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Epidemiological Investigation and Genome Analysis of Duck Circovirus in Southern China

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Abstract: Duck circovirus (DuCV), a potential immunosuppressive virus, was investigated in Southern China from March 2006 to December 2009 by using a polymerase chain reaction (PCR) based method. In this study, a total of 138 sick or dead duck samples from 18 different farms were examined with an average DuCV infection rate of ~35%. It was found that ducks between the ages of 40～60 days were more susceptible to DuCV. There was no evidence showing that the DuCV virus was capable of vertical transmission. Farms with positive PCR results exhibited no regularly apparent clinical abnormalities such as feathering disorders, growth retardation or lower-than-average weight. The complete genomes of 9 strains from Fujian Province and 1 from Zhejiang Province were sequenced and analyzed. The 10 DuCV genomes, compared with others genomes downloaded from GenBank, ranged in size from 1988 to 1996 base pairs, with sequence identities ranging from 83.2% to 99.8%. Phylogenetic analysis based on genome sequences demonstrated that DuCVs can be divided into two distinct genetic genotypes, Group Ⅰ (the Euro-USA lineage) and Group Ⅱ (the Taiwan lineage), with approximately 10.0% genetic difference between the two types. Molecular epidemiological data suggest there is no obvious difference among DuCV strains isolated from different geographic locations or different species, including Duck, Muscovy duck, Mule duck, Cheery duck, Mulard duck and Pekin duck.

Key words: Duck circovirus(DuCV); Epidemiological investigation; Genome; Phylogenetic analysis

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Circovirus are small, non-enveloped viruses composed of icosahedra particles with a diameter of 17-26.5 nm and a circular single-stranded DNA genome, approximately 2kb in size [9, 28]. Currently, the family *Circoviridae* comprises two genera *Gyrovirus* and *Circovirus*. To date, the genus *Gyrovirus* contains only one member, the chicken anaemia virus (CAV) [17]. Within the genus *Circovirus* there are several

members, including porcine circovirus types 1 and 2 (PCV 1 and PCV 2) [4], psittactine beak and feather disease virus (BFDV) [16], pigeon circovirus (PiCV, also known as columbid circorus, CoCV) [13,27], canary circovirus (CaCV) [14, 26], goose circovirus (GoCV) [21, 27], duck circovirus (DuCV) [8], raven circovirus (RaCV) [22], starling circovirus (StCV) [11], finch circovirus (FiCV), gull circovirus (GuCV) [25], ostrich (*Struthio camelus*) (OCV) [3]and the recently identified circoviruses infecting mute swan (*Cygnus olor*) (SwCV) [7].

Duck circovirus (DuCV) was first reported in Germany in 2003[8, 20]. Since then, DuCV has subsequently been reported in Hungary [5], Taiwan [2] and the USA [1]. We reported the first detection of DuCV in the mainland China in Fujian Province [6], since then DuCV has been reported in Shandong (SD-LY0701), Guangdong [12], Zhejiang (this study) and Jiangsu (JS-AQ0901) provinces. To better understand the characteristics of DuCV in Southern China, we report here the detection of DuCV at different farms in Southern China, and the complete nucleotide sequences of 10 strains. Comparative analysis was conducted with DuCVs reported from Taiwan, Shangdong, Guangdong, USA and Germany.

MATERIALS AND METHODS

Samples collection

A total of 138 ducks were collected from 18 different duck farms located in different areas of Fujian, Zhejiang and Guangdong Province in Southern China from March 2006 to December 2009 (Table 1). In general, there was no obvious clinical abnormities among the sampled duck flocks, although sporadic deaths and growth retardation in a small number of ducks were often observed.

