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Phylogenetic and recombination analysis of genomic sequences of PCV2 isolated in Korea

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Abstract The complete genomic sequences of 13 PCV2 viruses obtained between 2005 and 2007 were analyzed in order to determine their phylogenetic relationship and identify possible recombination events between PCV2a and PCV2b. Twelve PCV2b viruses and one PCV2a virus were identified by phylogenetic analysis. Notably, two PCV2b viruses (PF163 and C7201-1) were shown to belong to the 1B subgroup of PCV2b, which had not been previously reported in Korea. Theses two viruses were also predicted to be possible recombinants between PCV2a (the minor parent) and PCV2b (the major parent) by the RDP program ($P < 0.01$). A recombination site was predicted to exist in ORF1 of both viruses. This additional evidence of PCV2 recombination in Korea further supports the important role of recombination in genetic evolution.

Keywords PCV2 · PCV2a · PCV2b · Recombination · ORF1 · Korea

Introduction

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, circular, single-stranded DNA virus of the family Circoviridae [16]. PCV2 is suspected to be associated with a clinical condition observed in pigs shortly after weaning and fattening known as postweaning multisystemic wasting syndrome (PMWS) [10], which was first isolated in western Canada in 1998 [5]. In a review paper, Opriessnig et al. maintained that PMWS is a kind of PCV2-associated diseases (PCVAD). In the United States, the most common clinical signs of PCVADs are pneumonia and systemic disease [18]. This complex clinical manifestation makes it difficult for practitioners to diagnose PCVAD because multiple differential factors must be considered.

The PCV2 genome is 1,768 bp in size [9, 15], and consists of ORF1 (945 bp), ORF2 (702 bp), and ORF3, which are mainly associated with apoptosis [12]. Recent studies have proposed that PCV2 is divided into two main genotypes with different terminologies [4, 7, 17]. In the present study, PCV2a (Group 2) and PCV2b (Group 1) were used to classify the two different genotypes. Although a cross-sectional study by Grau-roma suggested that PCV2b was more closely correlated with PMWS than PCV2a [8], animal experiments revealed that there were no type-specific differences in virulence, and infection with either type conferred cross-protection [19]. In genetic analysis, PCV2a and PCV2b were shown to have five subgroups (2A, 2B, 2C, 2D, and 2E) and three subgroups (1A, 1B, and 1C), respectively [17]. In Korea, phylogenetic analysis of PCV2 isolated between 1999 and 2006 showed that PCV2a and PCV2b co-circulated together, especially subgroup 2E of PCV2a and subgroups 1A and 1C of PCV2b [1].

Recently, several studies have presented evidence of recombination in PCV2. First, Olvera et al. [17] suggested that members of the PCV2b-1B subgroup could be recombinants, and in the USA and China, natural recombinants between PCV2a and PCV2b were identified [11, 13]. Both natural recombination events occurred at ORF1, which is mainly associated with viral replication. Furthermore, Cheung et al. [3] found that recombination

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could also naturally occur within ORF2 (Capsid gene). As recombination events can affect the characteristics of the virus, such as virulence, host range, attenuation, and so on, continuous monitoring of this event should be followed to evaluate the evolution of the virus. Until now, recombination between two PCV2 genotypes has only been reported in the USA and China. Therefore, the aim of this study was to identify recombinant viruses arising from PCV2a and PCV2b strains in Korea, and to further analyze whether the recombination events were consistent with previously reported data.

Materials and methods

Samples

PCV2 positive samples were selected from 40–100-day-old pigs obtained from different regions and different farms in Korea from 2005 to 2007. These pigs showed mild to severe PMWS clinical signs and macroscopic lesions. The pigs were killed and the organs (heart, lung, lymph nodes, spleen, liver, kidney, and tonsil) were collected, minced, and pooled into a 20% suspension with DMEM. The DNA was then extracted from the supernatant of each suspension using a commercial DNA extraction kit (DNeasy Tissue Kit, QIAGEN) according to the manufacturer's instructions.

