Detection and genetic characterization of porcine circovirus 4 (PCV4) in Guangxi, China

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Abstract

Porcine circovirus type 4 (PCV4), a novel circovirus, was first detected in pigs with porcine dermatitis and nephropathy syndrome (PDNS) in China. This study investigated the frequency of porcine circovirus 4 (PCV4) in pigs in Guangxi Province, China, from 2015 to 2019 and its genome diversity. Thirteen of 257 (5.1%) samples were positive for PCV4, 9 of 13 (69.2%) PCV4-positive samples were coinfected with PCV2 or PCV3, and one PCV4-positive sample was coinfected with both PCV2 and PCV3. Similar to other PCVs, PCV4 contains two major ORFs and a stem loop (TTCAGTATTAC). Multiple sequence alignments showed that the PCV4 genome shares 25.3-73.8% nucleotide similarity with other representative circovirus genomes. Interestingly, the PCV4 Cap protein shares relatively high homology (approximately 50%) with the PCV2 Cap protein and has multiple highly homologous peptides. Multiple amino acid sequence alignments of the Cap protein revealed that PCV2 and PCV4 have multiple highly homologous antigen sites and identical receptor binding sites. Therefore, PCV2 and PCV4 may have cross-protective immunogenicity. Phylogenetic analysis showed that PCV4 is closely related to mink circovirus and bat-associated circovirus. In summary, this was the first seroprevalence and genetic investigation of PCV4 in Guangxi Province, China. The results provide insights into the epidemiology and pathogenesis of this important virus.

Title

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Running Head

Prevalence of novel porcine circovirus 4 (PCV4) in pig populations

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Summary

Porcine circovirus type 4 (PCV4), a novel circovirus, was first detected in pigs with porcine dermatitis and nephropathy syndrome (PDNS) in China. This study investigated the frequency of porcine circovirus 4 (PCV4) in pigs in Guangxi Province, China, from 2015 to 2019 and its genome diversity. Thirteen of 257 (5.1%) samples were positive for PCV4, 9 of 13 (69.2%) PCV4-positive samples were coinfected with PCV2 or PCV3, and one PCV4-positive sample was coinfected with both PCV2 and PCV3. Similar to other PCVs, PCV4 contains two major ORFs and a stem loop (TTCAGTATTAC). Multiple sequence alignments showed that the PCV4 genome shares 25.3-73.8% nucleotide similarity with other representative circovirus genomes. Interestingly, the PCV4 Cap protein shares relatively high homology (approximately 50%) with the PCV2 Cap protein and has multiple highly homologous peptides. Multiple amino acid sequence alignments of the Cap protein revealed that PCV2 and PCV4 have multiple highly homologous antigen sites and identical receptor binding sites. Therefore, PCV2 and PCV4 may have cross-protective immunogenicity. Phylogenetic analysis showed that PCV4 is closely related to mink circovirus and bat-associated circovirus. In summary, this was the first seroprevalence and genetic investigation of PCV4 in Guangxi Province, China. The results provide insights into the epidemiology and pathogenesis of this important virus.

KEYWORDS: Porcine circovirus type 4; Porcine; Epidemiology; Phylogenetic analysis

1. INTRODUCTION

Porcine circovirus (PCV), containing a small single-stranded, nonenveloped, closed circular DNA genome, has been reported as one of the smallest viruses and belongs to the genus *Circovirus* under the family *Circoviridae* (Tischer et al., 1982). Its genome contains two major open reading frames (ORF1 and ORF2), which encode a replication-associated protein (Rep) and a structural protein (capsid protein, Cap), respectively. Specifically, Cap contains multiple cell epitopes that are associated with virus neutralization (Cao et al., 2018; Mayr et al., 1968).

Three major genotypes of PCV have been reported. Porcine circovirus type 1 (PCV1) was first identified as a contaminant in a pig kidney cell culture (PK-15) and is considered nonpathogenic (Cao et al., 2018). However, PCV2 and PCV3 are the causative agents of multiple clinical diseases in swine and result in substantial economic losses for the pig industry worldwide (Meng, 2013; Palinski et al., 2017; Sun et al., 2019; Wang et al., 2019). In a recent report, porcine circovirus type 4 (PCV4), a novel and genetically divergent porcine circovirus, was first revealed in Hunan, China. PCV4 is suspected to be associated with severe clinical disease involving respiratory signs, enteric signs and porcine dermatitis and nephropathy syndrome (PDNS) (Zhang et al., 2019).