DNA extractions

DNA was isolated from a mixture of the lung, liver, spleen and fabricius bursa of each sample using a DNeasy Blood & Tissue kit (Qiagen, Germany) according

Table 1. Detection of DuCV from different regions in Southern China*

Area		Farm	DuCV Ratio	GenBank No.	Total
Fujian	Minhou	1	4/7	GQ423740	21/37(56.76%)
		2	5/8	GQ423741	
		3	8/12	GQ423742	
		4	0/5	none	
		5	4/5	GU168779	
	Changle	1	5/10	GQ423743	5/16(31.25%)
		2	0/6	none	
	Zhangpu	1	4/7	GQ423747	4/12(33.33%)
		2	0/5	none	
	Fuqing	1	8/15	GQ423744	12/24(50%)
		2	0/10	none	
		3	4/9	GQ423745	
	Putian	1	4/10	GQ423746	4/20(20%)
		2	0/10	none	
Zhejiang	Wenzhou	1	3/4	GQ334371	3/7(42.86%)
		2	0/3	none	
	Jinhua	1	0/5	none	0
Guangdong	Foshan	1	0/7	none	0

* From DuCV positive farms, only one complete genome sequence was determined from each farm, which was used in the final phylogenetic analysis.

to the manufacturer's instructions. Total DNA was precipitated and used for DuCV examination, and each DNA sample was defined as a virus strain.

Primers used for Epidemiological investigation

A primer pair was designed according to Chen[2], with the following sequences for forward: 5'-ATATT ATTACCGGCGC(C/T) TGTA-3' and reverse: 5'-TC AGGAATCCCTG(A/C)AGGTGA-3'. The targeted amplificon is a 228-bp segment of DuCV genome.

Epidemiological investigation for field samples

A total of 138 clinic samples were examined using a GoTaq Green Master Mix (Promega, USA) according to the manufacturer's instructions. The PCR mixture (50μL) contained 25μL GoTaq Green Master Mix, 22μL distilled water, 1μL primers (20 pmol each) and 1 μL of template DNA. PCR condition was 1 cycle of 94 ℃ for 5 min; 35 cycles of 94 ℃ 30 s (melting), 54 ℃ 30 s (annealing), and 72 ℃ 30 s (extension) and a final extension period of 72 ℃ for 5 min. The PCR product was separated by electrophoresis in a 1.5% agarose gel. The specific PCR products were purified using an agarose gel DNA purification kit (Takara, Dalian) according to the manufacture's instructions. Purified DNA samples were cloned using a pMD18-T kit (TaKaRa, Dalian) and then trans- formed into *Escherichia coli* DH5α cells. The positive recombinant clones were sequenced to confirm the sequences.

Vertical transmission investigation

The embryonated eggs of Ducks, Muscovy ducks and Mule ducks (20 for each), non-embryonated budgerigar eggs (15 each) were collected in Fujian Province, together with a mixture of the lung, liver and spleen of newly hatched animals (15 each). These samples were analyzed for the presence of DuCV DNA using the same method as described above.

Primers used for amplification the complete genomes

DuCV was examined using a GoTaq Green Master Mix (Promega, USA) according to the manufacturer's instructions using the following primer pair 5'-CAAT GGCGAAGAGCGGCAACTACT-3' (forward) and 5'-AGCTGCCCAAGTGTTTAATCCCT-3' (reverse). The reaction mixtures contained 1 μL of template DNA. PCR thermal cycles were denaturation at 95 ℃ for 5 min, 35 cycles each comprising 94 ℃ for 50 s (melting), 55 ℃ for 30 s (annealing), and 72 ℃ extension 90 s (elongation) of amplification and a final extension period of 72 ℃ for 10 min, although small variations in the annealing temperature were used according to the manufacturer's directions. The DuCV-sepecfic PCR product was cloned into pMD18-T and sequenced. Based on the determined sequence, another primer pair 5'- CCCAATAAAC TACTGAGACGAA-3' (forward) and 5'- GAA CATG AACATAATCTTCAAAGG -3' (reverse) was used to amply the remaining genomic sequences by inverse-PCR. Also, the DuCV-sepecfic PCR product was cloned into pMD18-T and sequenced.

Analysis of the DuCV complete genome sequences

The complete genome sequences of DuCV were obtained by assembling sequences of the continuous overlapping PCR products using the Lasergene software package (DNASTAR Version 7.0 Madison, WI, USA). Nucleotide sequence alignments were performed using the ClustalW method with the MegAlign program in the Lasergene software package.

