	95.3 (82)	98.7 (22)	95.3 (83)	96 (70)	96.1 (68)	95.1 (86)
France (PCV2b)			95.3 (83)	96.2 (66)	90.8 (162)	96.1 (68)	95.4 (81)	96.3 (65)	96.2 (67)	95.4 (80)	96.3 (65)	95.7 (76)	96.2 (66)	99.6 (7)	99.5 (8)	96.2 (66)
DK1987 (PCV2c)				94.6 (94)	91.8 (145)	94.5 (96)	93.8 (109)	94.3 (100)	94.5 (96)	93.7 (111)	94.7 (93)	93.9 (107)	94.6 (94)	94.9 (90)	94.9 (90)	94.6 (94)
BDH (PCV2d)					91.7 (147)	99.7 (5)	94.7 (92)	99 (16)	99.6 (6)	94.8 (91)	99.9 (1)	95.1 (86)	99.8 (2)	95.9 (72)	95.8 (73)	99.6 (6)
45358 (PCV2e)						91.5 (150)	90.6 (166)	92.2 (138)	91.6 (148)	90.5 (168)	91.7 (146)	90.6 (166)	91.7 (147)	90.6 (166)	90.6 (166)	91.6 (148)
KNU-1929							94.9 (90)	98.9 (19)	99.4 (9)	94.9 (89)	99.7 (4)	95.2 (84)	99.7 (5)	95.8 (74)	95.7 (75)	99.4 (9)
KNU-1930								94.7 (93)	94.7 (93)	99.2 (13)	94.8 (91)	99.4 (10)	94.7 (92)	95.1 (86)	95.2 (84)	94.8 (91)
KNU-1931									98.9 (18)	94.7 (92)	99.1 (15)	95 (87)	99 (16)	95.9 (71)	95.9 (72)	98.9 (19)
KNU-2013										94.7 (92)	99.7 (5)	95 (87)	99.6 (6)	95.8 (73)	95.8 (74)	99.4 (10)
KNU-2014											94.9 (90)	99.7 (5)	94.8 (91)	95.1 (85)	95.3 (83)	94.7 (92)
KNU-2015												95.1 (85)	99.9 (1)	95.9 (71)	95.9 (72)	99.7 (5)
KNU-2016													95.1 (86)	95.4 (81)	95.4 (80)	95 (88)
KNU-2017														95.9 (72)	95.8 (73)	99.6 (6)
KNU-2018															99.4 (10)	95.8 (73)
KNU-2019																95.8 (74)
KNU-2020																

