

Research note: genetic diversity of duck circoviruses circulating in partial areas of Guangdong province, southern China

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ABSTRACT Duck circovirus (DuCV) is the smallest known virus in waterfowl that infects both domestic and wild duck. Infected ducks often show stunted growth and immunosuppression, which increases the rate of secondary infection with other pathogens. In this study, 270 liver tissue samples were collected to screen the presence of DuCV in Guangdong province, China, and the complete genome sequences were recovered and systematically analyzed. Genetic analyses revealed that sequences determined in this study shared 81.6% to 100.0%

genome-wide pairwise identity with previously identified DuCV genomes. Phylogenetic analyses showed that 2 DuCV genotypes with a high infection rate were co-circulating in duck population in Guangdong province, and extensive recombination events have occurred during the evolution of DuCV. Our results expand upon the knowledge regarding the genetic diversity and evolution of DuCV, and also indicate that extensive genetically divergent DuCV are co-circulating in the duck populations in Guangdong, southern China.

Key words: duck circovirus, genetic diversity, recombination events, southern China

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INTRODUCTION

Duck circovirus (DuCV) belongs to the genus *Circovirus* in the family of *Circoviridae*. There are several members in this genus, such as porcine circovirus types 1 and 2 (PCV 1 and PCV 2) (Faurez et al., 2009), and goose circovirus (GoCV) (Soike et al., 1999). DuCV is a nonenveloped, single-stranded circular DNA virus with an icosahedral structure (Soike et al., 1999). The genome of DuCV is about 2 kb in length and contains 3 main open-reading frames (ORFs), ORF1, ORF2, and ORF3 (Hattermann et al., 2003). ORF1 encodes the replication-associated protein (Rep) on the virion sense strand, ORF2 encodes the capsid protein (Cap) on the complementary sense strand, and ORF3 is embedded backwards within the sequence of ORF1 which encodes ORF3 protein with apoptotic function (Hattermann et al., 2003). The intergenic regions between the middle of ORF1 and ORF2 contain a stem-loop, which is the initiation site of viral replication (Hattermann et al., 2003). In addition, DuCV has been divided into 2

genotypes (DuCV-1 and DuCV-2) based on the phylogenetic analysis of the complete genome and ORF2, and DuCV-1 and DuCV-2 have been further divided into 4 and 3 subtypes, respectively (Ji, et al., 2020).

DuCV was first reported in mallard ducks with a poor physical condition in Germany in 2003 (Hattermann et al., 2003). Thereafter, cases of ducks infected with this virus have been reported in Hungary (Fringuelli et al., 2005), Taiwan (Ting et al., 2021), and mainland China (Ji et al., 2020). Ducks infected with DuCV often present with feather growth disturbance, growth retardation, poor body condition and low body weight, and histopathological examination of the bursa of Fabricius often showed lymphocytopenia, necrosis and histiocytosis, which causes immunosuppression (Soike et al., 2004). Additionally, the disease progression is further complicated by co-infection with infected other bacterial, viral and fungal pathogens.

DuCV has been confirmed in several countries, including mainland China, where it has a wide geographic distribution (Ji et al., 2020). Recently, the impacts of DuCV infections on duck farming in central, eastern, and southern China have become increasingly serious (Ji et al., 2020; Liu et al., 2020). Guangdong province is one of the largest duck breeding and consumption areas in China. DuCV was reported to have a prevalence rate of 20% to 53.3% in Guangdong province in 2018 and 2019. In this study, duck liver samples were collected from 4

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regions in Guangdong province and analyzed to determine the prevalence rate of DuCV in Guangdong province. Moreover, the complete genome sequences were recovered, and the phylogenetic relationship and recombination events were also analyzed. The results of this study not only expand upon the knowledge regarding the genetic diversity and evolution of DuCV, but also indicate the high genetically divergent DuCV strains co-circulating in the duck population in Guangdong, China.

MATERIALS AND METHODS

Sample Collection and Processing

During October 2020 to August 2021, many diseased ducks were sent to the animal hospital of Foshan University for disease diagnosis by four duck farms located in Foshan, Qingyuan, Zhaoqing, and Jiangmen in Guangdong province. A total of 270 liver samples were collected by the veterinarians under the oral permission of owners, and the procedures for sampling and sample processing were approved by the Ethics Committee of Foshan University. Samples collection were performed total of five times in each farm in October 2020, and April, May, July, and August 2021, respectively. Approximately 30 mg of liver sample was individually homogenized in 500 μ L of sterile phosphate-buffered saline solution (PBS, pH = 7.02, GIBCO), and total DNA was extracted from 200 μ L of homogenate supernatant using a DNA extraction kit (OMEGA) according to the manufacturer's instructions. All of the extracted DNA samples were stored at -70°C for further detection of DuCV.

