VIROLOGICA SINICA, June 2012, 27 (3):154-164
DOI 10.1007/s12250-012-3214-4

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Complete Genome Sequence Analysis of Duck Circovirus Strains from Cherry Valley Duck*

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Abstract: To investigate molecular epidemiology of DuCV in Cherry Valley ducks in China, the complete genomes of six DuCV strains, which were detected from Cherry Valley ducks in China between 2007 and 2008, were sequenced. Sequence and phylogenetic analysis were carried out to compare these six strains with another 27 DuCV strains from Mulard duck, Muscovy duck, Pekin ducks and Mule duck. The analysis showed that the six DuCV strains exhibited typical genetic features of the family of DuCV, such as a stem-loop structure, three major open reading frames (Rep, Cap and ORF3), four intergenic repeats and the conserved motifs for rolling circle replication and for the dNTP binding domain located in the Rep protein. Phylogenetic analysis of the nucleotide sequences of the complete genome and Cap gene of these strains together with those that have been previously published demonstrated two distinct DuCV genotypes. The DuCV strains with complete genomes containing 1988 and 1989 nucleotides clustered in genotype A, whereas the strains with complete genomes containing 1991, 1992, 1995 and 1996 nucleotides lay in genotype B. The six DuCV strains from Cherry Valley ducks were divided into the two groups. The results of the study provides some insight into the variation of DuCVs in Cherry Valley ducks.

Key words: Cherry Valley duck; Duck circovirus; Complete genome; Phylogenetic analysis

Members of the Circoviridae family are small, non-enveloped, spherical viruses which contain circular, single-stranded DNA genomes. This family comprises two genera, Gyrovirus and Circovirus[27,28]. Chicken anemia virus (CAV) is the only member of the Gyrovirus[1], while the Circovirus genus has many members including Porcine circovirus type Ⅰ (PCV-1) and Porcine circovirus type Ⅱ (PCV-2)[8,18], psittacine beak and feather disease virus (BFDV)[4], Gull circovirus (GuCV)[30], pigeon circovirus (PiCV) or columbid circovirus (CoCV)[17, 21, 33], canary circovirus (CaCV)[22,29], goose circovirus (GoCV)[24], Duck circovirus (DuCV)[9], Finch circovirus (FiCV) [23], and so on.

DuCV was detected from a mulard duck for the first time in German and the complete genome was

Received: 2012-07-25, Acceted: 2012-05-02
* Foundation items: the grant of Shandong Province Higher Educational Science and Technology Program (J08LF07) and Shandong Provincial Natural Sciences Fund (Q2006D04).
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sequenced[9,25]. Later, DuCV were successively identified from some sick Muscovy ducks in Hungary[6], Taiwan of China[5] and China[11], Pekin ducks in America[3], and Cherry Valley ducks in China[35]. The birds infected by DuCV were in a poor body condition and showed marked feather dystrophy with haemorrhagic shafts to many feathers, especially in the dorsal part. Histopathological examination of the dorsal skin revealed a mainly heterophilic inflammatory infiltration of the follicular and perifollicular tissue. The gross pathological appearance of the internal organs was attributable to a concurrent infection by Riemerella anatipestifer. Histopathological examination of bursal tissue demonstrated lymphocytic depletion, necrosis and histiocytosis[25]. Since there is no in vitro culture method for DuCV detection, the study of DuCV mostly depends on molecular approaches, such as PCR, real time PCR or nucleotide sequencing[5-7, 9, 11, 25, 31, 35].

In this study, we sequenced the complete genome of six DuCV strains which were found in Cherry Valley ducks in China between 2007 and 2008. Sequence and phylogenetic analysis were carried out to compare the six strains with other DuCVs, including strains from Mulard duck, Muscovy duck, Pekin ducks and Mule duck. The results from this study will aid a better understanding of DuCV in Cherry Valley ducks.

MATERIALS AND METHODS

Virus samples

The bursa of Fabricius (BF) of Cherry Valley ducks were collected from six commercial farms in Shandong Province of China between 2007 and 2008. These ducks, aged between 4 and 8 weeks, showed feathering disorders, poor body condition and low weight under field conditions.

DNA extraction

BF (0.1g) was homogenized with 500 μL sterile 0.9% NaCl. DNA was extracted using DNAzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, homogenate (500 μL) was combined with 2× volume DNAzol reagent and incubated at room temperature for 10 min followed by centrifugation at 10, 000 rmp for 10 min.

Primers

Based on the published sequence of DuCV, four pairs of primers were designed to amplify four overlapping fragments that covered the complete genome of DuCV[11]. Sequences of the four pairs primers were:

P1f5'-TTACCGGCGCTTGTACTC-3'/P1r5'-TACT TGTTTTCGGCGGGA-3',

P2f5'-ACGCAACGTGATTGGAAG-3'/P2r5'-CAT TACGCCATGGGCATG-3',

P3f5'-CCAATAAACTACTGAGAC-3'/P3r5'-ATC GGCGTGCATATCGTG-3' and

P4f5'-CGTAGCCTTCGTCTTCTG-3'/P4r5'-GGT ATGTCGACTCTTTGG-3'.