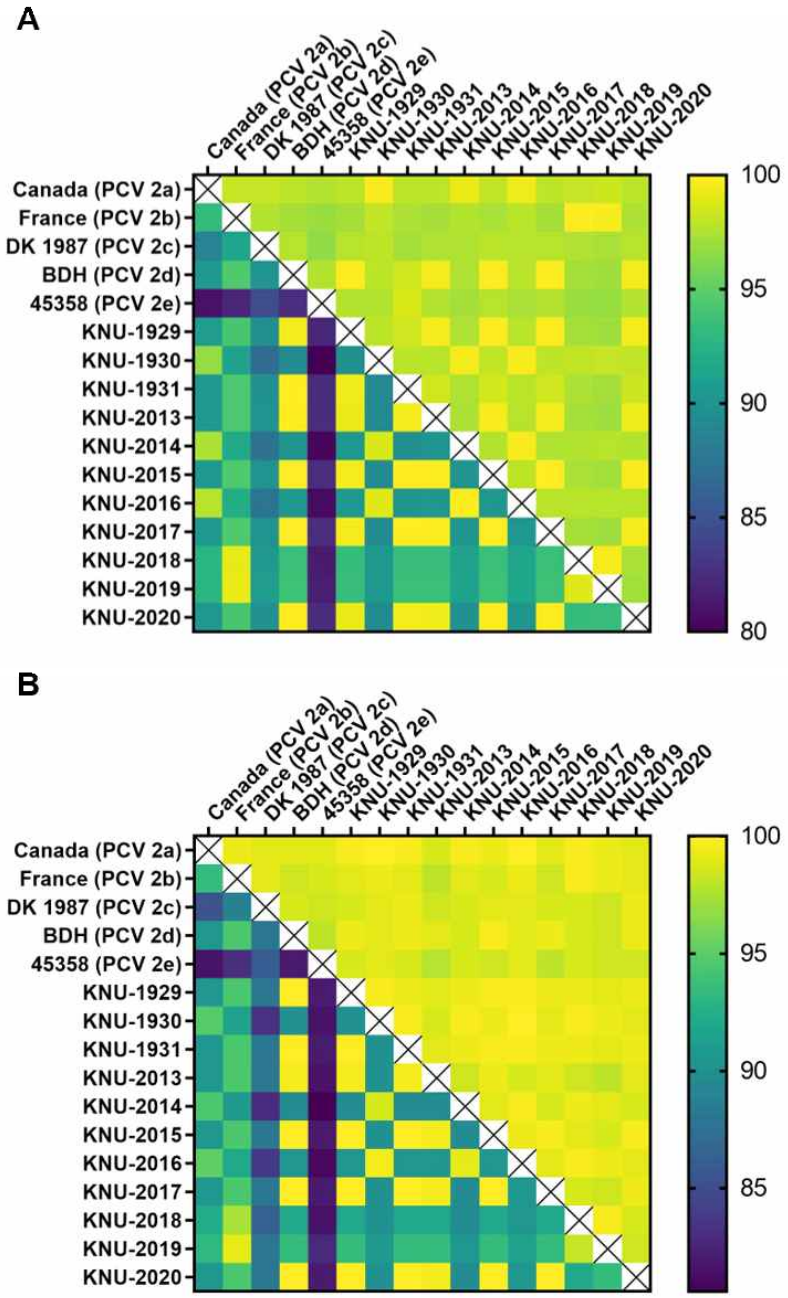

Fig. 6. Heatmap of the ORF1 and ORF2 nucleotide (A) and amino acid (B) identity of the Jeju PCV2 variants and genotype-representative PCV2 strains. Yellow gradient bar represents the scale of similarity percentage.

Table 7. Comparison of the ORF1 and ORF2 sequences of the Jeju PCV2 variants and genotype-representative PCV2 strains