Detection and Complete Genome Amplification of DuCV

Using the extracted DNA as a template, all samples were screened for DuCV by using common DuCV detection primers as previously described (Banda et al., 2007). Distilled water was used as the negative control for PCR amplification. PCR products with an expected size of 480 bp were considered to be positive for DuCV. Furthermore, the complete genome of DuCV were amplified from the positive samples using the primer pairs F2 (5'-TCCGGATCCGAAAAATCCAAATAC-3') and R2: (5'-CCCGGATCCGGAAGTGGACCAAC-3') (Zhang et al., 2013b). All of the PCR products amplified by each of the primer sets were purified using a gel extraction kit (TaKaRa, Dalian, China), cloned into the pMD19-T vector (TaKaRa, Dalian, China), and transformed into *Escherichia coli* competent cells. Positive inserts were determined by PCR, and 5 positive clones were submitted for sequencing conducted by Sangon Biotechnology Company (Shanghai, China). To prevent contamination, the PCR reaction mixture and template DNA was prepared in a separate room using dedicated pipets and barrier tips.

Sequence Comparison and Recombination Analysis

Sequence assembly and manually editing was performed using the SeqMan program (DNASTAR, Madison, WI), and the nucleotide (nt) sequence identities were calculated by the MegAlign program available within the Lasergene software package (version 7.1, DNASTAR).

Before phylogenetic analysis, potential recombination events involved in the evolutionary history of circoviruses were assessed using RDP version 4.70 (Martin et al., 2015). The RDP analysis was performed with default settings using seven detection methods (RDP, GENECONV, bootscan, maximum chi square, Chimera, SISCAN, and Distance Plot). Putative recombination events were identified with a Bonferroni corrected P value cutoff of 0.05, and only sequences with significant evidence of recombination ($P < 0.05$), namely, (1) detected by three or more methods and (2) confirmed by phylogenetic analysis, were accepted as credible recombination events.

Phylogenetic Analysis

Maximum-likelihood (ML) trees were constructed using MEGA version 7.0, based on the best-fit nt substitution model General Time Reversible (GTR) nucleotide substitution model and optimized parameters of gamma (Γ)-distribution and proportion of invariable sites (i.e., GTR + Γ + I), which was determined using jModel Test. Bootstrap values were calculated from 100 replicates, and the phylogenetic trees were mid-point rooted for the purpose of clarity only.

RESULTS AND DISCUSSION

Prevalence of DuCV in Partial Areas of Guangdong Province

The liver tissues of 270 domestic ducks were collected from different duck farms in Guangdong province to detect circovirus in ducks. After PCR screening, sequencing, and BLAST analysis, 69 DNA samples were determined as positive for DuCV. The overall prevalence of DuCV in duck populations in Guangdong province was 25.6% (69/270), while the positive rates of DuCV infection in Foshan, Qingyuan, Zhaoqing, and Jiangmen were 25.8%, 36.0%, 20.6%, and 23.3%, respectively. The prevalence of DuCV detected herein was consistent with a previous study conducted in Guangdong province (Liu et al., 2020). Moreover, BLAST analysis of the sequences obtained in the initial detection showed that 49 and 20 virus strains were determined as genotype DuCV-1 and genotype DuCV-2, respectively, which also demonstrated that DuCV-1 has a wider distribution than DuCV-2. Collectively, these data not only demonstrated a high DuCV infection rate in the duck population in Guangdong province, but also that DuCV-1 is the predominant genotype circulating within Guangdong.

Recombination Events Involved in the Evolution of DuCV

The 177 complete genome sequences of DuCV retrieved from GenBank and the 22 sequences obtained in this study were subjected to recombination analysis that was performed by RDP software. Nine statistically supported recombination events were detected in 26 of the 199 DuCV sequences, suggesting the ubiquitous presence of recombination in the evolutionary history of DuCV (Figure 1). Noticeably, all recombinant sequences were identified in China, indicating the extensive genetic diversity of DuCV circulating in China. More importantly, four recombinant sequences associated with three recombination events (events 3, 5, and 10) were determined in this study, and four sequences identified herein were determined as the major and/or minor parent sequence (Figure 1), suggesting that duck populations in Guangdong province may play an important role in the evolution of DuCV. Guangdong is the intersection of the international migratory bird routes through China. Previous study has reported the presence and recombination events of circovirus in migrating wild ducks (Niu et al., 2018), which suggested that wild birds play important roles in the worldwide transmission, the gene flow, and the occurrence of genetic recombination events of circovirus.