Polymerase chain reaction (PCR)

A Taq PCR Master Mix Kit (QIAgen, Germany) was used to amplify four segments of DuCV following the manufacturer's instructions. Briefly, 2 μL of extracted DNA was added to the reaction mixture with final concentrations of 2.5 mmol/L $MgCl_2$, 1×PCR buffer, 0.3 mmol/L dNTPs, 0.5 μL of each primer (100 ng/μL) and 0.2 μL of Taq DNA polymerase (5 U/μL) per 25 μL. DNA was amplified for 33 cycles with the following parameters: a first denaturation at 94 ˚C for 5 min, followed by denaturation at 95 ˚C for 50 s, annealing at 55 ˚C for

40 s and elongation at 72 ˚C for 1 min; and a final extension at 72 ˚C for 10 min. PCR products were visualized in a 1.0% agarose gel and stained with ethidium bromide.

Cloning and sequencing of the genomic sequences

PCR products were purified using a Gel Purification Kit (Invitrogen, USA), and the purified PCR-products were TA-cloned into pMD18-T vector (TaKaRa, China) and transformed into a DH5a competent cell according to the manufacturer's protocol. To avoid base mismatching resulting from PCR, three clones were selected to sequence for each fragment. The nucleotide (nt) sequences of the positive clones were determined using T7 and SP6 sequence primers from a commercial service (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd).

Sequence analysis of the genome sequence

The complete genome sequences of the six DuCV strains examined in this study have been deposited into GenBank under accession numbers EU022374, EU022375 and GU131340 to GU131343. The accession numbers of all sequences used in this study are given in Table 1. Nucleotide and amino acid sequence

Table 1. General information and accession number of DuCV isolates used in this study

Isolate	Accession number	Host	Origin	Genome (nt)	Reference
AY228555	AY228555	Mulard duck	Germany	1996	[14]
TC1/2002	AY394721	Muscovy duck	Taiwan, China	1988	[18]
TC2/2002	DQ166836	Muscovy duck	Taiwan, China	1988	[18]
TC3/2002	DQ166837	Muscovy duck	Taiwan, China	1988	[18]
TC4/2002	DQ166838	Muscovy duck	Taiwan, China	1988	[18]
FJ0601	EF370476	Muscovy duck	China	1988	[19]
33753-52	NC007220	Pekin duck	U.S.A.	1991	[20]
FJ0701	GQ868757	Muscovy duck	China	1988	Liu and Jiang unpublished
MH25	EF451157	Muscovy duck	China	1995	[22]
HZ09	EU344802	Muscovy duck	China	1989	Fu and Huang unpublished
LJ33	EU344803	mule duck	China	1995	Fu and Huang unpublished
MH06	EU344804	mule duck	China	1995	Fu and Huang unpublished
MH11	EU344805	Muscovy duck	China	1988	Fu and Huang unpublished
YS07	EU344806	Cherry valley duck	China	1992	Fu and Huang unpublished
ZC03	EU344807	Muscovy duck	China	1992	Fu and Huang unpublished
MH02/07	EU499309	mule duck	China	1988	Shi and Huang unpublished
PT07	EU499310	Muscovy duck	China	1988	[23]
LJ07	EU499311	Muscovy duck	China	1995	Shi and Huang unpublished
WS-GD01	FJ554673	Muscovy duck	China	1988	Li et al. unpublished
Zhejiang	GQ334371	Muscovy duck	China	1995	Wan and Huang unpublished
FujianZQ300	GQ423740	Muscovy duck	China	1996	Wan and Huang unpublished
FJPT09	GQ423741	Muscovy duck	China	1988	Wan and Huang unpublished
FJMH207	GQ423742	Muscovy duck	China	1995	Wan and Huang unpublished
FJCL311	GQ423743	Muscovy duck	China	1988	Wan and Huang unpublished
FJFQ315	GQ423744	Muscovy duck	China	1988	Wan and Huang unpublished
FJFQ312	GQ423745	Muscovy duck	China	1988	Wan and Huang unpublished
FJPT60	GQ423746	Muscovy duck	China	1988	Wan and Huang unpublished
LY0701	EU022374	Cherry valley duck	China	1988	This paper
WF0701	EU022375	Cherry valley duck	China	1991	This paper
WF0801	GU131340	Cherry valley duck	China	1995	This paper
WF0802	GU131341	Cherry valley duck	China	1988	This paper
WF0803	GU131342	Cherry valley duck	China	1991	This paper
WF0804	GU131343	Cherry valley duck	China	1988	This paper

alignments were performed by the Clustal W method with Megalin 5.08 (DNAStar Inc., USA). Phylogenetic trees were constructed from the aligned 33 strains' nucleotide sequences and polyprotein amino acid sequences of DuCV and by the Clustal W method with 1000 bootstrap replicates.

RESULTS

Genomic organization analysis of the six DuCVs

The complete genome sequences of the six DuCV strains from Cherry Valley ducks in this study contained 1988, 1991 and 1995 nucleotides (Table 1) respectively. Like other members of the DuCV family, the genomes of the six strains contained two major ORFs encoding replicase protein (Rep) and capsid protein (Cap). Two minor ORFs were identified in the complementary strand. The ORFC2 was observed in three DuCV strains (WF0701, WF0801 and WF0803), whereas the ORF3 was observed in all the six DuCV strains. The other two minor ORFs were identified only in the viral strand of WF0701 (Table 2). The initiation codons of the two major ORFs varied in the six DuCV strains. In the intergenic region between Rep and Cap genes, there existed a loop-stem structure with the nonanucleotide motif TATTATTAC. In the up and down streams of the nucleotide sequence, there were two inverted repeat sequences (10 bp in length). After the stem-loop structure, two tandem arranged direct repeats of the hexamer GTACTC were found from nt 11 to 17 and 18 to 23. As in all the other DuCV strains, the genomes of the six strains contained four copies of repeat sequences (CACTTGGGCAG or CACTTGGCAG).