Similar to the PCV genome, the PCV4 genome is also circular single-stranded DNA and contains two major open reading frames. Genomic and phylogenetic analyses revealed that PCV4 has the closest relationship to mink circovirus (MiCV), which is associated with enteric disease (Zhang et al., 2019). However, knowledge about the infection rate and pathogenicity of this virus is limited. Consequently, we became interested in understanding the seroprevalence of PCV4, preferably using a molecular approach that facilitates any necessary genetic analyses.

2. MATERIALS AND METHODS

2.1 Sample information

From 2015 to 2019, tissue samples (n = 93) and serum samples (n = 164) of pigs were collected from 15 swine farms in Nanning, Guilin, Fangchenggang, Beihai, Baise, and Hezhou in Guangxi Province, China (Figure 1). Of these, 17 tissue samples and 11 serum samples were from swine with severe clinical signs, including respiratory disease, lymphadenopathy and PDNS. This study received animal ethics approval (No. Xidakezi2000138) from Guangxi University (see ethics approval and consent to participate).

2.2 DNA isolation and polymerase chain reaction (PCR)

The viral genome was extracted from swine serum or tissue using the EasyPure Viral DNA/RNA Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Two primer pairs were

designed based on the reference sequences of the PCV4/HNU-AHG1-2019 strain (NO. MK986820.1), and published primers and protocols were used to detect PCV4 (Table S1). The PCR mixture contained 2 μL of extracted DNA, 2 μL of primer pairs (10 μM), 25 μL of 2×Phanta Max Master Mix (Vazyme, Nanjing, China), and 21 μL of DNase/RNase-Free water. The PCR amplification conditions were as follows: predenaturation for 3 min at 95 °C; followed by 35 cycles of 15 s at 95 °C, 15 s at 62 °C, extension for 1 min at 72 °C; and a final extension for 5 min at 72 °C. Subsequently, the PCR products were separated using 1.2% agarose gel electrophoresis of DNA and cloned into a pMD18-T vector (Takara Co. Dalian). The recombinant vectors were amplified in *Escherichia coli* (*E. coli* , DH5α) for sequencing.

2.3. Multiple sequence alignment and phylogenetic analysis

The genome sequences of PCV4 obtained in this study have been deposited in GenBank under the accession numbers MT311852–MT311854. Multiple sequence alignments were carried out using the Megalign program within the Lasergene package (DNAStar software, DNASTAR Inc.), and phylogenetic relationships were assessed with MEGA software (version 7). Support for the phylogenetic relationships was determined by bootstrapping (1000 replicates). In the present study, the method described by Zhang et al. was used to divide the clades of PCV4 (Zhang et al., 2019).

3. RESULT AND D ISCUSSION

Farm workers and all species of farm animals can be infected by circovirus (Li et al., 2011). Multiple studies have indicated that three circovirus species can infect pigs. PCV2 and PCV3 are recognized as the main pathogens in PCV-associated disease (PCVAD) and cause severe economic loss to the swine industry worldwide (Liu et al., 2019; Wang, Cao, et al., 2019; Wen et al., 2018). Recently, a novel and genetically divergent circovirus, PCV4, was considered to be a new pathogen of respiratory disease, diarrhea and PDNS (Zhang et al., 2019).

In the present study, with reference to the method of Zhang et al. (Zhang et al., 2019), a TaqMan® real-time PCR assay was used to detect PCV4 in clinical samples collected between 2015 and 2019 from Guangxi Province, China. The results showed that 5.1% (13/257) of the porcine samples collected from six cities were PCV4 positive. Eight of 93 tissue samples were PCV4 positive, and 5 of 164 serum samples were PCV4 positive. Six porcine samples with PDNS and two porcine samples with respiratory disease were PCV4 positive. Coinfection of pigs with PCV2 and PCV3 is known to exacerbate disease severity. Further detection showed that 9 of 13 (69.2%) samples were coinfected with PCV2 (n=7) or PCV3 (n=3). Sample NN88 from Nanning was coinfected with PCV2 and PCV3 at the same time.