Complete sequencing of the PCV2 genome

A pair of primers was designed based on the genomic sequence of the Canadian PCV2 strain (GenBank accession number: AF027217): forward primer, 5′ GAG AAT TCA ACC TTA ACC TTT C 3′, and reverse primer, 5′ GTG AAT TCT GGC CCT GCT CC 3′. In order to obtain the complete genome of PCV2, a 1,778 bp DNA fragment was amplified from extracted DNA by PCR with TaKaRa Ex Taq™ polymerase (TaKaRa, Japan) using this pair of primers. The PCR consisted of an initial step at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 54.5°C for 1 min, extension at 72°C for 2 min 10 s, and a final extension at 72°C for 12 min. The PCR product containing the complete PCV2 genome was ligated into a pMD18-T Vector (Takara, Japan) and transformed in *Escherichia coli* JM109 competent cells. The recombinant plasmids were verified by PCR and sequencing (Macrogen Inc., Seoul, Korea). For sequencing, the universal primer set consisting of M13F (−20), 5′ GTA AAA CGA CGG CCA GT 3′ and M13R (−20), 5′ GCG GAT AAC AAT TTC ACA CAG G 3′ was used. These primer sequences flanking the recombination site between the PCR product and pMD18-T vector are commercially available from Macrogen Inc.

Sequence analysis

A total of 13 full genomic sequences were obtained (Table 1) and aligned with 19 reference genomic sequences from PCV1, PCV2a, and PCV2b using the Clustal X (Ver. 1.81) program. Consensus neighbor-joining (NJ) and maximum parsimony (MP) trees were constructed with 1,000 bootstrap replication, using the MEGA version 3.1 program. In the case of NJ tree construction, Kimura 2-parameter model was used. The nucleotides and deduced amino acids were also analyzed using a BioEdit program (Ver. 7.0.5.3)

Recombination analysis

The recombination between PCV2a and 2b was analyzed by the recombination detection program (RDP) [13]. Briefly, this program identifies recombinants from a group of aligned DNA sequences using a number of recombination detection and analysis algorithms. As the input of a large number of sequences in RDP analysis results in increased noisy recombinant events [14], previously known recombinant sequences and interfering reference sequences were excluded from RDP analysis. Hence, in this study, a total of 10 PCV2 sequences were included as references for RDP analysis; PCV2a (DQ104421, AY874165, AY874166, EU148506), PCV2b (EU450584, AY732494, AY321994, AY484411, AY321983), and PCV1 (AY193712).

Table 1 Information on PCV2 full genomes used in this study

I.D.	GenBank Accession No.	Age of pigs (day)	Year	Type[a]
2351-1	FJ905466	40	2005	PCV2b
PF65-1	FJ905464	50	2005	PCV2b
CP5544-2	FJ905461	50	2005	PCV2b
CP5491-2	FJ905462	50	2005	PCV2b
PF163[b]	FJ905469	60	2005	PCV2b
C5461	FJ905465	100	2005	PCV2b
M787-2	FJ905460	40	2007	PCV2b
C7201-1[b]	FJ905470	50	2007	PCV2b
C7155	FJ905463	70	2007	PCV2b
P757	FJ905459	70	2007	PCV2b
e320-1	FJ905467	No information	2007	PCV2b
P710-1	FJ905468	No information	2007	PCV2b
C7189	FJ905471	40	2007	PCV2a

[a] Genotyping of PCV2 using neighbor-joining analysis

[b] Possible PCV2 recombinants identified in this study

Results

Phylogenetic analysis of 13 PCV2 full genomic sequences

In agreement with data previously reported by An et al. [1], PCV2a and PCV2b were both detected in tissue samples from pigs showing signs of PMWS. The NJ tree showed that the PCV2b viruses in this study were predominantly included in the PCV2b-1A subgroup, and that the PCV2a virus (C7189) was in the PCV2a-2E subgroup in which all previously reported Korean PCV2a viruses are included (Fig. 1). However, a different pattern of phylogeny was observed in the MP tree. The viruses that make up the PCV2b-1C subgroup in the NJ tree were branched as an out-group in the MP tree, and did not belong to either the PCV2a or PCV2b subgroups. Interestingly, two strains, PF163 and CP7201-1, did not belong to the PCV2b-1A or 1B subgroups in the NJ tree, although they were shown to be in the PCV2b group. However, in MP tree analysis, the PF163 and C7201-1 strains were grouped with the PCV2b-1B subgroup.