Genotyping by complete genome sequences

As shown in Fig. 1A, all 33 available complete genome sequences were clustered into two clear genetic groups, strongly supported by 100% bootstrap values. The nucleotide identities in DuCV complete genomes within the same group were at least 91.9%, whereas those between members of the two groups did not exceed 86.8% (Table 3). We labelled the genetic groups DuCV genotypes A and B. Genotype A, represented by nineteen DuCV strains with genomes of 1988 and 1989 nucleotides, included three DuCV strains (LY0701, WF0802 and WF0804) examined in this study and another sixteen viruses from Taiwan

Table 2. Genomic organization analysis of the six DuCV isolates in this study

Isolate	genotype	ORF Rep/V1	ORFV2	ORFV3	ORF Cap/C1	ORFC2	ORF3/C3	The first intergenic region (nt)	The second intergenic region (nt)	Nonamer sequence	Hexamer sequence
LY0701	A	297[a] (nt 33-926)[b]	—	—	257 (nt 1924-1151)	—	98 (nt 399-103)	96	224	TATTATTAC	ACTCCG ACTCCA
WF0802	A	297 (nt 33-926)	—	—	257 (nt 1924-1151)	—	98 (nt 399-103)	96	224	TATTATTAC	ACTCCG ACTCCA
WF0804	A	292 (nt 48-926)	—	—	257 (nt 1924-1151)	—	98 (nt 399-103)	111	224	TATTATTAC	ACTCCG ACTCCA
WF0701	B	292 (nt 48-926)	—	—	257 (nt 1928-1155)	120 (nt 1737-1375)	78 (nt 399-163)	110	228	TATTATTAC	ACTCCG ACTCCG
WF0803	B	292 (nt 48-926)	—	—	257 (nt 1928-1155)	120 (nt 1737-1375)	78 (nt 399-163)	110	228	TATTATTAC	ACTCCG ACTCCG
WF0801	B	292 (nt 49-927)	70 (nt 1373-1585)	85 (nt 1661-1918)	257 (nt 1932-1159)	120 (nt 1741-1379)	78 (nt 400-164)	111	231	TATTATTAC	ACTCCG ACTCCG

[a]Number of amino acids encoded by ORF; [b]nt position of ORF. The first intergenic region located between the 5' ends of *rep* and *cap*. The second intergenic region located between the 3' ends of *rep* and *cap*.