To analyze the genetic relationship between PCV4 strains and other representative circoviruses, three complete genome sequences (GX2020/NN88, GX2020/GL69, GX2020/FCG49) of PCV4 were obtained. All strains in this study had genomes that were 1770 bp long. Similar to PCVs, the three Guangxi strains of PCV4 also contain two major ORFs. ORF1 encodes the Rep protein (296 amino acids), and ORF2 encodes the Cap protein (228 amino acids). The amino (N) terminus of the Rep protein contains 3 conserved amino acid motifs: RCR motif I (FTLNN), RCR motif II (PHLQG) and RCR motif III (YCSK). The C terminus contains the dNTP binding site GVGKS. The PCV4 genome has two noncoding intergenic regions (IRs) between the 5'- and 3'- ends of the two major ORFs. A stem loop was detected in the genome of PCV4, and it contains 11 bases (TTCAGTATTAC) instead of the 9 bases previously reported (Figure 2) (Zhang et al., 2019).

Multiple sequence alignment of the PCV4 strains in this study showed that the sequences in their genomes shared 5.1%–73.8% nucleotide identity with all available reference sequences in the complete genome. Furthermore, the PCV4 strains in this study shared 98.5%–99.1% and 99.7%–99.8% nucleotide identity with other PCV4 strains and each other, respectively. An amino acid sequence comparison showed that the Rep protein and Cap protein the PCV4 strains in this study shared 5.1%–80.5% and 17.8%–70.6% identity with those of all available reference strains. Interestingly, multiple sequence alignment results based on three different methods have confirmed that PCV4 shares the highest homology with MiCV (73.8% for the complete genome, 80.5% for the Rep protein and 70.6% for the Cap protein). However, multiple sequence alignment

results showed that PCV4 shares relatively low nucleic acid and amino acid identity ([?] 50%) with PCV1, PCV2, and PCV3 (Table 1).

In circoviruses, Cap is the sole structural protein and contains immunologically important epitopes associated with virus neutralization (Lekcharoensuk et al., 2004; Meng, 2013). Therefore, it has been the main target for vaccines. At present, commercial vaccines against PCV2 have been introduced worldwide, and they have been considered a successful story in veterinary vaccinology (Park et al., 2019). The Cap protein of PCV4 shares a low amino acid sequence similarity (<30%) with that of PCV3. Thus, cross protection seems unlikely. Interestingly, the Cap protein of PCV4 has relatively high homology (approximately 50%) with those of PCV1 and PCV2. Several previous studies have reported the antigenic site (Lekcharoensuk et al., 2004; Mahe et al., 2000; Shang et al., 2009) and receptor binding site (^{98}IRKVKV^{103}) of the Cap protein of PCV2 (Misinzo et al., 2006). In this study, multiple sequence alignment analysis of the amino acid sequence of the Cap protein revealed that PCV2 and PCV4 have multiple highly homologous antigen sites and identical receptor binding sites (Figure 3). Therefore, PCV2 and PCV4 may have cross-protective immunogenicity.

To promote understanding of the genetic relationship between the different strains identified in the present study, a phylogenetic tree was constructed using the maximum likelihood method based on the complete genome and the amino acid sequences of the Rep and Cap proteins. Interestingly, the results were similar using the different algorithms. The results confirm that PCV4 had a close relationship to MiCV and bat-associated circovirus, followed by PCV1 and PCV2. PCV4 and PCV3 are not in the same branch and are distant from each other (Figure 4). Limited numbers of sequences, however, resisted independent evolution analysis on PCV4. Furthermore, multiple studies have indicated that PCV2 and PCV3 are transmitted to nonporcine hosts, possibly through cross-species transmission routes (Song et al., 2019; X. Wang et al., 2018; J. Zhang et al., 2018). Can PCV4 also infect nonporcine hosts? Further studies are needed to answer this question.

Overall, this research is the first report to detect PCV4 in Guangxi Province, China, and to obtain three complete genomic sequences. Our results combined with those of other reports suggest that PCV4 may commonly circulate within swine herds in South China. This research detected PCV4 in swine with PDNS, and whether PCV4 infection is related to PDNS still needs further research to confirm. Further studies are warranted to elucidate the prevalence and pathogenesis of this novel circovirus.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest regarding the research, authorship, and/or publication of this article.