A total of 23 sequences were used for homology analysis and phylogeny construction (Table 2). Sequence

Table 2. List of all DuCVs used in this study

GenBank No.	Host	Genome size(nt)	Area	DuCV Strains	Reference
NC-005053	Mullard Duck	1996	Germany	Ger	Hatterman *et al.* [8]
NC-007220	Pekin Duck	1991	USA	USA	Banda *et al.* [1]
AY394721	Muscovy Duck	1988	Taiwan, China	TC1/2002	Chen *et al.* [2]
DQ166836	Muscovy Duck	1988	Taiwan, China	TC2/2002	Chen *et al.* [2]
DQ166837	Muscovy Duck	1988	Taiwan, China	TC3/2002	Chen *et al.* [2]
EF370476	Muscovy Duck	1988	Fujian, China	FJ0601	Jiang *et al.* [10]
EF451157	Muscovy Duck	1995	Fujian, China	MH25	Fu *et al.* [6]
EU022374	Duck	1988	Shandong, China	SD-LY0701	Not available
EU344806	Cheery Valley Duck	1992	Fujian, China	YS07	Not available
EU499310	Muscovy Duck	1988	Fujian, China	PT07	Shi *et al*. [19]
EU499311	Muscovy Duck	1995	Fujian, China	LJ07	Shi *et al.* [18]
FJ554673	Muscovy Duck	1988	Guangdong, China	WS-GD01	Li *et al.* [12]
GU014543	duck	1995	Jiangsu, China	JS-AQ0901	Not available
GQ334371	Muscovy Duck	1995	Zhejiang, China	ZJ01	this study
GQ423740	Muscovy Duck	1996	Fujian, China	ZQ300	this study
GQ423741	Muscovy Duck	1988	Fujian, China	PT09	this study
GQ423742	Muscovy Duck	1995	Fujian, China	MH207	this study
GQ423743	Muscovy Duck	1988	Fujian, China	CL311	this study
GQ423744	Muscovy Duck	1988	Fujian, China	FQ315	this study
GQ423745	Muscovy Duck	1988	Fujian, China	FQ312	this study
GQ423746	Muscovy Duck	1988	Fujian, China	PT60	this study
GQ423747	Duck	1995	Fujian, China	ZZ302	this study
GU168779	Grimaud Muscovy Duck	1995	Fujian, China	ZQ290	this study

* Three pairs (NC005053 and AY228555, DQ100076 and NC007220, NC006561 and DQ166838) had 100% sequence identity. The Hungary virus only had partial genomes sequence (AJ964962), and hence not included in our final analysis. GenBank accession numbers highlighted in bold indicate those derived from the current study.

alignment and phylogenetic analysis were performed using MEGA 4 and the Neighbor-Joining method [23]. The numbers on the branches represented bootstrap support for 1 000 replicates. The sequence of goose circovirus (GenBank accession number GU320569) was used as the out-group.

Epidemiological investigation

The age ranges of the 138 samples are detailed in Table 1 and Table 3. 11 out of 46 samples tested positive for ages of 0 to 4 weeks, 26 of 65 samples for 4 to 8 weeks, and 12 of 27 samples for those more than 8 weeks. Overall the DuCV infection rate was 35.5%. The results suggested that ducks at the age of more than 4 weeks were more susceptible to the DuCV infection（$P<0.05$）. It is interesting to note that ducks in most PCR-positive farms exhibited no apparent clinical abnormality, indicating that the infection of DuCV may be subclinical. Meanwhile, no DuCV-specific amplicon was obtained from all the vertical transmission samples.

Genomes organizations and overall similarity

The 10 DuCVs sequenced in this study had genome length varying from 1988 to 1996 nt (Table 1). The sequences were deposited in GenBank (see table 2 for accession numbers). The overall genome nt identity of the strains ranged between 83.2% to 99.8 % (data not

Table 3. Age distribution of DuCV positive ducks isolated in this study

Days (weeks)	0<d≤4	4<d≤8	d>8	All
Samples in all	46	65	27	138
Positive	11	26	12	49
Positive rate (%)	23.91	40	44.44	35.51

shown).