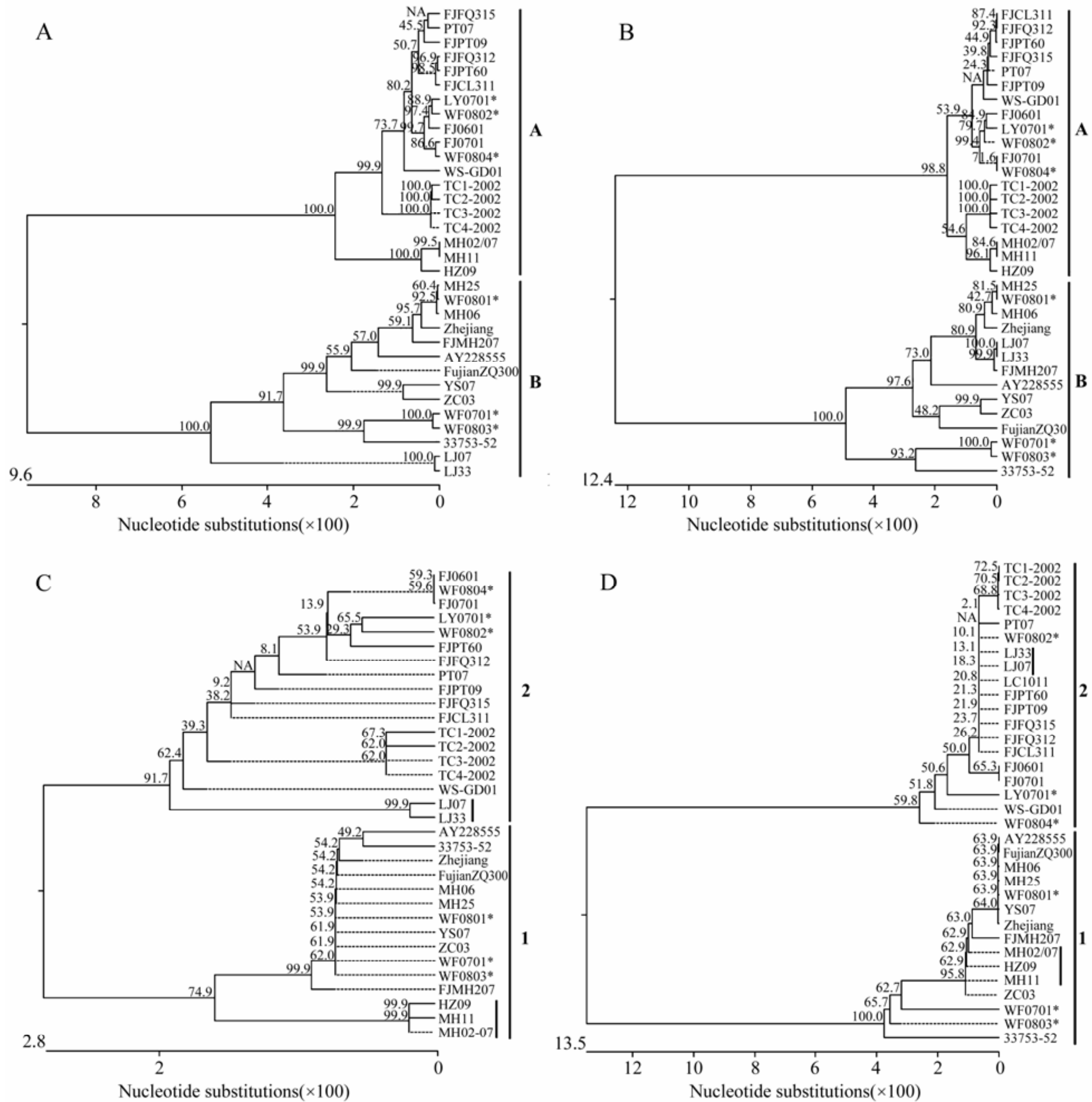

Fig. 1. The phylogenetic trees of DuCV isolates based on the nucleotide sequences of complete genome (A), the complete nucleotide sequences of Cap gene (B), the complete amino acid sequences of Rep gene (C) and the complete amino acid sequences of ORF3 gene(D). Numbers at nodes indicate bootstrap percentages obtained after 1 000 replicates. Genotypes are indicated on the right side of the trees. The bar indicates genetic distance and *, the strains sequenced in this study.

Table 3. Percentage nucleotide (nt) and amino acid (aa) identity in the complete genome and Cap gene within and between DuCV genotypes, Rep gene and ORF3 gene

Comparison	Complete genome	Cap	
	nt	aa	nt
Within genotype A	94.6-100	96.1-100	96-100
Within genotype B	91.8-99.9	95.7-100	88.8-100
Between genotypes A and B	82.4-86.8	86.4-89.5	77.3-80.2

and China[5,11,31]. Genotype B contained fourteen DuCV strains whose genome were 1991, 1992, 1995 and 1996 nucleotides and the other three DuCV strains (WF0701, WF0801 and WF0803) determined in this study and another sixteen viruses from Germany, America and China[3,7,9] (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

Genotyping by Cap sequences

The Caps of all the 33 DuCVs consisted of 794 nucleotides and encoded 257 amino acid residues. The phylogenetic trees constructed by the Cap (Fig. 1B) nucleotide sequences of 33 DuCVs further confirmed the existence of the same two genotypes defined by the complete genome analysis, which contained the same DuCVs as the complete sequences. The nucleotide and amino acid identities in Cap region within the same genotype were from 88.8% to 99.7%, whereas those between members of the two genotypes were from 80.2% to 89.5%, respectively (Table 3). Comparisons of deduced amino acid sequences of the 33 available Cap revealed there were 22 genotype-associated alterations in capsid protein between the two genotypes of DuCVs (Fig. 2).