Strain name (Genotype)	Nucleotide/amino acid identity (%) (No. of nucleotide/amino acid difference)															
	Canada	France	DK1987	BDH	45358	KNU-1929	KNU-1930	KNU-1931	KNU-2013	KNU-2014	KNU-2015	KNU-2016	KNU-2017	KNU-2018	KNU-2019	KNU-2020
Canada (PCV2a)		98.1/99.3 (17/2)	98.1/99 (17/3)	97.9/99 (19/3)	97.6/99 (22/3)	97.9/99.6 (19/1)	99.6/100 (3/0)	97.9/99.6 (19/1)	97.9/98.7 (19/4)	99.2/99.6 (7/1)	98/99.3 (18/2)	99.3/100 (6/0)	97.9/99 (19/3)	98.1/99.6 (17/1)	98.3/99.3 (16/2)	97.8/99 (20/3)
France (PCV2b)	93.2/93.5 (47/15)		97.6/99 (22/3)	97.2/98.4 (26/5)	96.9/98.7 (29/4)	97.2/99 (26/3)	98/99.3 (18/2)	97.4/99 (24/3)	97.2/98 (26/6)	97.6/99 (22/3)	97.3/98.7 (25/4)	97.8/99.3 (20/2)	97.2/98.4 (26/5)	99.8/99.6 (1/1)	99.6/99.3 (3/2)	97.4/99 (24/3)
DK1987 (PCV2c)	88.6/85.4 (80/34)	91.3/88.8 (61/26)		97.7/98.7 (21/4)	96.6/98.4 (32/5)	97.7/98.7 (21/4)	97.9/99 (19/3)	97.2/99.3 (26/2)	97.5/98.4 (23/5)	97.5/98.7 (23/4)	97.8/99 (20/3)	97.7/99 (21/3)	97.7/98.7 (21/4)	97.5/98.7 (23/4)	97.3/98.4 (25/5)	97.7/99.3 (21/2)
BDH (PCV2d)	90.5/90.5 (66/22)	94.4/94.4 (39/13)	89.9/88 (71/28)		97.6/98 (22/6)	99.7/99.3 (2/2)	97.9/99 (19/3)	98.4/99.3 (15/2)	99.5/99 (4/3)	97.5/98.7 (23/4)	99.8/99.6 (1/1)	97.8/99 (20/3)	99.7/99.3 (2/2)	97.2/98.7 (26/4)	97/98.4 (28/5)	99.5/99.3 (4/2)
45358 (PCV2e)	80.9/81.5 (137/44)	82/83.1 (129/40)	84.5/86.1 (111/33)	82.5/81.9 (125/43)		97.6/98.7 (22/4)	97.6/99 (22/3)	98.7/98.7 (12/4)	97.6/97.7 (22/7)	97.2/98.7 (26/4)	97.7/98.4 (21/5)	97.3/99 (25/3)	97.6/98 (22/6)	96.9/98.7 (29/4)	96.8/98.4 (30/5)	97.6/98.4 (22/5)
KNU-1929	90.8/90.5 (64/22)	94.1/94.4 (41/13)	89.6/88 (73/28)	99.5/100 (3/0)	82.1/81.9 (128/43)		97.9/99.6 (19/1)	98.4/99.3 (15/2)	99.5/99 (4/3)	97.5/99.3 (23/2)	99.8/99.6 (1/1)	97.8/99.6 (20/1)	99.7/99.3 (2/2)	97.2/99.3 (26/2)	97/99 (28/3)	99.5/99.3 (4/2)
KNU-1930	96.7/94.8 (23/12)	91.1/91.4 (62/20)	86.8/83.3 (93/39)	89.3/90.1 (75/23)	80/81.5 (143/44)	89.6/90.1 (73/23)		97.9/99.6 (19/1)	97.8/98.7 (20/4)	99.5/99.6 (4/1)	98/99.3 (18/2)	99.6/100 (3/0)	97.9/99 (19/3)	98/99.6 (18/1)	98.1/99.3 (17/2)	98/99 (18/3)
KNU-1931	90.4/90.5 (67/22)	94.3/94.4 (40/13)	89.7/88 (72/28)	99.8/100 (1/0)	82.4/81.9 (126/43)	99.4/100 (4/0)	89.1/90.1 (76/23)		98.4/99 (15/3)	97.5/99.3 (23/2)	98.5/99.6 (14/1)	97.8/99.6 (20/1)	98.4/99.3 (15/2)	97.4/99.3 (24/2)	97.2/99 (26/3)	98.3/99.3 (16/2)
