

CASE REPORT

Detection of Porcine circovirus 3 from captured wild boars in Korea

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Abstract

Porcine circovirus 3 (PCV3) is a newly discovered ssDNA virus. The virus was first reported in pigs suffering from several clinical syndromes, including porcine dermatitis and nephropathy syndrome, reproductive disorders, respiratory disease and myocarditis. PCV3 was recently reported in wild boars with high prevalence as well. In this study, 266 wild boar anal swab, feces, nasal swab and whole blood samples were collected from three mainland provinces and one island province (Chungbuk, Gangwon, Gyeonggi, Jeju) of South Korea between 2019 and 2020 including 119 from male, 142 from female and 5 undetermined. PCV3 was diagnosed targeting conserved rep (replication associated protein) gene region using Direct PCR and sequencing. Out of 266 tested samples, 15 were positive for PCV3 with detection frequency at 5.6%. Among 266 samples tested, we obtained 14 partial rep gene sequences and one complete genome sequence of PCV3 with a genome size of 2000nt. Here we present the evidence of PCV3 circulation in Korean wild boars.

KEYWORDS

korea, porcine circovirus 3, wild boar

1 | INTRODUCTION

Circoviridae known to be the smallest autonomously replicating animal virus capable of infecting wide range of animals to birds. They have a circular, ambisense genome ranging from 1.7–2kb in size. Circoviruses have inversely arranged two major open reading frames (ORFs) encoding for

replication associated protein (rep) and capsid protein (cap) which are separated by a 3' intergenic region between stop codons and a 5' intergenic region between start codons. Porcine circoviruses are known to infect pigs and there are four types including the very recent report of Porcine circovirus 4. Meanwhile porcine circovirus 1 (PCV1) has no pathogenicity in pigs, Porcine circovirus 2 (PCV2) is responsible for causing Porcine

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circovirus associated disease or postweaning multisystemic wasting syndrome (PMWS). PCV3 was recently discovered porcine circovirus and was first reported in USA (Palinski et al., 2016). The presence of virus was subsequently reported in other Countries including Brazil (Tochetto et al., 2018), China (Ku et al., 2017; Shen et al., 2018), Colombia (Vargas-Bermudez et al., 2019), Denmark (Franzo, Legnardi, Hjulsager, et al., 2018), Germany (Fux et al., 2018), India (Bera et al., 2020), Japan (Hayashi et al., 2018), Korea (Kim et al., 2018; Kwon et al., 2017), Malaysia (Tan et al., 2020), Poland (Stadejek et al., 2017), Russia (Yuzhakov et al., 2018), Spain (Saporiti et al., 2020), Sweden (Ye et al., 2018) and Thailand (Kedkovid et al., 2018). Since the first report, PCV3 was detected in the presence of severe diseases including porcine dermatitis and nephropathy syndrome (PDNS), reproductive disorders, respiratory signs and myocarditis and PCV3 genomes were also detected in asymptomatic animals which speculates the interpretation of this virus as potential causative agent of the disease though circoviruses are considered to be immune suppressive and increase in disease severity with co-infecting pathogens (Shulman & Davidson, 2017). Besides swine populations, PCV3 was also detected in wild boars with the first report of PCV3 detection in wild boar reported in Italy (Franzo, Tucciarone, et al., 2018) and subsequently reported in other countries such as Brazil (Varela et al., 2020), Germany (Prinz et al., 2019) and Spain (Klaumann et al., 2019).

Wild boars are susceptible to several pathogens with potential for transmission to humans and animals. In addition, many viral diseases present in domestic pigs can also affect wild boars and these animals may act as a disease reservoir (Meng et al., 2009). Wild boar populations are seeming to be increasing around the world, hence larger number of animals are available for infection with viruses as well as higher possibility of transmission between species (Vetter et al., 2020). From 1978 to 2016 (38 years), the index of wild boar abundance (estimated through 127 game survey sites in South Korea), increased from 1.1 to 5.6 (509% increase), and the wild boar hunting harvest in South Korea rose from 18,345 in 2012 to 57,971 in 2017 (Jo & Gortázar, 2020).

The aim of this study was to understand the prevalence and genetic characteristics of Porcine circovirus 3 in captured Korean wild boars.