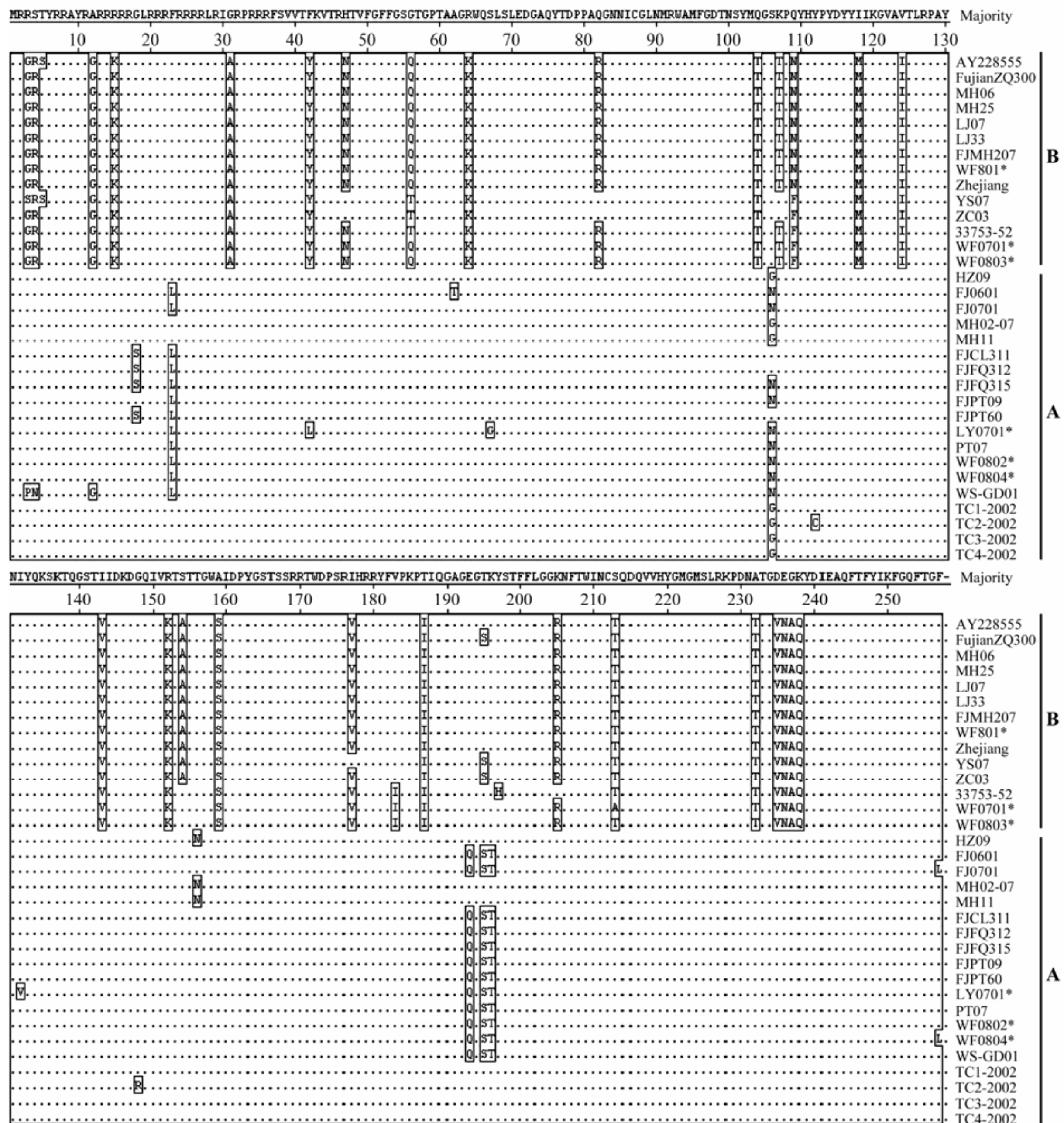

Fig. 2. Deduced amino acid sequence comparisons of the 33 DuCV strains in capsid protein. Amino acids identical to the majority are represented by dots, nonidentical amino acids are indicated by single letters, and the strains sequenced in this study are marked by stars.

Sequence analysis of the Rep sequence

In the six DuCV strains, the Reps of four DuCVs consisted of 879 nucleotides and encoded 292 amino acid residues, whereas the Reps of the other two DuCVs (LY0701 and WF0802) consisted of 894 nucleotides and encoded 297 amino acid residues (Table 2). The phylogenetic trees constructed by the Rep (Fig. 1C) amino acid sequences of 33 DuCVs showed the existence of two genotypes, but these differed from the two genotypes defined by the complete genome and Cap gene analysis. The nucleotide and amino acid identities in Rep region within the same genotype were from 91.9% to 100% and from 96.6% to 100%, whereas those between members of any of the two genotypes were from 87.1% to 93.7% and from 92.6% to 96.6%, respectively (Table 4). The conserved amino acid motifs associated with rolling circle replication (I, II, and III) were indicated by shadowed boxes, and the dNTP binding domain (A) was similarily indicated (Fig. 3).

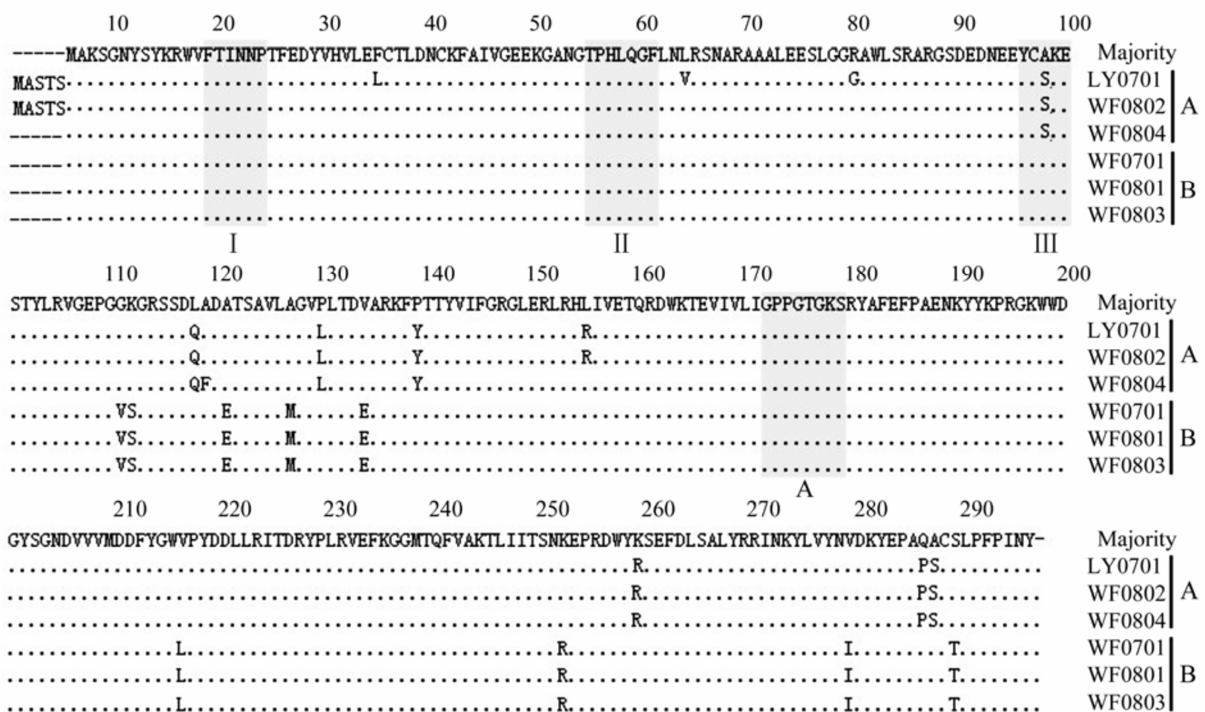

Fig. 3. Deduced amino acid sequences of the Rep proteins from the six DuCVs. Conserved amino acid motifs associated with rolling circle replication (I, II, and III) are indicated by shadowed boxes, and the the dNTP binding domain (A) is similarily indicated. A dot indicates a position where the amino acid is identical to that of the majority. Alignments were prepared using Megalin Clustal W Method (Dnastar Inc.).

