Extensive genetic diversity and recombination events identified in goose circoviruses circulating in partial areas of Guangdong province, Southern China

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ABSTRACT Circoviruses represent a group of small viruses with circular single-strand DNA genome that infect a wide range of both domesticated and wild animals. Domesticated geese infected with circovirus have been confirmed in many parts of the world, and is considered to cause immunosuppression and facilitate the secondary infections caused by other pathogens. In the present study, extensive genetically diversified goose circoviruses (**GoCVs**) were identified in the liver samples of domesticated geese from Guangdong province, southern China. Genetic analysis revealed that the sequences generated in this study shared 81.5 to 99.7% genome-wide pairwise identity with previously identified GoCV genomes. More importantly, nine recombination events were identified among all known complete genomome sequences of GoCV including those obtained herein, and the majority was determined associate with the sequences identified from Guangdong province, suggesting that recombination is the primary driver for the diversification of GoCVs. Additionally, purifying selection was the dominant evolutionary pressure acting on the genomes of GoCVs, and the ORF C1 gene of GoCV showed a higher genetic variation than ORF V1 gene. These results expand the knowledge about the genetic diversity and evolution of GoCV, and also indicate extensive genetically divergent GoCV strains were co-circulating in goose population in partial areas of Guangdong province, southern China.

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

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INTRODUCTION

Circoviruses, belonging to the genus *Circovirus* in the family *Circoviridae* represent a group of non-enveloped small viruses with circular single-strand DNA genome about 2 kb in length (Breitbart et al., 2017; Rosario et al., 2017). The genomes of circoviruses have ambisense organization containing 2 major open reading frames (**ORF**): ORF V1 encoding the replication-associated protein (**Rep**) on the virion sense strand, and ORF C1 encoding the capsid protein (**Cap**) on the complementary sense strand (Breitbart et al., 2017; Rosario et al., 2017). Members of the genus *Circovirus* are known to infect various vertebrates, including mammals (Li et al., 2010; Baekbo et al., 2012; Lian et al., 2014), birds (Todd, 2004), and fishes (Lőrincz et al., 2011). Circoviruses are considered to cause immunosuppression in infected animals, which is associated with the apoptosis of lymphocytes (Todd et al., 2001a; Todd, 2004; Opriessnig et al., 2020), and further facilitate the secondary infections caused by other bacterial, viral, and fungal pathogens (Todd, 2000, 2004).

Many circoviruses, such as goose circovirus (**GoCV**), duck circovirus (**DuCV**), pigeon circovirus (**PiCV**), mute swan circovirus (**SwCV**), and Beak and feather diseases virus (**BFDV**) have been identified from a wide variety of bird species (Soike et al., 1999; Todd et al., 2001b; Hattermann et al., 2003; Halami et al., 2008; Julian et al., 2013). GoCV was first described in a commercial flock with a history of increased mortality and stunting syndrome in Germany (Soike et al., 1999). Histopathological examination revealed the multisystem infection of GoCV in domesticated geese, with the presence of virus in the bursa of Fabricius, spleen, thymus, bone marrow, liver, kidney, lung, and heart, and the infected geese often shows nonspecific clinical symptoms such as feather disorders, diarrhea, and growth retardation (Guo et al., 2011). Recently, GoCV has also been detected in wild geese (Stenzel et al., 2015). Since its first discovery in Germany in 1999, GoCV infection in domesticated geese has been reported in other countries or regions in European and Far East (Chen et al., 2003;

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Cságola et al., 2012; Niu et al., 2018; Stenzel et al., 2018). In mainland China, GoCV infection in domesticated geese was firstly confirmed in Zhejiang province, eastern China (Yu et al., 2007). Thereafter, it has been confirmed the presence in 6 provinces of China, with an overall prevalence of 21.7% (Niu et al., 2018).

China is a large country of goose breeding, which always occupies the first place in the world, and Guangdong province is the largest goose breeding and consumption area in China. In this study, extensive genetically diversified goose circoviruses (GoCVs) were identified in the liver samples of domesticated geese from Guangdong province, southern China, which expand the knowledge about the genetic diversity and evolution of GoCV, and also indicate highly genetic divergent GoCV strains were co-circulating in goose population in partial areas of Guangdong province, southern China.