KNU-2013	90.5/90.5 (66/22)	94.3/94.4 (40/13)	89.9/88 (71/28)	99.7/99.5 (2/1)	82.4/81.5 (126/44)	99.2/99.5 (5/1)	89.3/90.1 (75/23)	99.5/99.5 (3/1)		97.4/98.4 (24/5)	99.6/99.3 (3/2)	97.7/98.7 (21/4)	99.5/99 (4/3)	97.2/98.4 (26/5)	97/98 (28/6)	99.3/99 (6/3)
KNU-2014	97.4/94.4 (18/13)	91.8/90.9 (57/21)	87.2/82.9 (90/40)	90/89.7 (70/24)	80.3/80.6 (141/46)	90.3/89.7 (68/24)	98.7/98.7 (9/3)	89.8/89.7 (71/24)	90/89.7 (70/24)		97.6/99 (22/3)	99.6/99.6 (3/1)	97.5/98.7 (23/4)	97.6/99.3 (22/2)	97.7/99 (21/3)	97.6/98.7 (22/4)
KNU-2015	90.5/90.5 (66/22)	94.4/94.4 (39/13)	89.9/88 (71/28)	100/100 (0/0)	82.5/81.9 (125/43)	99.5/100 (3/0)	89.3/90.1 (75/23)	99.8/100 (1/0)	99.7/99.5 (2/1)	90/89.7 (70/24)		97.9/99.3 (19/2)	99.8/99.6 (1/1)	97.3/99 (25/3)	97.1/98.7 (27/4)	99.6/99.6 (3/1)
KNU-2016	97.7/95.2 (16/11)	92.1/91.8 (55/19)	87.5/83.7 (88/38)	90.3/90.5 (68/22)	80.5/81 (140/45)	90.5/90.5 (66/22)	98.9/99.5 (7/1)	90.1/90.5 (69/22)	90.3/90.5 (68/22)	99.7/99.1 (2/2)	90.3/90.5 (68/22)		97.8/99 (20/3)	97.8/99.6 (20/1)	97.8/99.3 (20/2)	97.8/99 (20/3)
KNU-2017	90.5/90.5 (66/22)	94.4/94.4 (39/13)	89.9/88 (71/28)	100/100 (0/0)	82.5/81.9 (125/43)	99.5/100 (3/0)	89.3/90.1 (75/23)	99.8/100 (1/0)	99.7/99.5 (2/1)	90/89.7 (70/24)	100/100 (0/0)	90.3/90.5 (68/22)		97.2/98.7 (26/4)	97/98.4 (28/5)	99.5/99.3 (4/2)
KNU-2018	92.7/92.2 (51/18)	99.1/97.4 (6/6)	90.4/86.3 (67/32)	93.5/91.8 (45/19)	81.4/81.5 (133/44)	93.3/91.8 (47/19)	90.5/90.1 (66/23)	93.4/91.8 (46/19)	93.4/91.8 (46/19)	91.2/89.6 (61/24)	93.5/91.8 (45/19)	91.5/90.5 (59/22)	93.5/91.8 (45/19)		99.6/99.6 (3/1)	97.3/98.7 (25/4)
KNU-2019	92.7/93.1 (51/16)	99.2/99.1 (5/2)	90.7/88 (65/28)	93.7/93.5 (44/15)	81.6/82.7 (132/41)	93.4/93.5 (46/15)	90.5/90.9 (66/21)	93.5/93.5 (45/15)	93.5/93.5 (45/15)	91.2/90.5 (61/22)	93.7/93.5 (44/15)	91.5/91.4 (59/20)	93.7/93.5 (44/15)	98.9/98.2 (7/4)		97.1/98.4 (27/5)
KNU-2020	90.3/90.5 (68/22)	94.1/94.4 (41/13)	89.7/88 (72/28)	99.7/100 (2/0)	82.4/81.9 (126/43)	99.2/100 (5/0)	89.3/90.1 (75/23)	99.5/100 (3/0)	99.4/99.5 (4/1)	89.7/89.7 (72/24)	99.7/100 (2/0)	90/90.5 (70/22)	99.7/100 (2/0)	93.3/91.8 (47/19)	93.4/93.5 (46/15)	