Recombination analysis using recombination detection program

The PF163 and C7201-1 strains were selected as recombinants, and the breakpoint of recombination was determined by GENECONV and SiSCAN methods, and statistically confirmed by BootScan, MaxChi, and 3Seq methods in the RDP program ($P < 0.01$). The major parent and minor parent were shown to be PCV2b (CP5544-2, 2005) and PCV2a (AY874165, 2005) with higher than 99% probability. The recombination graphs generated by bootstrap supports in BootScan analysis are presented in Fig. 2. The potential breakpoints were predicted to be at nt20 and nt368 in PF163 and at nt28 and nt398 in C7201-1 (Fig. 2).

'Nucleotide to nucleotide' comparison of the two PCV2 recombinants

As base to base analysis was important in order to prove that a recombination event had occurred [11], the possible breakpoints in the genomes of the two recombinants as

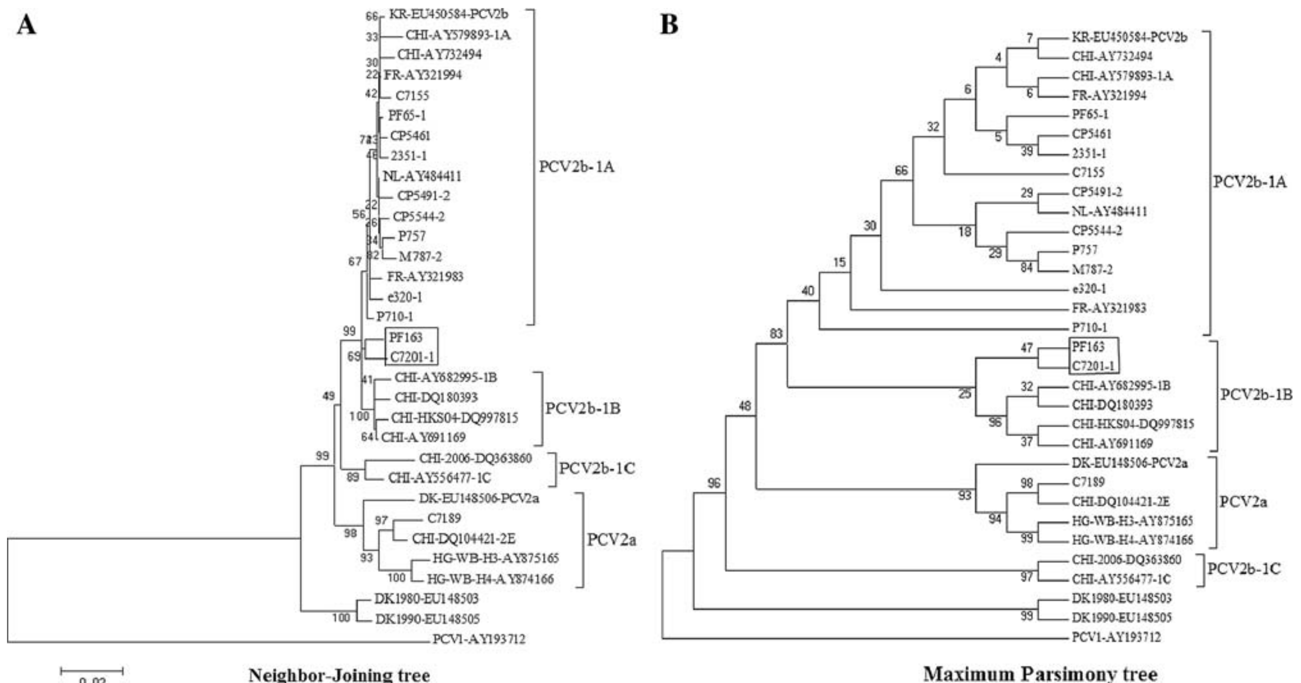

Fig. 1 Phylogenetic relationship of 13 PCV2 full genomes. PCV1 was used as an outgroup. Both PCV2a and PCV2b groups (genotypes 2 and 1) were analyzed by neighbor-joining (NJ) tree and maximum parsimony tree (MP). Previously known subgroups of PCV2b (PCV2b-1A, 1B, and 1C) are indicated on the dendrogram. The PCV2b-1C subgroup in the NJ tree was in a different clade and belonged to neither the PCV2a nor PCV2b subgroups in the MP tree. PF163 and C7201-1, which comprise a single subgroup among the PCV2b viruses in the NJ tree, were included in the PCV2b-1B subgroup in the MP tree. The PF163 and C7201-1 viruses are indicated by *boxes*