MATERIALS AND METHODS

Samples Collection and Processing

From June 2020 to August 2021, liver tissues of dead domesticated goose were randomly collected in Foshan, Qingyuan, Zhaoqing, Jiangmen, and Yangjiang cities of Guangdong province (Figure 1). Approximately 30 milligram of liver sample was individually homogenized in 500-μL sterile phosphate buffered saline solution (**PBS**, pH = 7.02, GIBCO), and total DNA was extracted from 200 μL supernatant from the homogenate using the DNA Extraction kit (OMEGA, Doraville, CA) according to the manufacturer's instructions. All extracted DNA samples were stored in −70°C for further detection of GoCV.

Screening and Complete Genome Recovery of GoCV

GoCV was screened using the broad-spectrum nested PCR targeting the ORF V1 gene of various avian circoviruses as previously described (Halami et al., 2008), and complete genomes of GoCV were recovered from the positive samples using a back-to-back primer pair GoCV-F (5'−CTSTCTCGWGCYCGGGGATCTGAC−3′) and GoCV-R (5′−CCAGGCTCTTCCTCCCAGCKWCT CTT−3′) described previously by Stenzel et al. (2018). Distilled water was used as the negative control among all the PCR amplification.

After 1% agarose gel electrophoresis, a PCR product with expected size of 350 bp was considered GoCV DNA positive. All PCR products amplified by each of the primer sets were purified using Gel Extraction kit (TaKaRa, Dalian, China), cloned into pMD19-T vector (TaKaRa), and transformed into *E. 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 preparation of PCR mix and template DNA added was prepared in a separate room, and aerosol-free pipette tips were used at each stage.

Sequence Comparison and Recombination Analysis

Sequence assembly and manually editing was performed using the SeqMan program (DNASTAR, Madison, WI), and the nucleotide (**nt**) sequence identity were calculated by MegAlign program available