The percent ORF1 identity was shown in the upper right and the percent ORF2 identity was presented in the lower left

Aligning the genomes of all PCV2 sequences revealed marked variation between the genotypes but clear similarities within the genotypes (Fig. 7). However, one PCV2d strain, KNU-1931, showed significant sequence diversity of the ORF1 gene, specifically in the first 300 nucleotides, compared with those of other PCV2d strains. This region of KNU-1931 had significant sequence homology to Korean and Chinese PCV2b strains, suggesting a potential natural recombination event. Phylogenetic analysis of the complete genome and ORF2 clearly defined the PCV2 strains into five genotype clusters, and all Jeju isolates were classified according to the respective genotypes (Fig. 8). However, the ORF1 gene-based phylogenetic analysis showed distinct tree topology and unique branches within the PCV2d cluster (Fig. 8C). Interestingly, the KNU-1931 strain was grouped with Korean and Chinese PCV2b strains (KU-1202-like), indicating the emergence of a novel recombinant strain (Fig. 8C).

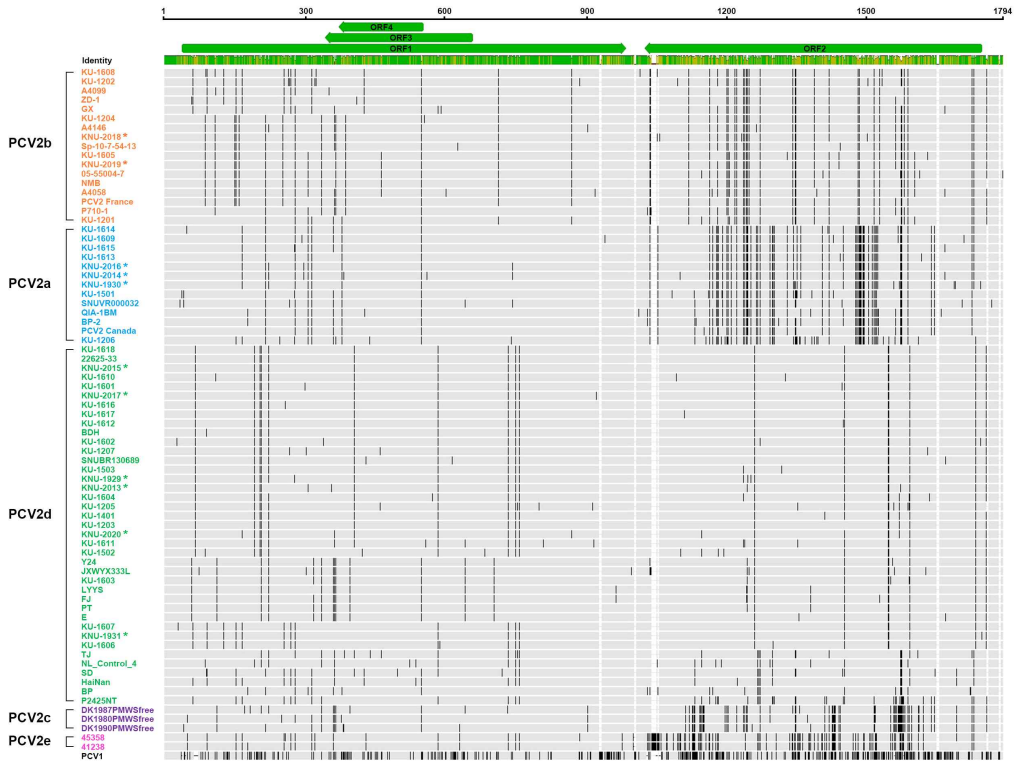

Fig. 7. Schematic diagram of multiple alignments of the PCV2 genome relative to the consensus sequence derived from 74 global PCV2 strains using Geneious software version 10.2.4. Genotypes of PCV2 are color-coded: PCV2a (blue), PCV2b (orange), PCV2c (purple), PCV2d (green) and PCV2e (pink). The Jeju PCV2 isolates identified during 2019 and 2020 in this study are marked with an asterisk (*). The top illustration depicts the genomic regions, with green arrows symbolizing the identified ORFs. Lightly shaded regions show homology to the consensus nucleotide sequence, and the vertical black bars represent variations from the consensus sequence. Thin horizontal dashed lines indicate deleted nucleotides.

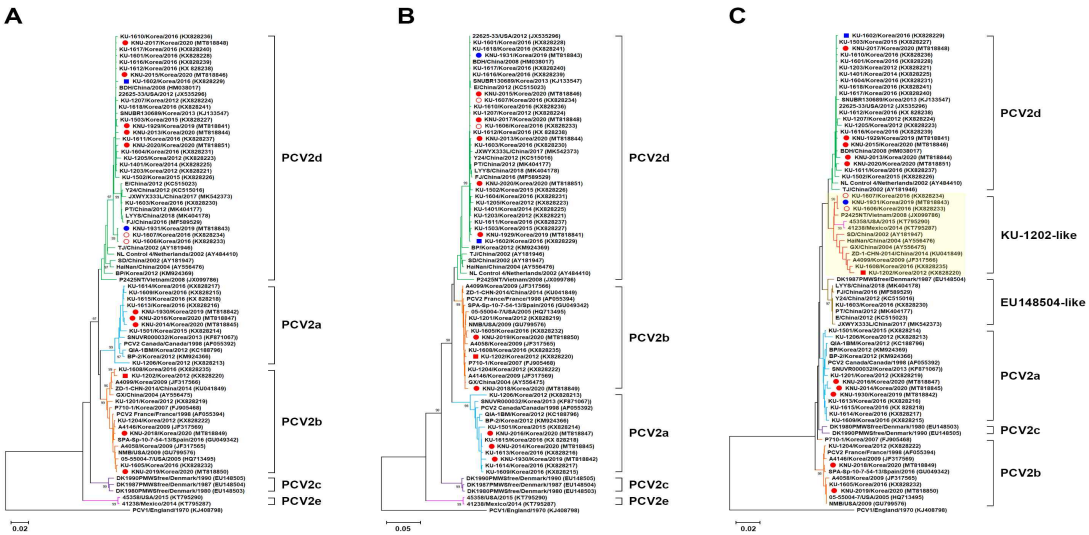

Fig. 8. Phylogenetic analysis based on the nucleotide sequences of the full-length genome (A), ORF2 (B), and ORF1 (C). The complete genome or corresponding gene sequences of a PCV1 strain were included as an outgroup. Multiple sequence alignments were performed using ClustalX, and phylogenetic trees were constructed from the aligned nucleotide sequences using the neighbor-joining method. The numbers at each branch indicate bootstrap values greater than 50% based on 1000 replicates. The names of the strains along with the country and year of isolation, GenBank accession numbers, and genotypes are shown. Red circles indicate the PCV2 strains identified in this study; a blue circle indicates the recombinant PCV2d strain identified in this study; a blue square indicates a major parental strain (PCV2d) identified in 2016; a red square indicates the minor parental strain (PCV2b) identified in 2012; open circles indicate the recombinant PCV2d strains identified in 2016. An ORF1-based KU-1202-like phylogenetic cluster containing KNU-1931 is shaded in yellow. Scale bars indicate nucleotide substitutions per site.