Table 4. Percentage nucleotide (nt) and amino acid (aa) identity in the complete Rep gene and ORF3 gene within and between DuCV genotypes

Comparison	Rep		ORF3	
	aa	nt	aa	nt
Within genotype 1	96.6-100	91.9-100	91.1-100	97-100
Within genotype 2	96.6-100	93.4-100	95.5-100	98.1-100
Between genotypes 1 and 2	92.6-96.6	87.1-93.7	73.4-81	88.6-91.1

Sequence analysis of the ORF3 sequence

The ORF3 was identified in all the 33 DuCV strains. Based on the complete amino acid sequences, the 33 ORF3 genes were clustered into the same two genotypes defined by the analysis of the complete amino acid sequence of Rep gene (Fig. 1D). The nucleotide and amino acid identities in ORF3 gene

within the same genotype were higher than 97% and 91.1%, whereas those between members of any of the two genotypes were only between 88.6% to 91.1% and 73.4% to 81%, respectively (Table 4). In this study, the ORF3 genes of three DuCVs (WF0701, WF0801 and WF0803) belonging to genotype 1 consisted of 237 nucleotides and encoded 78 amino acid residues, whereas the ORF3s of the other three DuCVs (LY0701, WF0802 and WF0804) belonging to genotype 2 consisted of 297 nucleotides and encoded 98 amino acid residues (Fig. 4).

DISCUSSION

Currently, there is a high prevalence of DuCV in Cherry Valley ducks. It has been reported that DuCV positive rate is 33.28% (247/742) in sick Cherry Valley ducks detected by PCR[35], and the DuCV antibody positive rate was 15.24% (246/1614) in Cherry Valley ducks without clinical syndromes tested by iELISA method[16]. In this report, the complete genome sequences of six DuCV strains from Cherry Valley ducks in China were obtained. Phylogenetic trees constructed from the nucleotide sequences of complete genome and Cap gene were in excellent agreement with one another and demonstrated the existence of two DuCV genotypes in all 33 complete genome sequences. In nineteen DuCV strains clustered in genotype A the genome contained 1988 and 1989 nucleotides, whereas for the other fourteen strains in genotype B the genome contained 1991, 1992, 1995 and 1996 nucleotides. The nucleotide identities for the complete genome within the same genotype were at