Genetic Diversity of DuCV Circulating in Partial Areas of Guangdong Province

The 22 complete genome sequences of DuCV obtained in this study have been deposited in GenBank under the accession numbers ON227536 to ON227557. The complete genome sequences of these virus strains ranged from 1989 to 1998 nt in length, and they shared 82.5% to 99.9% genome-wide pairwise identity with each other. They also exhibited 81.6% to 100.0% genome-wide pairwise identity with 177 complete genomes of DuCV retrieved from GenBank. In addition, phylogenetic trees clearly showed that these sequences could be divided into 2 genetic groups: DuCV-1 (1a, 1b, 1c, and 1d) and DuCV-2 (2a, 2b, and 2c) (Figure 2). Moreover, DuCV sequences identified herein were divided into 2 genotypes (DuCV-1b, DuCV-1a, and DuCV-2c). Specifically, 16 and 4 strains of virus classified into genotype DuCV-1b and DuCV-2c, respectively, were detected in all four farms, while 2 strains of virus belonging to the genotype DuCV-1a were only detected in duck farm located in Zhaoqing. These results suggest that genotype DuCV-1b was the most prevalent virus strains in these 4 duck farms, and also indicate the genetic diversity of DuCV circulating in duck population in Guangdong province. Noticeably, 7 viral sequences generated herein (ON227545, ON227537, ON227539, ON227536, ON227555–ON227557) formed monophyletic groups

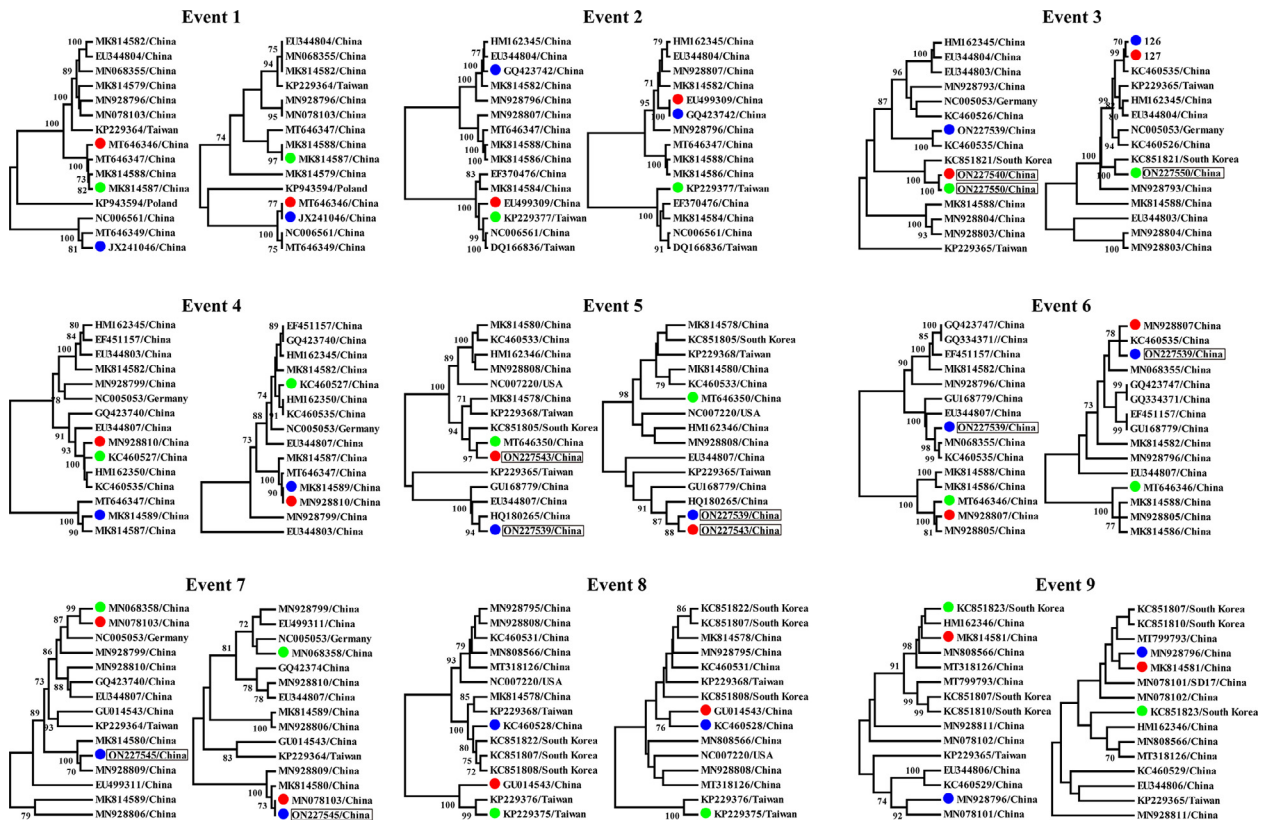


Figure 1. Phylogenetic trees illustrate the potential recombination events detected in this study. The dots marked in green, blue, and red indicate the potential major parent, minor parent, and recombinant sequences, respectively. Phylogenetic tree was reconstructed by using the UPGMA method available within the MEGA version 7.0. The numbers (>70) above branches indicate percent bootstrap values.