Genetic recombination analysis was performed using the RDP4 package to compare the KNU-1931 isolate and Korean strains to confirm the recombination event. Eight methods in the RDP4 platform (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq, and LARD) were utilized to identify recombination events and breakpoints; the results are summarized in Table 8. All eight modules indicated that KNU-1931 emerged as a consequence of recombination with high degrees of statistical support (average p-value, 9.004×10^{-5}). These results defined KU-1602 (PCV2d genotype) and KU-1202 (PCV2b genotype) as the major and minor parental sequences, respectively (Fig. 9A). Furthermore, a similarity plot also indicated the advent of KNU-1931 from natural inter-genotypic recombination between PCV2b (KU-1202) and PCV2d (KU-1602) strains circulating in South Korea (Fig. 9B). Two putative recombination breakpoints were detected at nts 1746 and 299, which correspond to nt 4 in the origin of replication (ori) and nt 249 in ORF1, respectively. These data pointed to the introduction of the region from ori to the 5′-end of ORF1 of the parental KU-1202 strain into the backbone of the parental KU-1602 strain. In addition, the minor parental region (nts 1746–1767 and 1–299) of KNU-1931 showed higher homology with KU-1202 (99.3%) than with KU-1602 (95.6%), whereas the major parental region (nts 300–1745) of KNU-1931 shared greater homology with KU-1602 (99.6%) than with KU-1202 (95.9%).

Table 8 Genetic recombination event of a PCV2 isolate detected by RDP 4 software

KNU-1931					
Detection method	Breakpoints (nt)		Major parent (Similarity)	Minor parent (Similarity)	*P*-value
	Beginning	Ending			
RDP	1746	299	KU-1602 (99.7%)	KU-1202 (99.4%)	6.508×10^{-4}
GENECONV					1.155×10^{-6}
BootScan					2.380×10^{-7}
MaxChi					1.452×10^{-5}
Chimaera					5.929×10^{-5}
SiScan					1.897×10^{-12}
3Seq					2.862×10^{-7}
LARD					4.047×10^{-7}

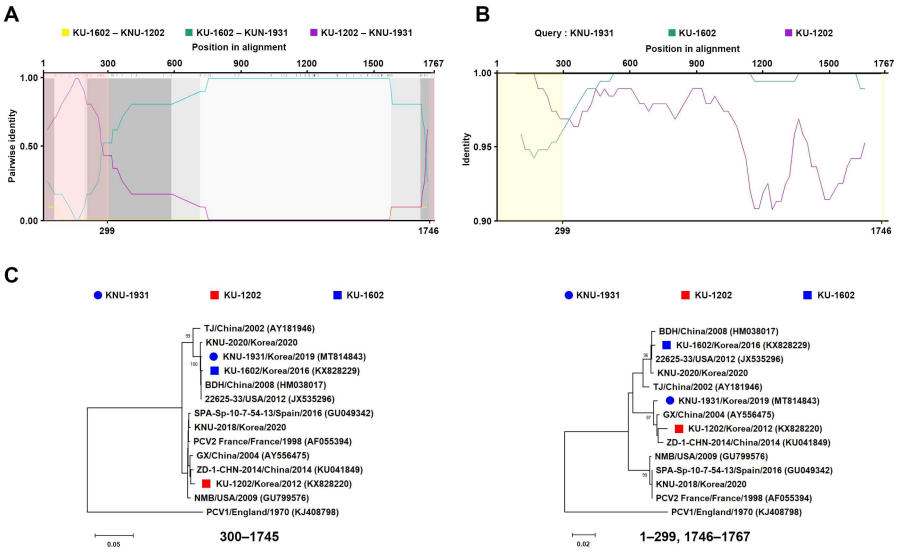

Fig. 9. Recombination analysis of KNU-1931. (A) Recombination detection. The x-axis indicates the genomic position, and the y-axis represents the pairwise identity between KNU-1931 and KU-1602; KNU-1931 and KNU-1202; or KU-1602 and KU-1202, illustrated using green, purple, and yellow lines, respectively. The beginning and end of the recombinant region are shaded red and labeled with position numbers. (B) Similarity plot analysis of KNU-1931 with KU-1602 (green) and KU-1202 (purple). The similarity plot was generated between KNU-1931 (query) and KU-1602 or KU-1202 using SimPlot v.3.5.1. with the two-parameter Kimura distance model and a window size of 200 bp and a step size of 20 bp. The x and y axes of the graph represent the nucleotide position (bp) and the percent nucleotide similarity, respectively. A yellow shaded area indicates the recombination region detected at nts 1746 - 1767 and 1 - 299, which encompass parts of the origin of replication (ori) and ORF1. (C) Phylogenetic trees of the major and minor parental regions of KNU-1931. The major parental region of KNU-1931 was closely related to the corresponding region of KU-1602, whereas the minor parental region was most closely related to the corresponding region of KU-1202.