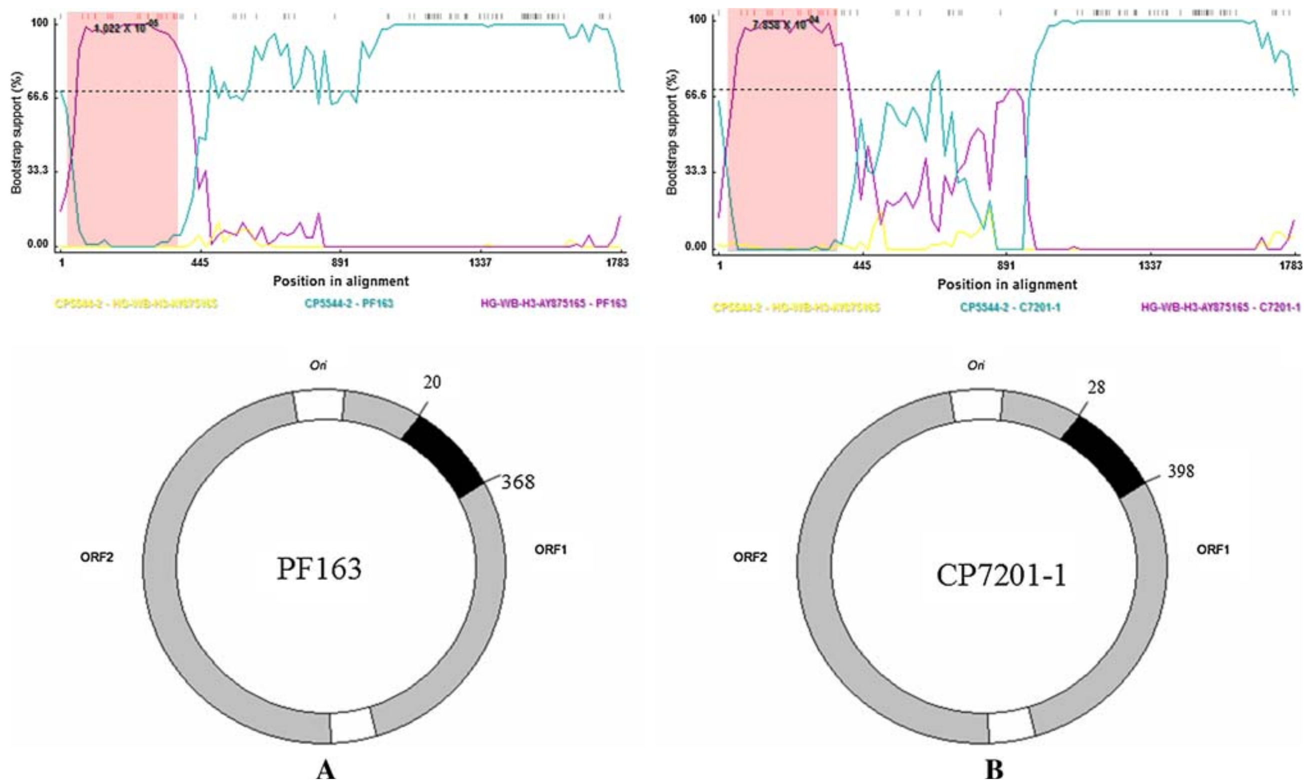

Fig. 2 Recombination analysis of PF163 and CP7201-1 viruses. The possible breakpoints in the recombination event between PCV2a (AY874165) and PCV2b (CP5544-2) were estimated by GeneCov and SiSCAN methods, and confirmed by BootScan, MaxChi, and 3Seq methods in the RDP program ($P < 0.01$). **a** BootScan analysis of the possible breakpoint in PF163 and its location in ORF1 in the PCV2 genome. **b** BootScan analysis of the possible breakpoint in CP7201-1 and its location in ORF1 in the PCV2 genome. The *black region* in ORF1 is the region that is potentially replaced by PCV2a in a PCV2b backbone

determined by RDP analysis were also analyzed by comparison with the genomes of the major and minor parent strains (Fig. 3). The nucleotide sequences within or outside of the breakpoints were almost identical to the sequences of PCV2a and PCV2b, respectively. The nucleotide sequences between the two breakpoints were most consistent with the minor parent, PCV2a (AY874165). In the region outside of the breakpoints, the nucleotide sequences were most similar to the major parent, PCV2b (CP5544-2). Further sequences (nt701–nt1768) containing ORF2 region of PF163 and C7201-1 were also similar to PCV2b (CP5544-2). The *Ori* site was relatively conserved both in PCV2a and PCV2b in this study, and a possible breakpoint might be extended to this *Ori* site. In fact, the breakpoint assumed by the BootScan method alone was extended to *Ori* site; from nucleotide 1,764 to 383 in PF163 and nucleotide 5 to 398 in C7201-1.