2 | MATERIALS AND METHODS

In Korea, wild boars are listed in the harmful wild animals. Therefore, the wild boar samples were obtained from the captured wild boars through Korea Wildlife Institute. The type of samples: anal swab, feces, nasal swab and whole blood were transported in the virus transport medium (Noble Biosciences™, Korea) collected between 2019 and 2020 in three mainland provinces (Chungbuk, Gangwon, Gyeonggi) and one island

province (Jeju). The collected sample was stored at 4°C until they were analysed. For each animal, information including sex, estimated weight, collection date and accurate sampling locations were carefully noted with latitude and longitude of the collection site.

DNA was extracted from 200µl of sample using QIAamp® DNA Mini Kit according to manufacturer's instructions. DNA was tested for the presence of PCV3 using a Direct PCR method with the following primers which targets the conserved rep gene region of Porcine circovirus 3: PCV3-F(5'-AAAGCCCGAAACACAGGTGGTGT-3') PCV3-R(5'-TTTTCCCGCATCCTGGAGGACCAAT-3')(Franzo, Legnardi, Centellegh, et al., 2018). The cycling conditions were: Initial denaturation of 95°C for 5mins, followed by 35cycles of amplification with denaturation at 95°C for 45secs, annealing at 57°C for 45secs, extension at 72°C for 45secs and final extension at 72°C for 5min. 2µl of extracted DNA and 1µl of 10µM of each primer was added to Maxime™ PCR PreMix kit (iNtRON Biotechnology, Korea) and amplified using the above described thermal protocol. The expected PCR products were checked on 1X TBE agarose gel. Around 480bp size of amplicons were extracted from gel using QIAquick® Gel Extraction kit according to manufacturer's guidelines and sequenced by sanger sequencing method. Sequencing was performed at Cosmogenetech, Korea with PCV3-F & PCV3-R primers.

Inverse PCR was performed for the full genome amplification of porcine circovirus 3 with multiple primers (WB-33-F1 5'-GACTGAAGTTGCGGAGAAGATGCCTGCAG-3', WB-33-R1 5'-CCCTTGCAAGTGTGGGGTACCGCTTTTTCC-3', WB-33-F2 5'-GCAGTTGTATTCAAGCCAC-3', WB-33-R2 5'-CGTTACAAGTCCGTTCTC-3', WB-33-F3 5'-CACATGCAGGGCGTTTAC-3', WB-33-R3 5'-GGACCACAAACACTTGGCTC-3') were designed extending outward from the obtained partial sequences. The PCR was performed using QIAGEN® LongRange PCR Kit according to manufacturer's instructions. Then, the PCR generated overlapping fragments were assembled into consecutive sequence by using CAP contig assembly program of BioEdit software. Putative ORF with a minimum size of 150aa and coding capacity were predicted using NCBI ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). The map of Porcine circovirus 3 isolate WB-33 was drawn using the Geneious prime software. Potential Hairpin and stem loop structure was predicted using Mfold webserver (<http://unafold.rna.albany.edu/?q=mfold>). Phylogenetic analysis was conducted in MEGA X.

3 | RESULTS AND DISCUSSION

In total, among 266 samples (Table 1) tested 15 were positive for PCV3 with a frequency of detection at 5.6% which is similar

TABLE 1 Month-wise distribution of Positive samples from wild boars

Year	Month												Total
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
2019	-	-	-	0/1	0/5	0/11	2/45	1/12	0/5	3/53	0/7	1/18	7/157
2020	0/5	2/18	5/30	0/15	0/10	0/6	0/11	0/2	0/1	0/1	1/6	0/4	8/109