Lastly, additional evidence of recombination was provided by statistically incongruent phylogenetic trees constructed using the major and minor parental regions, as well as base-by-base comparisons at genetic marker positions. KNU-1931 clustered with KU-1602 (PCV2d) in the phylogenetic tree of the major parental region, whereas its minor parental portion was more closely related to that of KU-1202 (PCV2b) (Fig. 9C). The base-by-base comparisons indicated that the nucleotide sequence of KNU-1931 was identical to that of KU-1202 within the breakpoints but was homologous to KU-1602 beyond the breakpoints (Fig. 10).

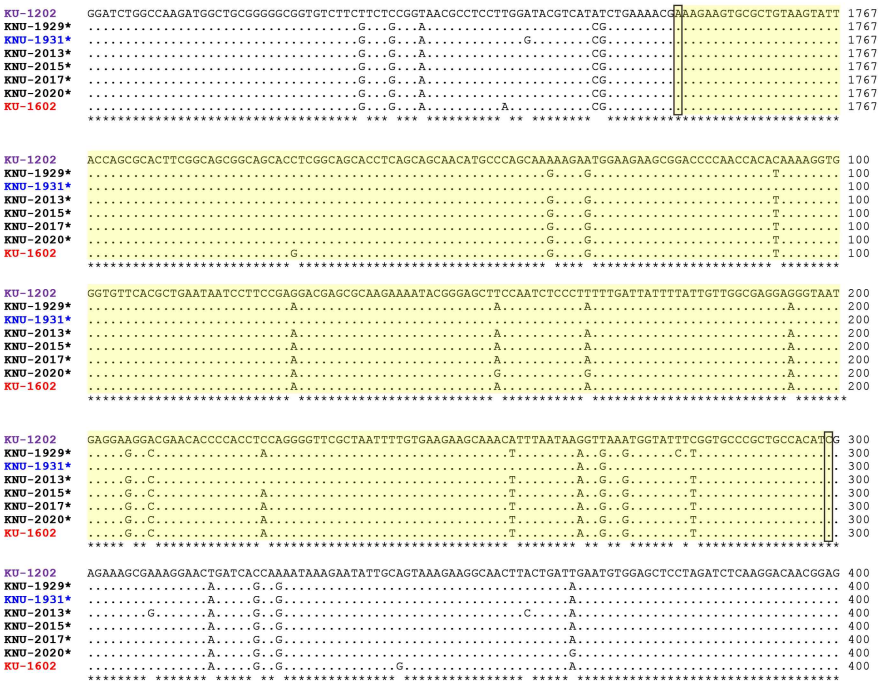

Fig. 10. Base-by-base nucleotide comparisons of the recombination fragment of KNU-1931 (blue) and potential minor (KU-1202; purple) and major (KU-1602; red) parents. The Jeju PCV2d strains identified in this study are marked with an asterisk (*). The recombination areas are shaded in yellow, and the solid boxes indicate the position of potential breakpoints.

Ⅳ. Discussion

PCV2 causes severe financial losses in the global swine industry and needs to be controlled for improvements to production performance. Since its discovery, PCV2 has exhibited extraordinary genetic diversity, and it comprises five distinct genotypes in the current classification. PCV2 has remained problematic and continued to evolve in Korean pig populations since it was first reported in 1999 [27]. Similar to findings from other PCV2-endemic pig-raising countries, retrospective studies have revealed the existence of two genotype shifts the first in 2002 from PCV2a to PCV2b and the second in 2012 from PCV2b to PCV2d and that PCV2a, PCV2b, and PCV2d have co-circulated in Korean pig herds [2, 19, 23]. Although single PCV2d infection was a common occurrence in mainland South Korea as well as Jeju Province, multiple genotypes of PCV2 have co-existed in the same swine herd, resulting in dual or triple infections [26]. In the present study, we confirmed that three genotypes of PCV2 comingle, and PCV2d is the most prevalent in Jeju Province of South Korea. Previously, more than 50% (8/14) of PCV2-positive pig farms in Jeju Province showed single infections with the PCV2d genotype [26]. However, all PCV2-positive cases in this study were co-infections with PCV2d and other genotypes in individual pigs, and single infections with one PCV2 genotype were absent in Jeju pig farms. This result might reflect the low number (n = 8) of farms tested in the current study as compared to a prior study (n = 14). Nevertheless, our data indeed indicate that minor genotypes of PCV2 still co-circulate at the farm level, which could

not only contribute to altered pathogenicity of PCV2 but also facilitate viral recombination.