Comparison of deduced amino acids of ORF1 in two PCV2 recombinants

In order to determine whether these recombination events altered the amino acid sequences of the *replicase* gene, a total of 13 deduced amino acids sequences from ORF1 were compared without considering the splicing mechanism (Fig. 4). A single amino acid difference, ^{150}P → ^{150}L, was identified between PCV2a (C7189) and PCV2b viruses. Partial differences (^{34}E → ^{34}D, ^{105}I → ^{105}M) were only observed among viruses without genotype-specific changes (PCV2a and 2b). The amino acid sequence was highly conserved in ORF1 of PCV2a and PCV2b, and there were no obvious differences between the two possible recombinants (PF163 and C7201-1) compared to other sequences.

Discussion

There are two PCV2 genotypes, PCV2a and PCV2b, and each genotype can be divided into several subgroups [17]. Although subgroups 1A and 1C of PCV2b and subgroup 2E of PCV2a were previously identified in pigs diagnosed with PMWS in Korea [1], subgroup 1C of PCV2b was not detected in samples in the present study. However, the two PCV2 genomes that were identified in this study (PF163 and C7201-1) were shown to belong to subgroup 1B of PCV2 by MP tree analysis. This is the first report of a member of the

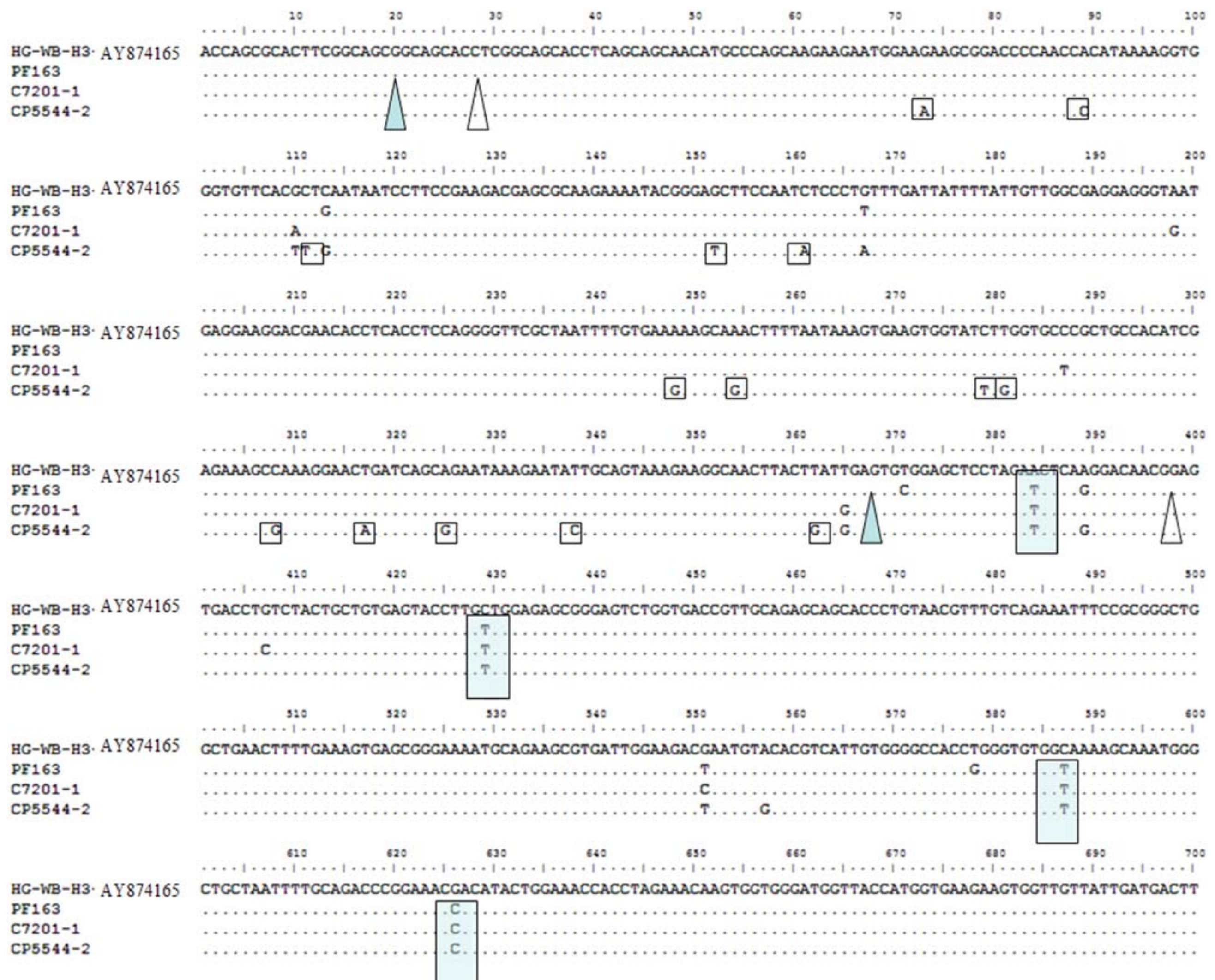

Fig. 3 Nucleotide comparison between possible recombinants and major/minor parent strains near the breakpoints. The *filled* and *hollow triangles* indicate the breakpoints of PF163 and C7201-1, respectively, as recombination sites. PCV2b (CP5544-2)-specific nucleotides are indicated by *small box* in the region inside of the breakpoints. In the region outside of the breakpoints, the nucleotide sequences, which were most similar to the major parent, PCV2b (CP5544-2), were indicated by *filled box*