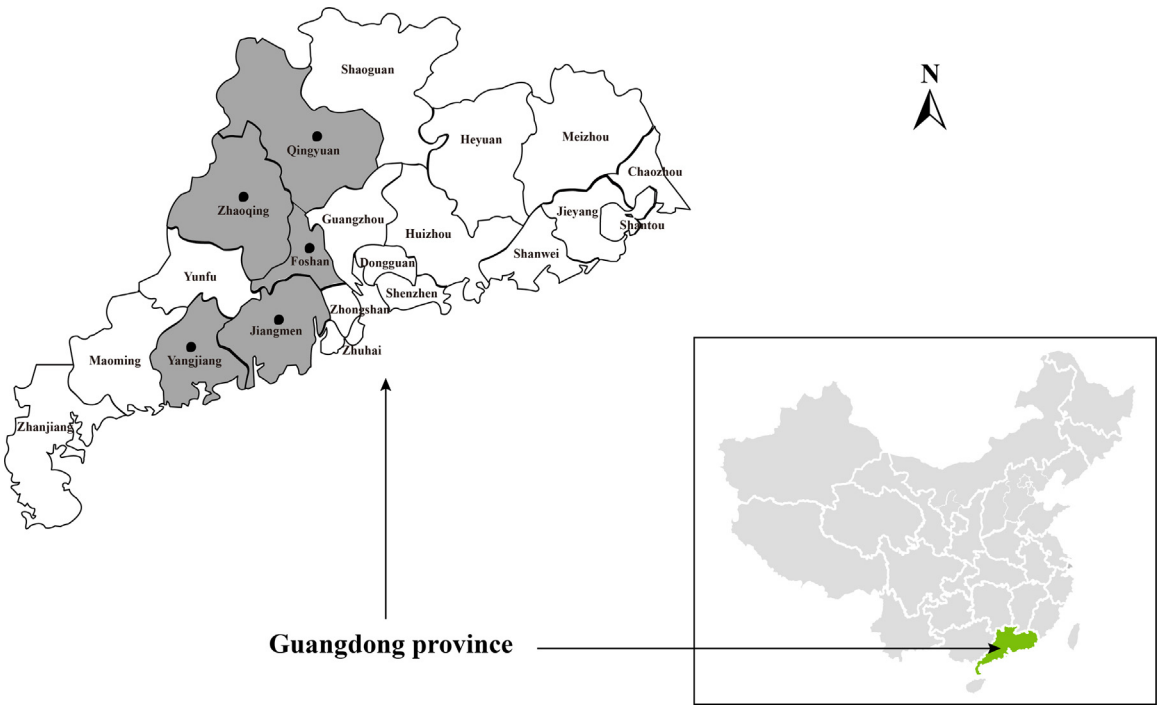

Figure 1. Geographic maps showing the location of sampling sites where the goose liver samples collected in this study. This map was plotted by combination of Surfer software version-4 (Golden Software, Golden, CO) and Adobe illustrator version CC2017 (Adobe, San Jose, CA). The black dots indicate the sampling regions in this study.

within the Lasergene software package (version 7.1, DNAstar).

Given that recombination events were identified commonly in circoviruses (Julian et al., 2013; Stenzel et al., 2014; Stenzel et al., 2018), potential recombination events involved in the evolutionary history of circoviruses were assessed using RDP version 4.70 (Martin et al., 2015) prior to phylogenetic analysis. The RDP analysis was performed with default settings using 7 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 ($P < 0.05$) of recombination, namely, 1) detected by 3 or more methods and 2) confirmed by phylogenetic analysis, were accepted as credible recombination events.

Phylogenetic Analysis

Maximum-likelihood (**ML**) trees were reconstructed using MEGA version 7.0 (Kumar et al., 2016), based on the best-fit nucleotide 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) determined using jModel Test (Posada, 2008). Bootstrap values were calculated from 1,000 replicates, and the phylogenetic trees were mid-point rooted for purposes of clarity only.

Selection Pressures Analysis

The existence of selective pressures along the genome was assessed by calculating the difference between nonsynonymous substitution (**d*N***) and synonymous site (**d*S***) rates for the aligned ORF V1 and ORF C1 genes using Single-Likelihood Ancestor Counting (**SLAC**) method as implemented in the Datamonkey web server (http://www.datamonkey.org). Since entropy takes into account the number of distinct viral variants, it is less susceptible than genetic distance to variation as a result of hypermutation (Wolinsky et al., 1996). Genetic diversity could occur under selection pressure, which displays the diversity level in numerical form by entropy, namely a higher number indicate relatively higher genetic complexity (Lourenço et al., 2018). Thus, the entropy was calculated based on amino acid sequence by using BioEdit as previously described (Ji et al., 2020). Additionally, codon-specific analyses of the entire Rep and Cap proteins coding region were also assessed using 3 methods performed in the Datamonkey web server: Fixed Effects Likelihood (**FEL**), Fast Unconstrained Bayesian AppRoximation (**FUBAR**), and Mixed Effects Model of Evolution (**MEME**). Only codons with significance of $P < 0.05$ or a posterior probability > 0.95 identified by 3 methods simultaneously were considered to subject to positive selection (Guo et al., 2020).

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The procedures for sampling and sample processing were approved by the ethics committee of Foshan University. All animals were treated in strict accordance with the Rules for the Implementation of Laboratory Animal Medicine (1998) from the Ministry of Health, China.

RESULTS

Detection and Genome Recovery of Circovirus in Geese

In this study, a total of 214 liver tissues of domesticated goose were collected in Guangdong province to detect the presence of GoCV. After nested PCR, sequencing and BLAST analysis, 76 DNA samples were determined to be GoCV DNA positive (Table 1). The positive rate of GoCV infection in 5 sampling sites ranges from 33.3 to 38.1%, with no significant difference ($P > 0.05$), and the overall prevalence of GoCV in geese in Guangdong province was 35.5% (76/214). Based on the BLAST results and different sampling sites, 37 positive samples were selected to recover the complete genomic sequences.