Sequence analysis indicated that there were two major open reading frames (ORFs) in the genome of DuCVs: one was *Rep*, coding for the replicating proteins, and the other was *Cap*, coding for the capsid protein. The *Rep* genes of SD-LY0701, FJ0601 [10], FQ312 and PT60 (this study) encode a 297-aa protein and start at 33 nt, and these four strains all have 1988-nt complete genome, while Reps from other strains were 292-aa. The Cap proteins of all strains were 257-aa in length. The 3' intergenic region, which is defined as being located between the termination codons of the major V1 and C1 ORFs, were about 110-111 nt for most DuCVs, but 96 nt for SD-LY0701, FJ0601, FQ312 and PT60.

Phylogenetic analysis of the complete genome of DuCVs

Based on the complete nucleotide sequences of 10 isolates in this study and 3 Taiwan strains, 1 German strain, 1 USA strain and others representative DuCVs (Table 2), a phylogenetic tree was constructed by the MEGA 4 Neighbor-Joining method. The sequence of GoCV was used as the out-group. The results showed that the DuCVs could be divided into two distinct genetic groups, the Euro-USA lineage (detected in Germany mullard duck and the USA Pekin duck) and the Taiwan lineage (detected in Taiwan Muscovy duck) (Fig. 1), with about 10.0% sequence identity difference between the two subgroups (data not shown).

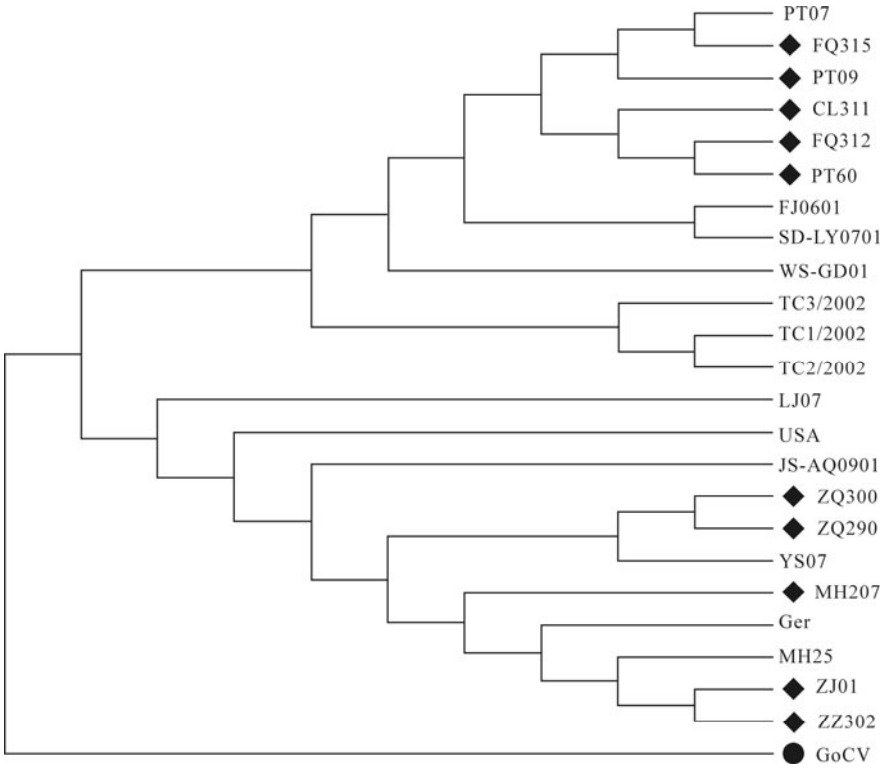

Fig. 1. Phylogenetic analysis of 23 DuCV genome nucleotide sequences. The tree is based on the DuCVs (Table 2) was constructed with the MEGA 4.0 Neighbor-Joining method. The GoCV was used as the out-group. The taxon Separation is 24 pixels, the tree width is 400 pixels, and the SBL is 0.46806491.

DISCUSSION

Because no *in vivo* culture system is available for the propagation of members in the genus *Circovirus* except for PCV1 and PCV2, polymerase chain reaction (PCR) based methods, including both real time PCR and nested PCR [2, 5, 7, 11], represent important tools for DuCV epidemiological investigation, and was the technology platform used for our current study.