AVAILABILITY OF DATA AND MATERIALS

The data set supporting the conclusions of this article is available in GenBank.

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TABLE 1 Identities (%) shared between porcine circovirus 4 (PCV4) and other members of the genus *Circovirus*

Name	PCV4	PCV4	PCV4
	The complete genome (%)	The Rep protein (%)	The Cap protein (%)
Bat-associated circovirus	44.6	76	41.5
Bat circovirus	65.3	52.6	28.8
Beak and feather disease virus	39.7	6.2	25.4
Canine circovirus	46.4	50.3	18.5
Columbid circovirus	41.6	48.6	23.2
Duck circovirus	40.9	49	24.8
Finch circovirus	42.7	49.1	27.1
Fox circovirus	46.7	52.4	18.5
Goose circovirus	44.6	47.9	23.6
Gull circovirus	38.3	45.7	26.2
Mink circovirus	73.8	80.5	70.6
Chicken anemia virus	25.3	5.1	17.8
Raven circovirus	40.7	47.4	25.6
Starling circovirus	43.9	50.5	23.2
Porcine circovirus 1	61.2	49.8	46.4
Porcine circovirus 2	60.9	48.3	50
Porcine circovirus 3	41.8	47.6	26

FIGURE 1 Geographical information for samples collected in Guangxi, China. Red stars indicate the geographical location of the samples.

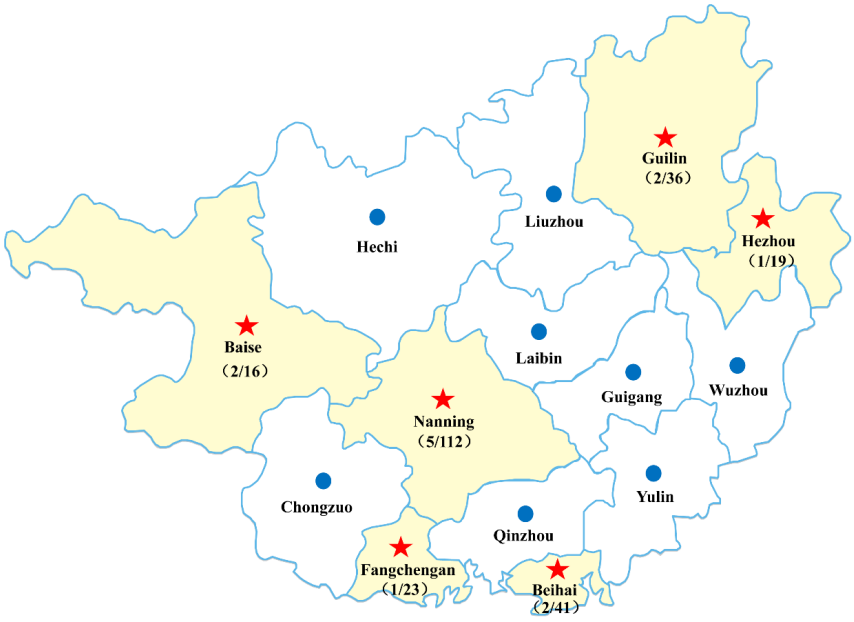
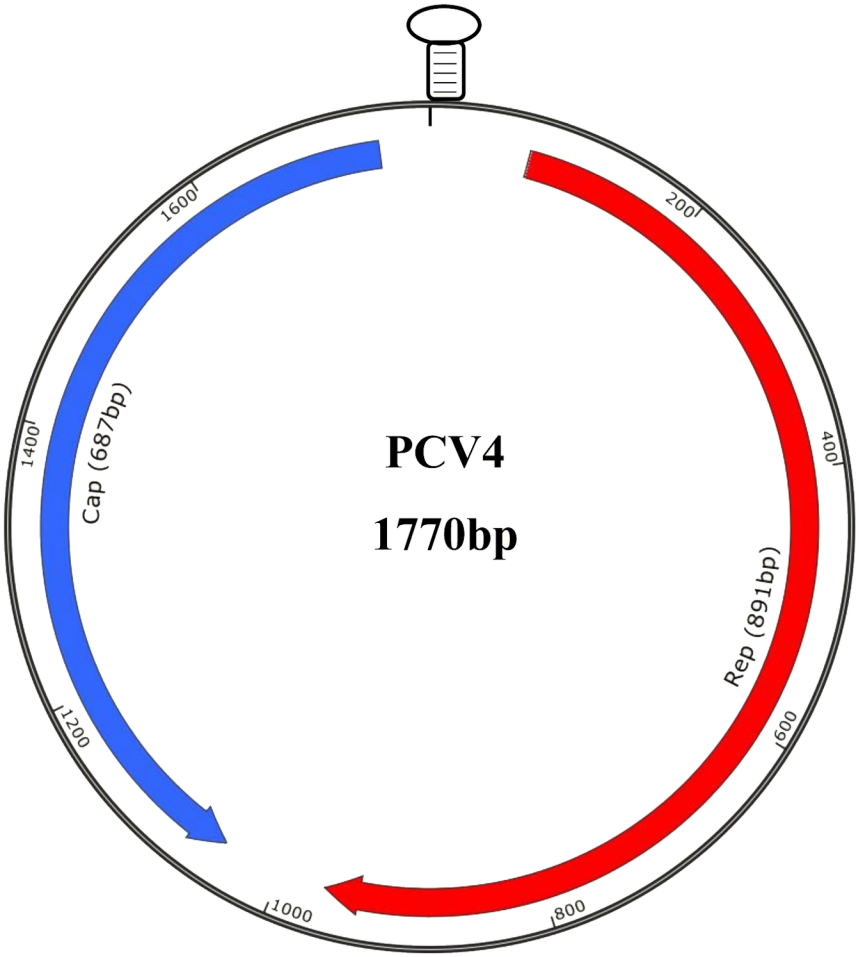