Comparative Genomic Sequence Analysis

The 37 complete genome sequences of GoCV strains determined in this study were deposited in GenBank under the accession numbers OL456394−OL456430. The genomes of these virus strains consisted of 1,820 to 1,822 nucleotides. Sequence comparison analyses shown that they shared 89.8 to 99.9% genome-wide pairwise identity with each other. Meanwhile, these sequences exhibited 81.5 to 99.7% genome-wide pairwise identity with other known GoCVs. Moreover, all the 37 sequences of GoCV obtained herein shared the highest genome-wide pairwise identity with sequences generated from goose in China (89.8−99.7%). In addition, sequences analysis showed that ORF V1 and ORF C1 genes of all the 139 GoCV strains, including 102 retrieved from GenBank and 37 generated in this study, shared 85.4 to 100% and 61.2 to 100% nucleotide sequence identity, respectively.

Table 1. Prevalence of GoCV in goose by location in Guangdong province.

Location	No. of individuals	No. of positive	Positive rate (%)
Foshan	52	19	36.5
Qingyuan	40	14	35.0
Zhaoqing	42	16	38.1
Jiangmen	36	12	33.3
Yangjiang	44	15	34.1
Total	214	76	35.5

Abbreviation: GoCVs, goose circovirus.

Table 2. A summary of the twelve recombination events detected using the RDP (R), GENECONV (G), BootScan (B), MaxChi (M), Chimaera (C), SiScan (S), and 3Seq (Q) methods implemented in the computer program RDP4.

Event	Recombinant region	Recombinant sequence	Major parent	Minor parent	Detection method	*P*-value
1	1798-1053	**MT831936,MT831921, MT831917, MT831913, OL456405, OL456430, OL456413, OL456421, OL456395, OL456397, OL456401, OL456407, OL456411**	**OL456429**	**OL456428**	RGMCSQ	5.749×10^{-4}
2	1691-982	KT808659, KT808654, KT808656, KT808652, KT808655, KT808651, KT808658	AF536932	KT808650	RGMCSQ	4.564×10^{-8}
3	1766-838	**OL456412**	**MT831937**	**OL456415**	RGMCSQ	4.183×10^{-6}
4	831-542	**OL456428**	**MT831937**	**OL456415**	RGMCSQ	4.183×10^{-6}
5	1765-920	KP229371, KP229374	AF536933	AF536939	RGMCSQ	3.368×10^{-4}
6	476-1113	**MT831911**	KT387277	**MT831921**	RGSQ	4.001×10^{-8}
7	476-1053	**MT831936, MT831921, MT831913, MT831917, OL456421, OL456395, OL456413, OL456397, OL456401, OL456407, OL456411, OL456405**	**OL456429**	**MT831931**	RGMSQ	7.415×10^{-4}
8	476-906	**OL456430, OL456424, OL456429**	KP203866	**MT831941**	RSQ	2.776×10^{-4}
9	61-752	KP203871, KP203870, KP203866, KP203867, KP203868, KP203869, KP229363, KP229370, KP229372, KP229373, AF536936, AF536938, AF536939	KT808661	KP203872	GCSQ	1.867×10^{-2}

The *P*-value is shown as the highest value estimated through the detection method shown in red, and only detection methods with associated *P*-values < 0.05 are shown.

The GenBank accession numbers in bold mean sequences identified in Guangdong province.

Recombination Analysis

After systematic screening the 102 complete genome sequences of GoCV retrieved from GenBank, and the 37 sequences obtained herein using RDP software, 9 statistically supported recombination events were detected within 39 of the 139 GoCV sequences with a transferred fragment size ranging from 16 to 49% of the genome (Table 2 and Figure 2). Additionally, 18 out of the 69 complete genome sequences of GoCV strains identified in Guangdong province were identified as recombinant, including 13 sequences determined herein. Moreover, six of the nine recombination events were determined associate with GoCV sequences identified in Guangdong province (Table 2). Noticeably, sympatric viruses recombination events (event 1, 3, 4, 5, 7) and allopatric viruses recombination events (event 2, 6, 8, 9) were simultaneously observed in sequences generated in Guangdong (Table 2).