1B subgroup of PCV2b in Korea. As members of the PCV2b-1B subgroup are suspected to be recombinants of PCV2a and PCV2b [17], and because a recently identified recombinant (HKS04) from China is also in the same PCV2-1B subgroup [13], we hypothesized that PF163 and C7201-1 might also be recombinants between PCV2a and PCV2b. As expected, theses two viruses were determined to be recombinants between PCV2b (CP5544-2, 2005) and PCV2a (AY874165, 2005) by RDP analysis and 'nucleotide to nucleotide' comparison.

In this study, evidence that supports natural recombination between PCV2a and PCV2b was obtained from samples from PWMS infected pigs in Korea. These recombinant viruses were found in pigs from different farms in different provinces (about 300 km apart) in 2005 and 2007, which means that the recombinant viruses had been circulating with other genotypes of PCV2 since the recombination occurred. Notably, the potential major parent (CP5544-2) of the two recombinant viruses is the same virus that was identified in 2005, when PCV2b was frequently detected in Korea. When PCV2b was first identified, the PCV2a strain was the predominant strain in Korea [1], but since that time, the PCV2b-1A groups have become predominant. This may indicate that recombination occurred around the time of the introduction of PCV2b, and that these recombinants entered the pool of PCV2 viruses circulating in Korea.

The main recombination site is predicted to lie in ORF1, which encodes the replicase protein in infected cells. This site is believed to be a favorable site for recombination