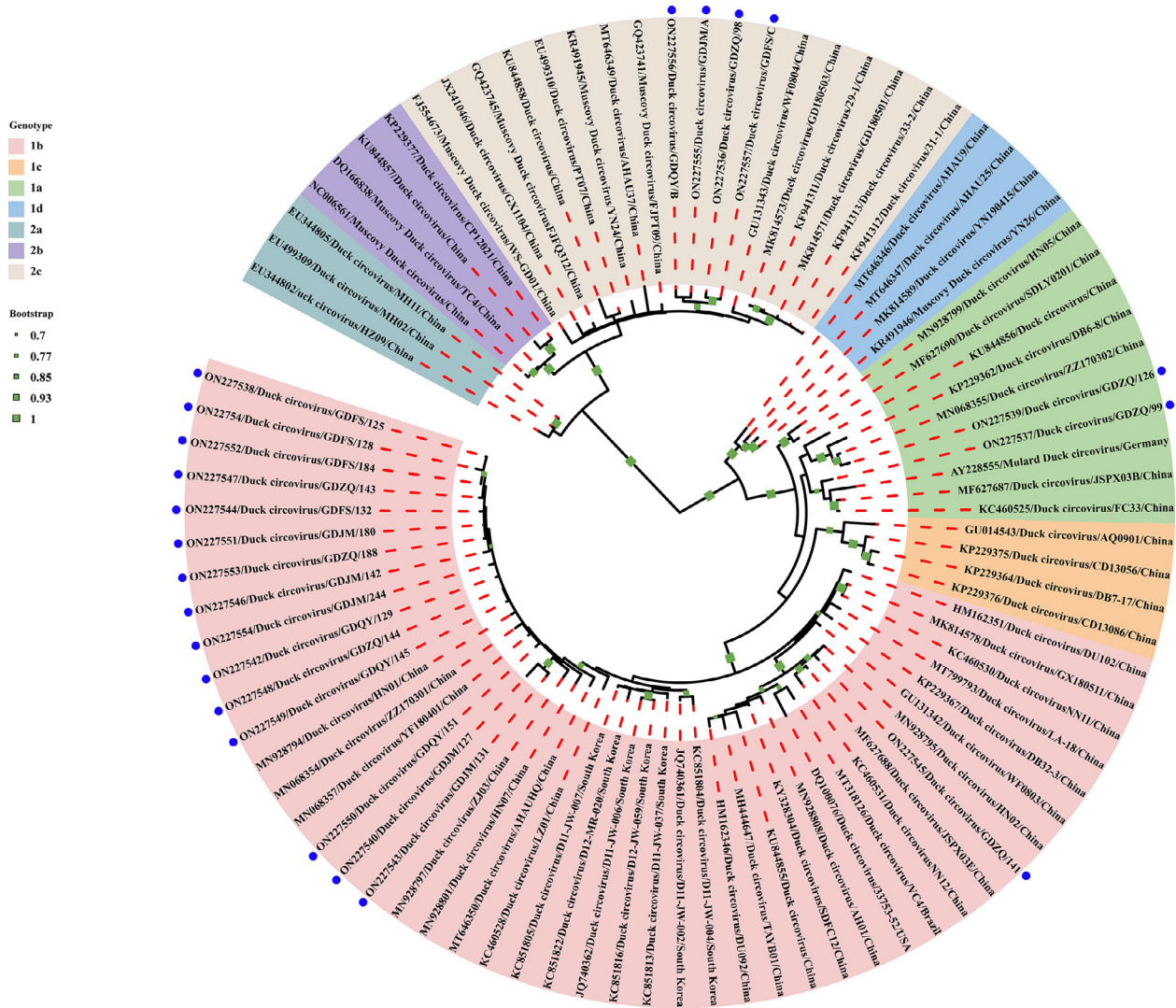


Figure 2. Phylogenetic tree reconstructed based on the complete genomic sequences of DuCVs. Bootstrap values reconstructed by using the maximum likelihood (ML) method available within the MEGA version 7.0 under GTR+ Γ +I model with 100 replicates of the alignment, and only bootstrap values >70% are shown at appropriate nodes. Blue dot indicates the sequence determined in this study.

with circovirus identified in wild ducks (KU844855, KU844856, and KU844855). The close phylogenetic relationships between circoviruses identified in domestic and wild ducks indicated that migratory birds may play an important role in the transmission and wide geographic distribution of circovirus.

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DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial

relationships that could be construed as a potential conflict of interest.

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