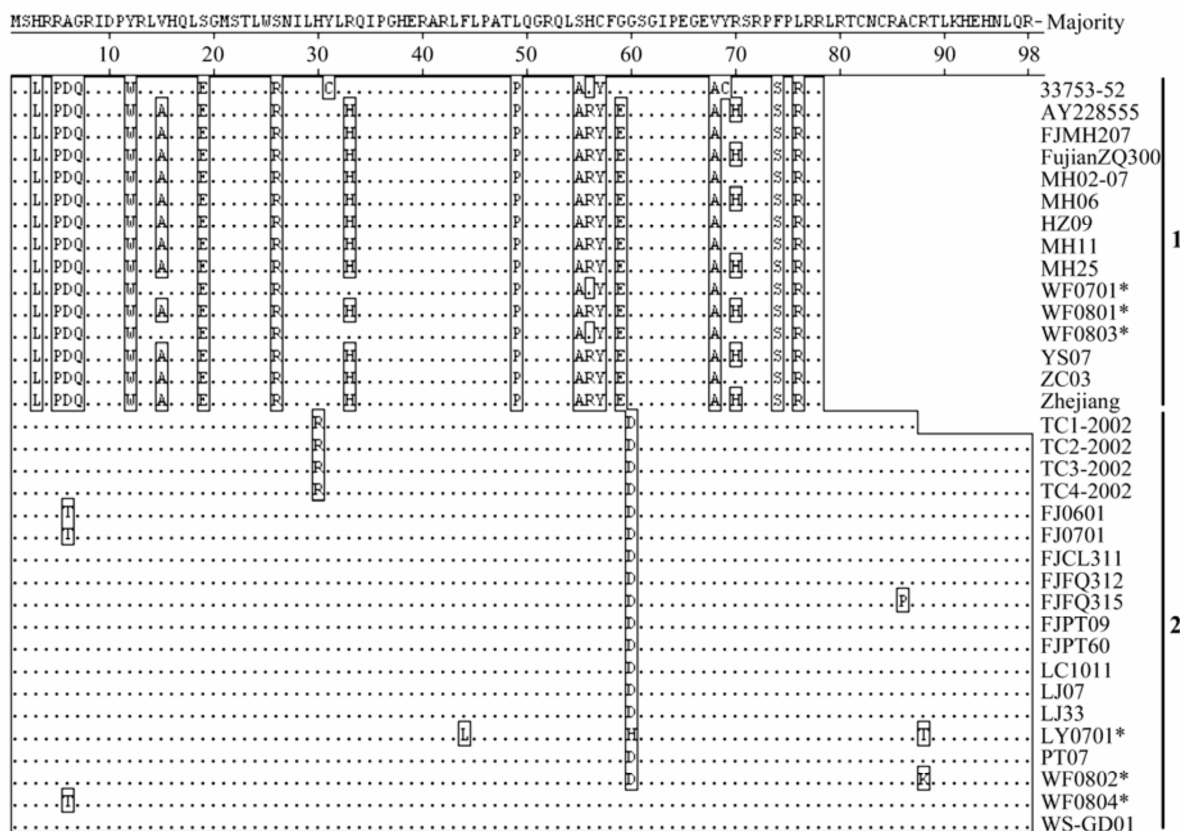

Fig. 4. Deduced amino acid sequence comparisons of the 33 DuCV strains in ORF3 protein. Amino acids identical to the majority are represented by dots, nonidentical amino acids are indicated by single letters, and the strains sequenced in this study are marked by stars.

least 91.9%, whereas those between members of the two groups did not exceed 86.8%. The nucleotide and amino acid identities in Cap region within the same genotype were at least 88.8% and 99.7%, whereas those between members of the two groups did not exceed 80.2% and 89.5%, respectively. The six DuCV strains obtained from Cherry Valley ducks included genomes of lengths 1988, 1991 and 1995 nucleotides, from both groups, showing that there were varieties of DuCVs in Cherry Valley ducks.

DuCV strains were obtained from Cherry Valley ducks, mulard ducks, Peking ducks, mule ducks and Moscovy ducks showing that it exists in all species of ducks. The same species and regions of ducks were infected by different genotype DuCVs and, the same genotype DuCVs appeared in different species and regions of ducks, so it may be concluded that there is no association between DuCV genotype and species or region.

It is well-known that virus structural proteins can self-assemble into virus-like particles (VLPs) which have native virus morphology and immunological properties[19, 20]. In the early studies, the Cap of PCV2 and BFDV expressed in insect cells from recombinant baculovirus, were able to self-assemble into VLPs[19, 26]. The Cap of BFDV also was found to bind to the viral DNA and be responsible for transporting the Rep into the nucleus[10]. There were 22 genotype-associated alterations in Cap between the two genotypes of DuCVs. Whether the amino acid alterations in Cap between the two genotypes cause the differences between serotypes and the other biological functions still necessitates further study.

Compared to the variable Cap protein, the Rep proteins are more conserved between all circoviruses[9]. In this study, between the two genotypes of DuCV, the amino acid identities of Rep ($\geqslant$ 92.6%) were clearly higher than that of Cap ($\geqslant$86.4%) and ORF3 ($\geqslant$73.4%) (Table 3 and 4). As founded in FJ0601 strain[11], the Rep gene of two DuCVs (LY0701 and WF0802) encoded 297 amino acid residues, and started at a different site to the other strains, with their Rep gene encoding 292 amino acids. Besides Rep and Cap genes, ORF3 was the third gene identified in all the DuCVs. The roles of ORF3 protein in the induction of apoptosis in PCV2-infected cells and determining the pathogenicity of the PCV2 have been previously demonstrated[2, 12-15]. In recent research, the ORF3 protein was proved to induce obvious apoptosis in Sf9 cells, so it was deduced that the ORF3 protein of DuCV might play an important role in viral pathogenesis via its apoptotic activity[34]. Based on the complete nucleotide and amino acid sequences, all the ORF3 genes of different DuCV strains were clustered into the two same genotypes, and the lengths of these genes in the two genotypes were different[32]. In this study, genotype-associated alterations in ORF3 gene between the two genotypes of DuCVs were shown in Fig. 4. Further work should be done to probe whether this difference can affect the DuCV biological characteristics, e.g. replication and pathogenicity.

Acknowledgements

This study was supported by the grant of Shandong Province Higher Educational Science and Technology Program (J08LF07) and Shandong Provincial Natural Sciences Fund (Q2006D04), China.

References

1. **Allan G M, Ellis J A.** 2000. Procine circoviruses: a review. **J Vet Diagn Invest**, 12: 3-14.