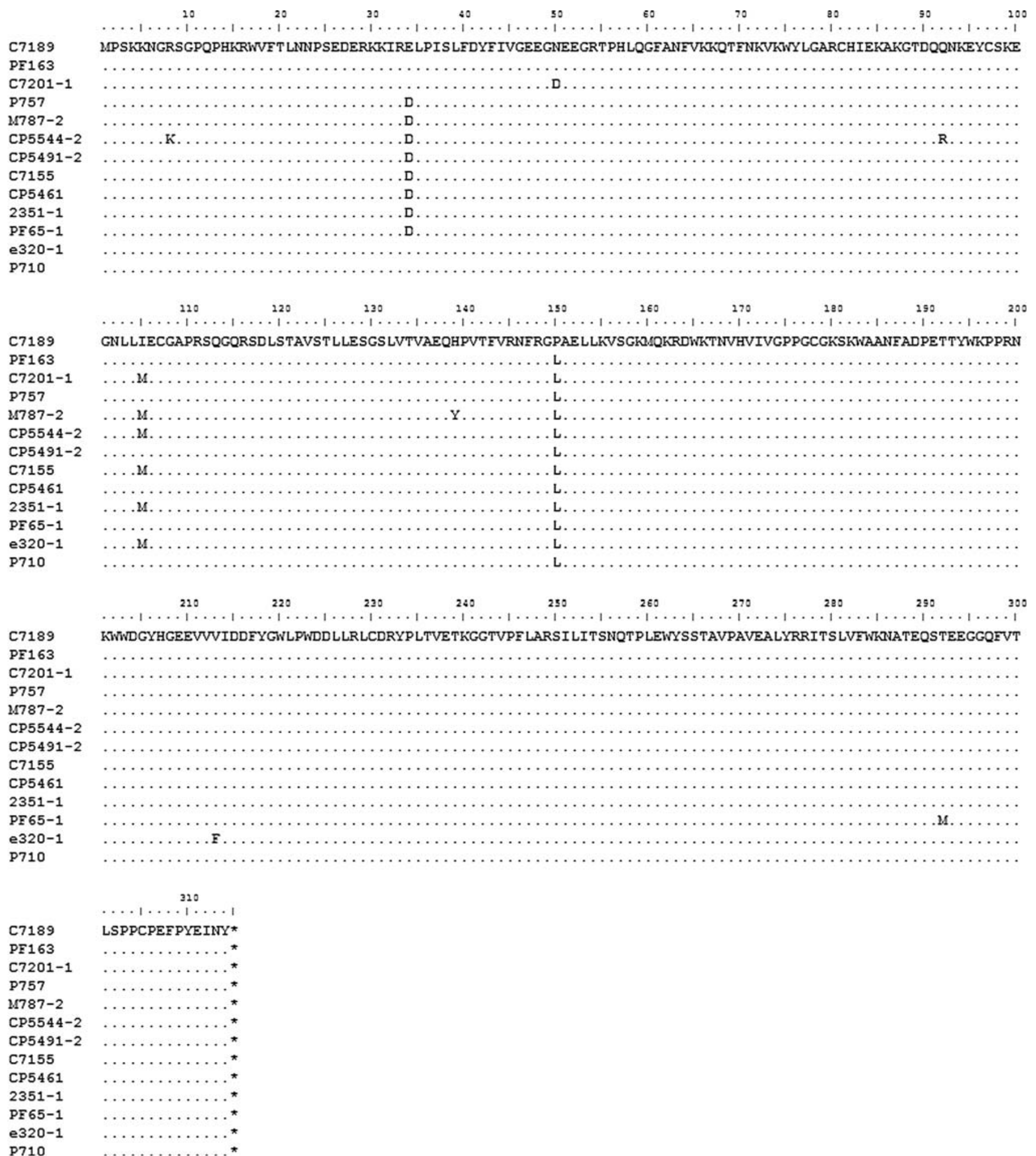

Fig. 4 Deduced amino acid sequences of ORF1 in Korean PCV2 full genomes. For the purpose of this study, splicing was not considered

because previous recombinants identified in the USA and China were also found to be recombined in ORF1 [11, 13]. It can be assumed that the relatively conserved nucleotide and amino acid sequences in ORF1 allow for easy recombination between two separate genotypes of PCV2. It is not clear how this recombination occurs or what effect it may have on the pathogenicity of the virus. Co-infection with PCV2a and PCV2b can occur in field conditions [2]. Although it has not been published, preliminary data from our laboratory also revealed that PCV2 DNA was

significantly higher in PCV2a and PCV2b co-infected pigs than in PCV2b-only infected pigs, without clinical differences. This elevation of PCV2 DNA replication in co-infected pigs might increase the probability of recombination between PCV2a and PCV2b. In another study, a chimera consisting of ORF1 of PCV1 and ORF2 of PCV2 was reported to be not pathogenic [6]. As natural recombination can occur in ORF2 as well as ORF1 region of PCV2 [3], pathogenic changes may be possible in the field by natural recombination.

Although previous reports have described PCV2 recombination events, there was no data to indicate whether this event commonly occurred in countries in which PCV2a and PCV2b are co-circulating. With the additional evidence of natural recombination between PCV2a and PCV2b in Korea presented here, it can be assumed that PCV2 recombination may play an important part the evolution of these viruses in nature.

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