FIGURE 2 Predicted genome organization of PCV4 (A) and the stem loop of PCV4 (B).

A B



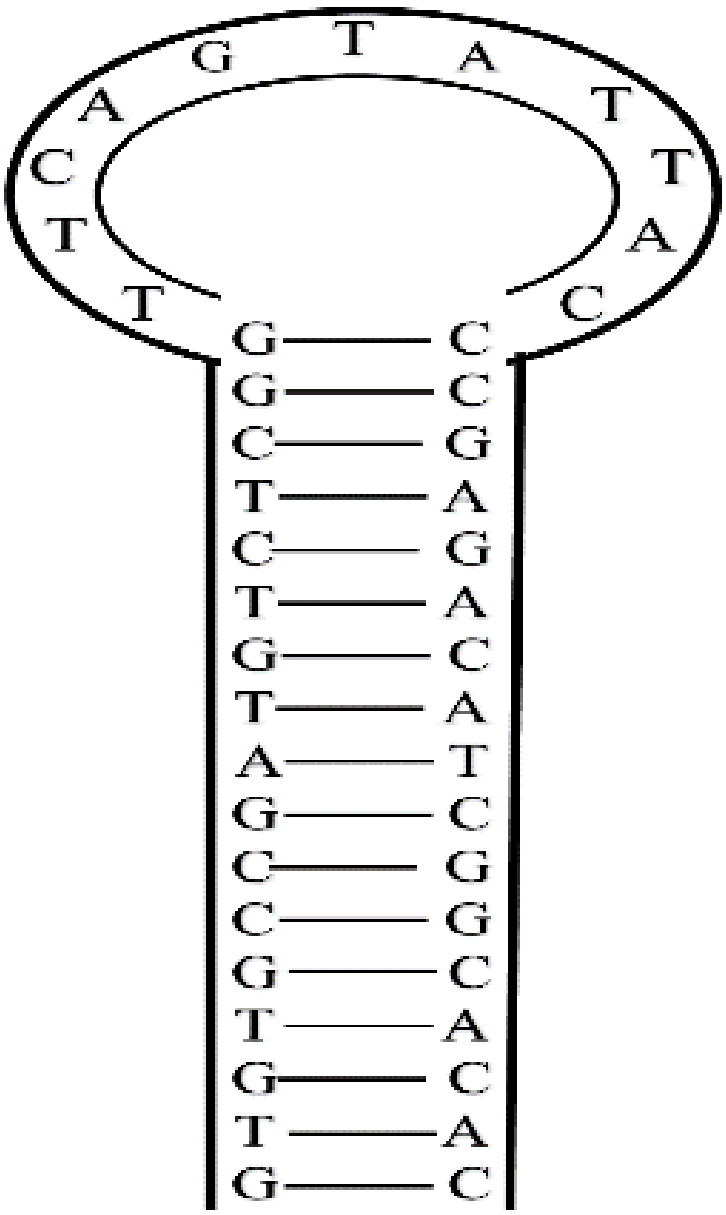


FIGURE 3 Multiple sequence alignment analysis of the amino acid sequence of the Cap protein in porcine circovirus 4 (PCV4) and PCV2. Black boxes indicate antigen sites, and yellow boxes indicate receptor binding sites.

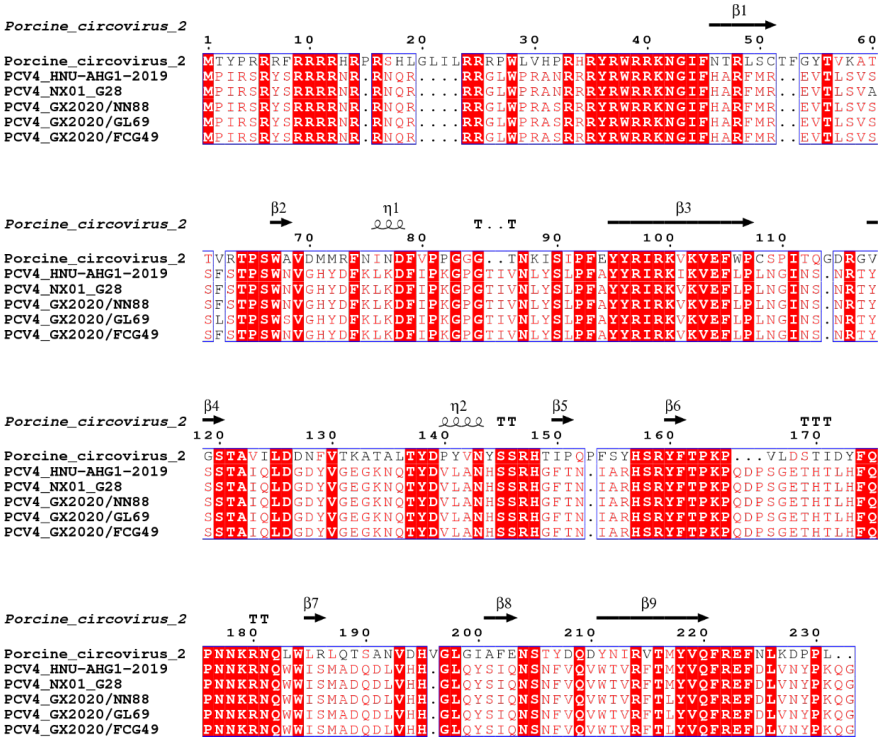


FIGURE 4 Phylogenetic analysis based on the complete genome (A) and the amino acid sequences of the Rep protein (B) and Cap protein (C).

A

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B

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`image6.emf` available at https://authorea.com/users/314652/articles/445036-detection-and-genetic-characterization-of-porcine-circovirus-4-pcv4-in-guangxi-china

C

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`image7.emf` available at https://authorea.com/users/314652/articles/445036-detection-and-genetic-characterization-of-porcine-circovirus-4-pcv4-in-guangxi-china

Supporting Information

Table S1. List of primer sequences used in this study.

Primer	Sequence (5'-3')	Reference
PCV4-F	GGAACGACAAGGACGACACTT	For detecting PCV4 (Zhang et al., 2010)
PCV4-R	CTTGAGGCTCTGGTATCTTATTGC	

Primer	Sequence (5'-3')	Reference
PCV4-TZ	FAM-CCGCCCTGAATGCCGGCAGCTCAATG-BHQ1	
PCV4-1-F	CAGTATTACCCGAGACATCGGCA	For genome sequencing, this study.
PCV4-873-R	GGGCTCTGATATCCACTTTCAGCTCCACAT	
PCV4-775-F	GTTCCATTGAGTTCGTGGCCAACGA	For genome sequencing, this study.
PCV4-115-R	GTGAAACAATATCTCTTCACGGGGTG	

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