The epidemiological investigation results showed the ducks at an age of more than 4 weeks were more susceptible to DuCV. Our results suggest that that infection of DuCV was mostly subclinical, which is different from previously published reports claiming obvious association of DuCV infection with clinical characteristics, e.g. a feathering disorder, poor body condition and low weight, which may induce damage to lymphoid tissue and immunosuppression [20, 28, 29]. There were also reports indicating that ducks positive for DuCV by PCR were more susceptible to co-infection with RA (*Riemerella anatipestifer*), *E. coli* (*Escherichia coli*), Duck Cholera (*Pasteurella multocida*), DHV-I (Duck hepatitis virus-I), AIV (Avian influenza virus), DPV (Duck parvovirus), Avian paramyxovirus type-1(APMV-1) and/or DRV (Duck reovirus) [30]. Due to the lack of a commercial vaccine at present, there is no effective measure to control the infection of DuCV and the associated co-infection of other pathogens. Previous studies found that BFDV DNA was detected in both embryonated eggs and non-embryonated budgerigar eggs, suggesting that the BFDV can be transmitted horizontally and vertically [15]. However, no DuCV-specific amplicon was obtained from the vertical transmission investigations.

Data obtained from this study and previous studies indicate that positive detection was observed in different species, including the Muscovy duck, the Mule duck, the Cheery Duck, the Mullard Duck and also the Pekin Duck in different regions and countries, suggesting that DuCV infection were ubiquitous, showing no species dependency.

In general, genetic variation of virus genome may lead to the change of their pathogenicity [28]. This study revealed an obvious difference in the Rep protein, with those of SD-LY0701, FJ0601, FQ312 and PT60 coding 297-aa whiles other strains coding for a 292-aa Rep protein due to a different translational initiation site. Further investigation is warranted to examine whether this difference will result in any biological changes, such as changes in replication or virulence.

However, there are several differences among the DuCV genomes. There are some similar regions among the genus *Circovirus*. A stem-loop structure (Table 4) with a highly conserved sequence of nine nucleotides nonamer "TA**T** TATT^{①}AC" (^{①}position 1) was found in the untranslated region between the two DuCV ORFs (5' intergenic regions) when all DuCV sequences in GenBank were compared (regardless of geographical origin and species). While the PCV1, PCV2,

Table 4. Homology of the nonamer sequence amongst circoviruses

Circovirus	BFDV	CaCV	**DuCV**	FiCV	GoCV	GuCV	PCV1	PCV2	PiCV	RaCV	StCV	SwCV
Nonamer	**TAG**	**CAG**	**TAT**	**TAG**	**TAT**	**TAG**	**TAG**	**AAG**	**TAG**	**GAG**	**CAG**	**TAT**
	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT	TAT
	TAC	TAC	TAC	TAC	TAC	TAC	TAC	TAC	TAC	TAC	TAC	TAC

GuCV, PiCV and FiCV had nine nucleotides "NA/N TATT①AC" (①position 1).When designing a diagnostic PCR test for the genus *Circovirus*, it is important to examine all available sequences of known isolates to avoid false negative results [9, 24], these genomic characteristics may be used to develop a broad-spectrum PCR method for identifying circovirus in the genus *Circovirus* from other avian species.

Analysis of the complete genomes sequences showed that there were two distinct groups located in two genotype branches of the phylogenetic tree: the Euro-USA lineage containing DuCV detected in Germany, USA, and the mainland China, which the genomes length were longer than 1988nt ; while the other is the Taiwan lineage, consisting with the strains found in the Taiwan area and the mainland China, which the genomes length were all 1988nt, including the coding 297-aa Rep protein strains SD-LY0701, FJ0601, FQ312 and PT60. The two lineages displayed an overall sequence difference of approximately 10.0% across the genome. The phylogenetic analysis demonstrated that there maybe two distinguish origin and evolution of DuCV, while these two lineage were co-circulating in mainland China. Earlier clinic samples and more species need to be examined to determine the exact origin.

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