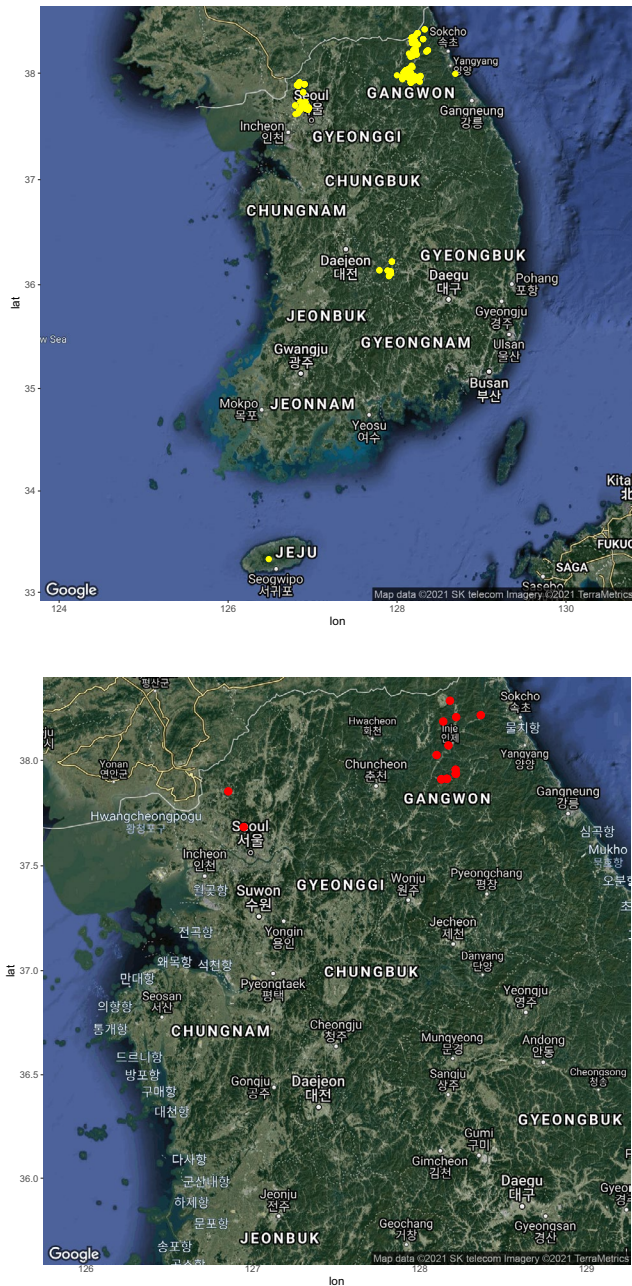


FIGURE 1 Geographical distribution of captured wild boar samples in Korea. Negative and positive samples have been represented in yellow and red, respectively. The map was constructed using the RStudio software

to studies conducted in China (Sun et al., 2018), Japan (Hayashi et al., 2018) and Spain (Klaumann et al., 2019). In this work, PCV3 was detected in whole blood, serum and nasal swab and in all age groups regardless of gender. Out of four tested provinces, positive samples were detected from Gangwon and Gyeonggi province (Figure 1). We obtained 14 partial replication associated protein gene sequences and one complete genome of porcine circovirus 3. Phylogenetic studies conducted to date suggests the very low genetic variability between strains indicating that the virus is relatively stable over the years. Fourteen partial rep gene sequences obtained

in this study showed more than 99% identity with each other and sequences from GenBank (Figure 3a).

The Porcine circovirus 3 isolate WB-33 complete genome encompasses 2000nt and deposited at GenBank under accession number MW168693. In comparison with other PCV3 complete genomes available at GenBank, the PCV3 isolate WB-33 sequence revealed more than 99% identity at nucleotide level. At amino acid level, the degree of identity was ranging from 97.98% to 99.19% for replication associated protein and 97.66% to 99.53% for capsid protein, respectively. The ORF1 encodes a 247 amino acid replication associated protein and the ORF 2 which is in opposite orientation to ORF1 encodes 214 amino acid capsid protein. A potential stem loop structure with a conserved nonanucleotide sequence atop TAGTATTAC which was described in other circoviruses as well and are considered to be responsible for initiating the rolling circle replication in circoviruses suggested by Andrew K. Cheung, 2006 was predicted (Figure 2).

To understand the evolutionary relationship of PCV3 isolate WB-33, it was compared with the representative members of circovirus (Figure 3b) and PCV3 genomes identified worldwide. PCV3 was classified into two main groups 'a' and 'b' and multiple sub clusters (a1, a2, b1, b2) based on the amino acid (aa) motifs coded by codon 122 of ORF1 and codons 24, 27, 77 and 150 of ORF2 (Fux et al., 2018; Li et al., 2018). Based on these motifs PCV3 isolate WB-33 was identified as PCV3b (Table 2). To date, most PCV3 sequences recovered from wild boars were classified as group b (Prinz et al., 2019; Varela et al., 2020), suggesting that this group may be more common in these animals. Complete genome sequence of PCV3 isolate WB-33 was compared with 83 complete genome sequences of PCV3 obtained from pigs and wild boars retrieved from GenBank (Figure 3c). PCV3 phylogenetic analysis showed that the PCV3 isolate WB-33 is closely related to PCV3 genomes recovered from wild boars in Brazil (Varela et al., 2020), Italy (Dei Giudici et al., 2020) and Spain (Klaumann et al., 2019) and sequences recovered from domestic pigs in China, Denmark, Germany and Italy.