2. **An D J, Song D S, Park B K.** 2008. Systemic cytokine profiles of mice vaccinated with naked DNAs encoding six open reading frame antigens of porcine circovirus type 2 (PCV2). **Res Vet Sci**, 85: 503-509.
3. **Banda A, Galloway-Haskins R I, Sandhu T S, *et al.*** 2007. Genetic analysis of a duck circovirus detected in commercial Pekin ducks in New York. **Avian Dis**, 51: 90-95.
4. **Bassami M R, Berryman D, Wilcox G E, *et al.*** 1998. Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. **Virology**, 249: 453-459.
5. **Chen C L, Wang P X, Lee M S, *et al.*** 2006. Development of a polymerase chain reaction procedure for detection and differentiation of duck and goose circovirus. **Avian Dis**, 50: 92-95.
6. **Fringuelli E, Scott A N, Beckett A, *et al.*** 2005. Diagnosis of duck circovirus infections by conventional and real time polymerase chain reaction tests. **Avian Pathol**, 34: 495-500.
7. **Fu G H, Cheng L F, Shi S H, *et al.*** 2008. Genome Cloning and Sequence Analysis of Duck Circovirus. **Chin J Virol**, 24: 138-143. (in Chinese)
8. **Hamel A L, Lin L L, Nayar G P.** 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. **J Virol**, 72: 5262-5267.
9. **Hattermann K, Schmmitt C, Soike D, *et al.*** 2003. Cloning and sequencing of duck circovirus (DuCV). **Arch Virol**, 148: 2471-2480.
10. **Heath L, Williamson A L, Rybicki E P.** 2006. The capsid protein of beak and feather disease virus binds to the viral DNA and is responsible for transporting the replication-associated protein into the nucleus. **J Virol**, 80: 7219-7225.
11. **Jiang S J, Zhang X X, Liu S N, *et al.*** 2008. Detection of Duck Circovirus by Polymerase Chain Reaction in Sick Ducks with Infectious Serositis. **Virol Sin**, 23: 265-271.
12. **Karuppannan A K, Jong M H, Lee S H, *et al.*** 2009. Attenuation of porcine circovirus 2 in SPF piglets by abrogation of ORF3 function. **Virology**, 383: 338-347.
13. **Karuppannan A K, Liu S, Jia Q, *et al.*** 2010. Porcine circovirus type 2 ORF3 protein competes with p53 in binding to Pirh2 and mediates the deregulation of p53 homeostasis. **Virology**, 398: 1-11.
14. **Liu J, Chen I, Du Q, *et al.*** 2006. The ORF3 protein of porcine circovirus type 2 is involved in viral pathogenesis *in vivo*. **J Virol**, 80: 5065-5073.
15. **Liu J, Chen I, Kwang J.** 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. **J Virol**, 79: 8262-8274.
16. **Liu S N, Zhang X X, Xie Z J, *et al.*** 2010. Development of an indirect ELISA for the detection of antibodies to duck circovirus in duck flocks. **Vet Microbiol**, 145: 41-46.
17. **Mankertz A, Hattermann K, Ehlers B, *et al.*** 2000. Cloning and sequencing of columbid circovirus (CoCV), a new circovirus from pigeons. **Arch Virol**, 145: 2469-2479.
18. **Mankertz A, Persson F, Mankertz J, *et al.*** 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. **J Virol**, 71: 2562-2566.
19. **Nawagitgul P, Morozov I, Bolin S R, *et al.*** 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. **J Gen Virol**, 81: 2281-2287.
20. **Noad R, Roy P.** 2003. Virus-like particles as immunogens.**Trends Microbiol**, 11: 438-444.
21. **Paré J A, Brash M L, Hunter D B, *et al.*** 1999. Observations on pigeon circovirus infection in Ontario. **Can Vet J**, 40: 659-662.
22. **Phenix K V, Weston J H, Ypelaar I, *et al.*** 2001. Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus Circovirus of the family Circoviridae. **J Gen Virol**, 82: 2805-2809.
23. **Shivaprasad H L, Hill L D, Todd D, *et al.*** 2004. Circovirus infection in a Gouldian finch (Chloebia gouldiae). **Avian Pathol**, 33: 525-529.
24. **Soike D, Koehler B, Albrecht K.** 1999. A circovirus-like infection in geese related to a runting syndrome. **Avian Pathol**, 28: 199-202.
25. **Soike D, Albrecht K, Hattermann K, *et al.*** 2004. Novel circovirus in mulard ducks with developmental and feathering disorders. **Vet Rec**, 154: 792-793.
26. **Stewart M E, Bonne N, Shearer P, *et al.*** 2007. Baculovirus expression of beak and feather disease virus (BFDV) capsid protein capable of self-assembly and haemagglutination. **J Virol Methods**, 141: 181-187.
27. **Todd D.** 2000. Circoviruses: immunosuppressive threats to

avian species: a review. **Avian Pathol**, 29: 373-394.

28. **Todd D.** 2004. Avian circovirus diseases: lessons for the study of PMWS. **Vet Microbiol**, 98: 169-174.
29. **Todd D, Weston J, Ball N W, *et al.*** 2001. Nucleotide sequence-based identification of a novel circovirus of canaries. **Avian Pathol**, 30: 321-325.
30. **Twentyman C M, Alley M R, Meers J, *et al.*** 1999. Circovirus-like infection in a southern black-backed gull (Larus dominicanus). **Avian Pathol**, 28: 513-516.
31. **Wan C H, Chen Z, Peng C X, *et al.*** 2010. Sequencing and analysis of genome of duck circovirus PT07. **Chin J Prev Vet Med**, 32: 235-237. (in Chinese)
32. **Wei Z, Zou J F, Lei Z, *et al.*** 2011. Cloning and sequence analysis of ORF3 gene of DuCV. **Chin J Prev Vet Med**, 33: 746-748. (in Chinese)
33. **Woods L W, Latimer K S, Niagro F D, *et al.*** 1994. A retrospective study of circovirus infection in pigeons: nine cases (1986–1993). **J Vet Diagn Invest**, 6: 156-164.
34. **Xiang Q W, Wang X, Xie Z J, *et al.*** 2012. ORF3 of duck circovirus: a novel protein with apoptotic activity. **Vet Microbiol**, DOI: 10.1016/j.vetmic.2012.03.045.
35. **Zhang X, Jiang S, Wu J, *et al.*** 2009. An investigation of duck circovirus and co-infection in Cherry Valley ducks in Shandong province, China. **Vet Microbiol**, 133: 252-256.