Genetic changes in viruses, including mutation and recombination, are the central driving forces of their evolution. PCV2 exhibits high rates of both nucleotide substitution and recombination [8, 32]. In particular, frequent inter-genotypic and intra-genotypic recombination play an important role in rapid PCV2 evolution and further affects viral classification [3, 10, 11, 17, 25, 29, 39, 40].

Our extensive recombination analysis uncovered that strain KNU-1931, identified in this study, is an inter-genotypic recombinant from the incorporation of a segment of KU-1202 (PCV2b) into the backbone of KU-1602 (PCV2d). The present analysis identified two potential recombination breakpoints at nts 1746 and 299, which are located in the distal region of ori and the proximal region of ORF1. The PCV genome primarily consists of two large ORFs, ORF1 and ORF2, located on the positive and complementary strands, respectively; the former is considered to be a hotspot of inter-genotypic recombination, while the latter is regarded as a favorable region of intra-genotypic recombination [3, 15, 16, 20, 32, 35]. Since ori is a relatively conserved region of the PCV2 genome, the possible breakpoint detected by RDP in this study might extend to this site. Given this probability, the ORF1 gene of a variant KNU-1931 strain would be a realistic target for natural inter-genotypic recombination between the PCV2b and PCV2d genotypes simultaneously circulating in South Korea.

In addition, we identified KU-1202 (PCV2b) and KU-1602 (PCV2d) as the minor and major parental strains of the KNU-1931 recombinant and were isolated in different provinces of mainland South Korea in 2012 and 2016, respectively. Considering this

geographic and temporal evidence, a natural inter-genotypic recombination event between PCV2b and PCV2d may have occurred around 2016 in the mainland and not on Jeju Island. Further investigation using Korean PCV2 sequences from the GenBank database confirmed the presence of two homologous recombinants, KU-1606 and KU-1607, from 2016 that shared high nucleotide identity (99.8%) with KNU-1931 in the present study (Fig. 7) and formed a monophyletic cluster with KNU-1931 in the PCV2d genotype (Fig. 8). These results suggest that the strain produced by the inter-genotypic recombination between PCV2b and PCV2d likely emerged in 2016 and then spread nationwide. Because the importation of live pigs or pork products from the mainland to Jeju Island is prohibited, we predict that such a recombinant variant was introduced to the Jeju pig herds by non-pig transmission sources, including traffic and humans, from the mainland.

Although vaccination is one of the effective strategies for control of PCV2 infection, the global epidemics of PCV2d might be connected with cases of vaccine failure [7, 34, 37]. Indeed, despite vaccination having taken place on all eight farms examined in this study, the animals suffered from clinical PCVAD under field conditions. Dual heterologous infection with PCV2a and PCV2b has been shown to induce more severe PCVAD than single infections [12]. Thus, co-infection with different PCV2 genotypes, including the recombinant PCV2d variant, appears to be associated with PCVAD in pigs with vaccine-induced immunity. Since the inter-genotypic recombination event could allow the virus to undergo large-scale genomic changes that could lead to the advent of novel variants with unusual traits or phenotypes, including modified pathogenicity, we cannot exclude the possibility that an emerging

recombinant variant might possess altered virulence and clinical manifestations in field circumstances. Therefore, further studies should be conducted to provide fundamental clues regarding the correlations between recombination and viral pathogenicity and to evaluate vaccine efficacy against the new recombinant variant. Considering the co-existence of multiple genotypes and the frequency of inter-genotypic recombination, we also need to improve the diagnostic and sequencing assays that are in use. Since the identification of recombinant PCV2b strains with a partial ORF1 of PCV2a after the first genotype shift [20], this is the first report to describe the existence of new PCV2d variants resulting from natural inter-genotypic recombination following the second genotype shift in South Korea. Recombination events are inevitable under conditions of different co-circulating PCV2 genotypes and will eventually cause antigenic modification, thereby triggering the advent of immune-escape variants. Therefore, continuous monitoring and surveillance of PCV2 evolution are of paramount importance to aid in the preparedness of effective measures against the emergence of novel variants or genotypes.

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