These results indicate that PCV3 genomic features do not support conclusions on its geographical and seasonal pattern of circulation, confirming the previous studies. Furthermore, genetic-based interpretations about PCV3 transmission between wild boars and domestic pigs may also be difficult considering its genomic stability in comparison with other single stranded DNA viruses. It is possible to assume that there is transmission between these animals due to increased wild boar populations and consequently higher probability of contact with domestic pigs. Therefore, the epidemiological role of these animals as possible reservoirs of PCV3 for domestic pigs should not be excluded and require further studies. As of those previous studies and this present study clearly validates that the wild boar is susceptible to PCV3 infection and the potential natural host for the circulation of PCV3. In conclusion, in this present study, we detected PCV3 in wild boars captured from Gyeonggi & Gangwon provinces and demonstrate the evidence of Porcine circovirus 3 circulation in Korean wild boar populations. Hence, our study explains

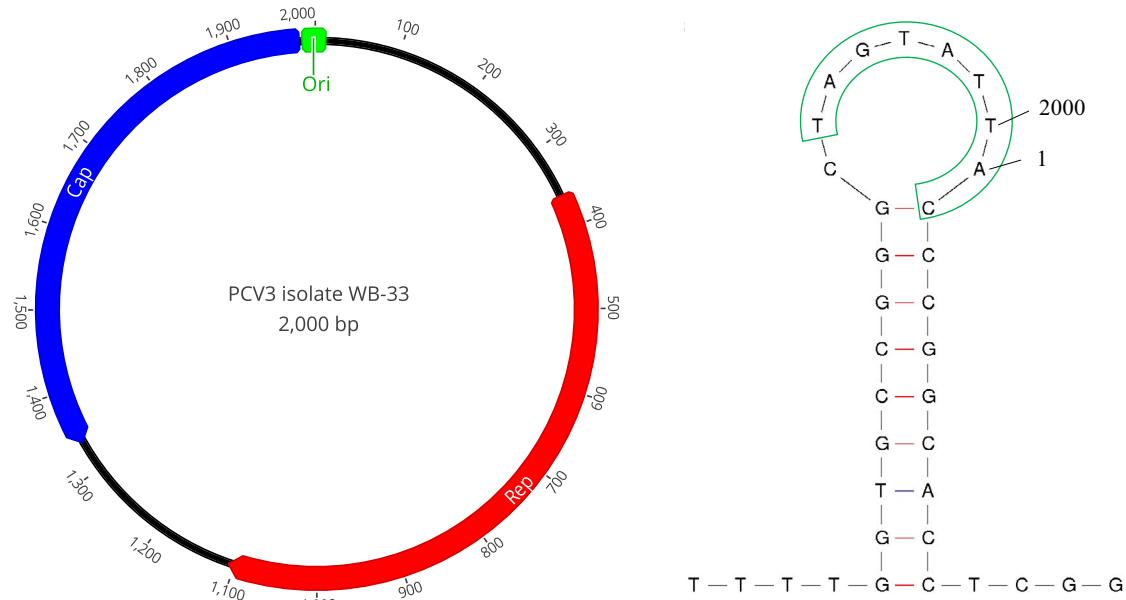


FIGURE 2 Predicted genome organizations of Porcine circovirus 3 isolate WB-33. The two major ORFs encoding putative replication-associated protein and capsid protein is shown in red and blue colour, respectively. origin of replication is shown in green colour. A potential stem loop structure was predicted using Mfold web server and the nonamer motif sequence is indicated

TABLE 2 Patterns for Porcine circovirus 3 grouping

Groups	ORF1		ORF2		
	122	24	27	77	150
a1	A	V	K	S/N	I
a2	S	A/V	K	S	I/L
b1	S/A	A/V	R	S	I/L
b2	S	A	R	T	L/I
WB-33	S	A	R	S	I

the paramount importance of further investigations on PCV3 presence in Korean wild boar populations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Gowtham Dhandapani: Conceptualization; Data curation; Formal analysis; Methodology; Writing-original draft. **Sun-Woo Yoon:** Investigation; Resources; Writing-review & editing. **Ji Yeong Noh:** Methodology; Validation. **Seong Sik Jang:** Methodology; Validation. **Sang-Hoon Han:** Resources. **Dae Gwin Jeong:** Funding acquisition; Investigation; Project administration; Resources. **Hye Kwon Kim:** Conceptualization; Funding acquisition; Investigation; Project administration; Supervision; Validation; Writing-review & editing.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. In Korea, wild boars are listed in the harmful wild animals and has been regularly hunted. Therefore, the wild boar samples were obtained from the hunted wild boars through Korea Wildlife Institute, consequently no ethical approval was required for the investigations.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at <https://www.ncbi.nlm.nih.gov/genbank/>, reference number MW168687 – MW168693 and MW561266 – MW561273.

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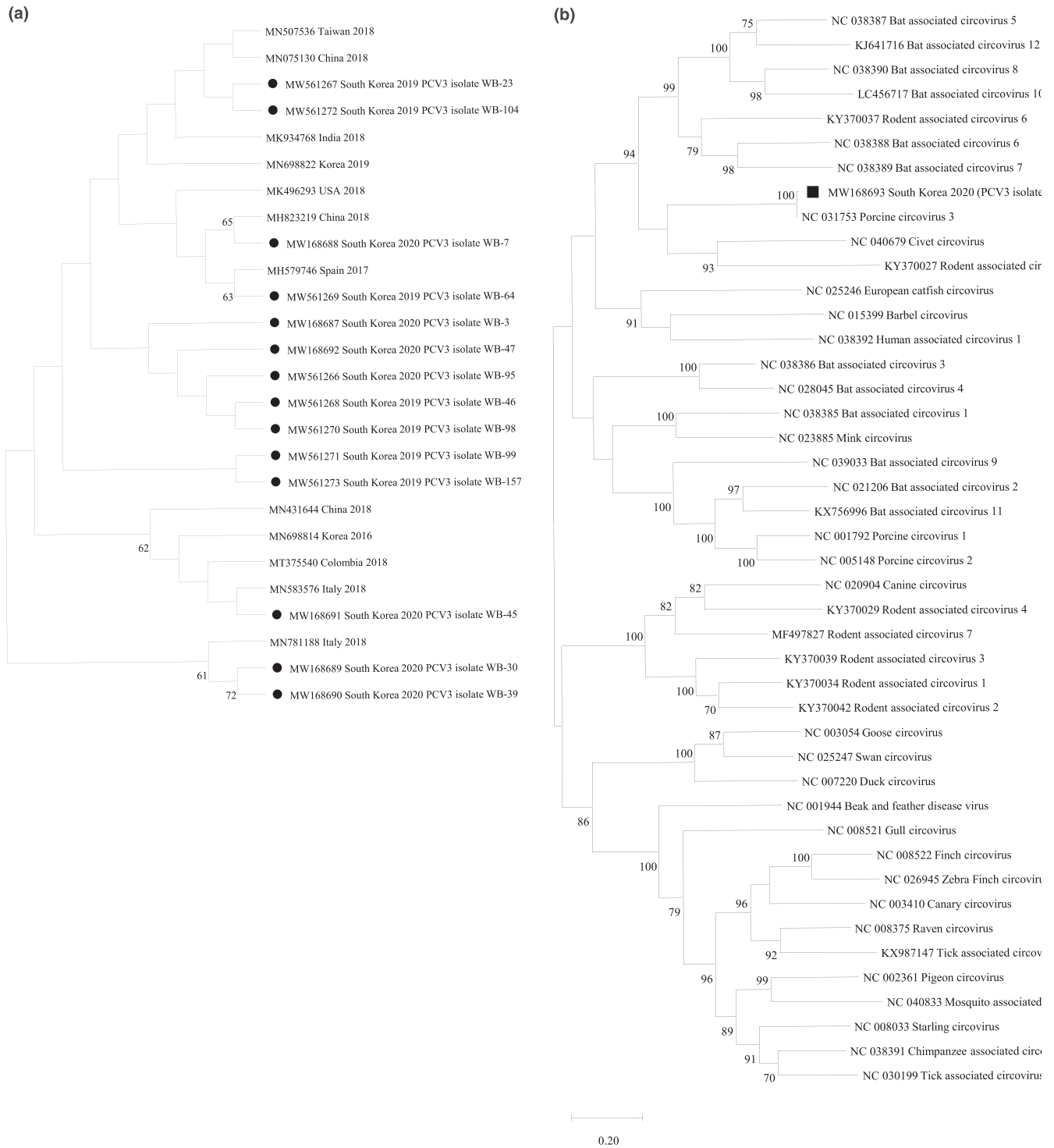


FIGURE 3 (a) Phylogenetic analysis of partial PCV3 rep gene sequences. The evolution history was inferred using maximum likelihood method based on Tamura-Nei model with 1,000 bootstrap replicates. Evolutionary analysis was conducted in MEGA X. Sequences obtained in this study shown in bold dots. (b) Evolutionary relationships of PCV3 isolate WB-33 with representative members of circovirus. The evolution history was inferred using maximum likelihood method based on Tamura-Nei model with 1,000 bootstrap replicates. Evolutionary analysis was conducted in MEGA X. Only bootstrap value >70 is shown. Sequence obtained in this study denoted in bold square. (c) Phylogenetic analysis of PCV3 genomes. The evolution history was inferred using maximum likelihood method based on Tamura-Nei model with 1,000 bootstrap replicates. Evolutionary analysis was conducted in MEGA X. Only bootstrap value >50 is shown. Green colour represents PCV3-a subtype and red colour represents PCV3-b subtype. 'WB' represents sequence obtained from wild boar. Sequence obtained in this study denoted in bold square

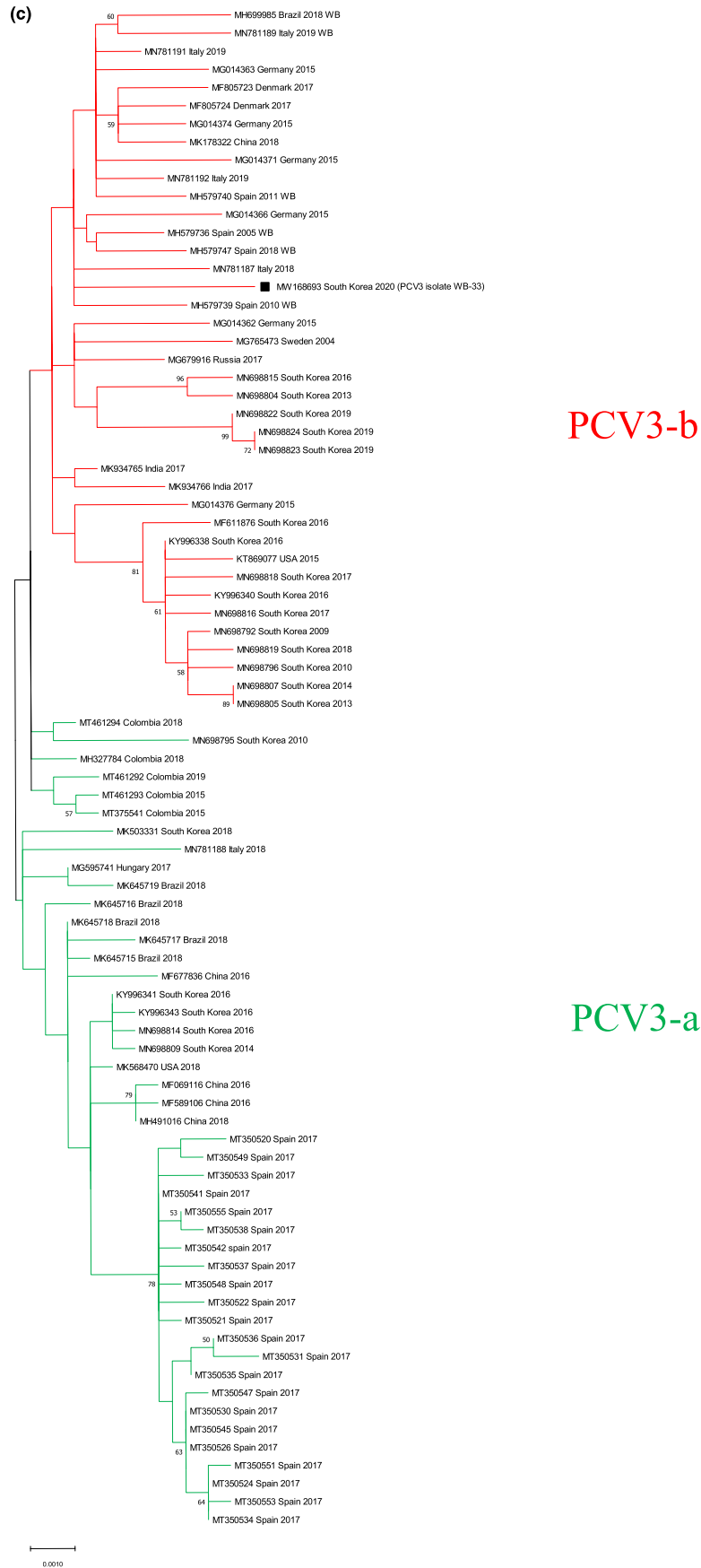


FIGURE 3 (Continued)

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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