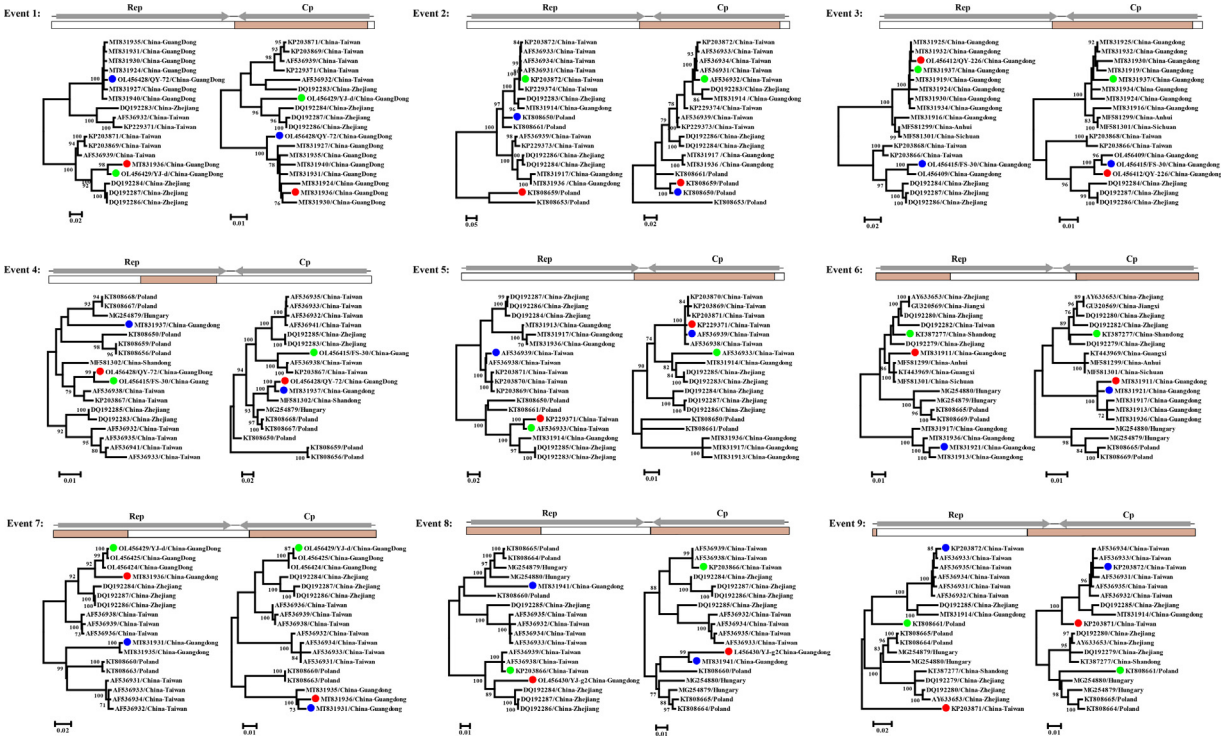

Figure 2. Phylogenetic trees illustrate the potential recombination events detected in this study. The orange indicates the sequences region deriver from major parent, while the white indicates the sequences region deriver from minor parent. The dots marked in green, blue, and red indicates the potential major parent, minor parent, and recombinant sequences, respectively. Phylogenetic tree was reconstructed by using the maximum likelihood (ML) method available within the MEGA version 7.0 under GTR+Γ+I model. The numbers (>70) above branches indicates percent bootstrap values.

Phylogenetic Analysis of GoCVs

Maximum-likelihood phylogenetic trees were reconstructed based on the representative complete genome sequences, and ORF V1 and ORF C1 gene sequences of GoCV from which all detected recombinant sequences were removed (Figures 3 and 4). In the complete genome tree, GoCV sequences identified in this study were clustered together with those viral sequences identified in China (Figure 3). Specifically, 21 GoCV sequences identified herein mainly presented the closest evolutionary

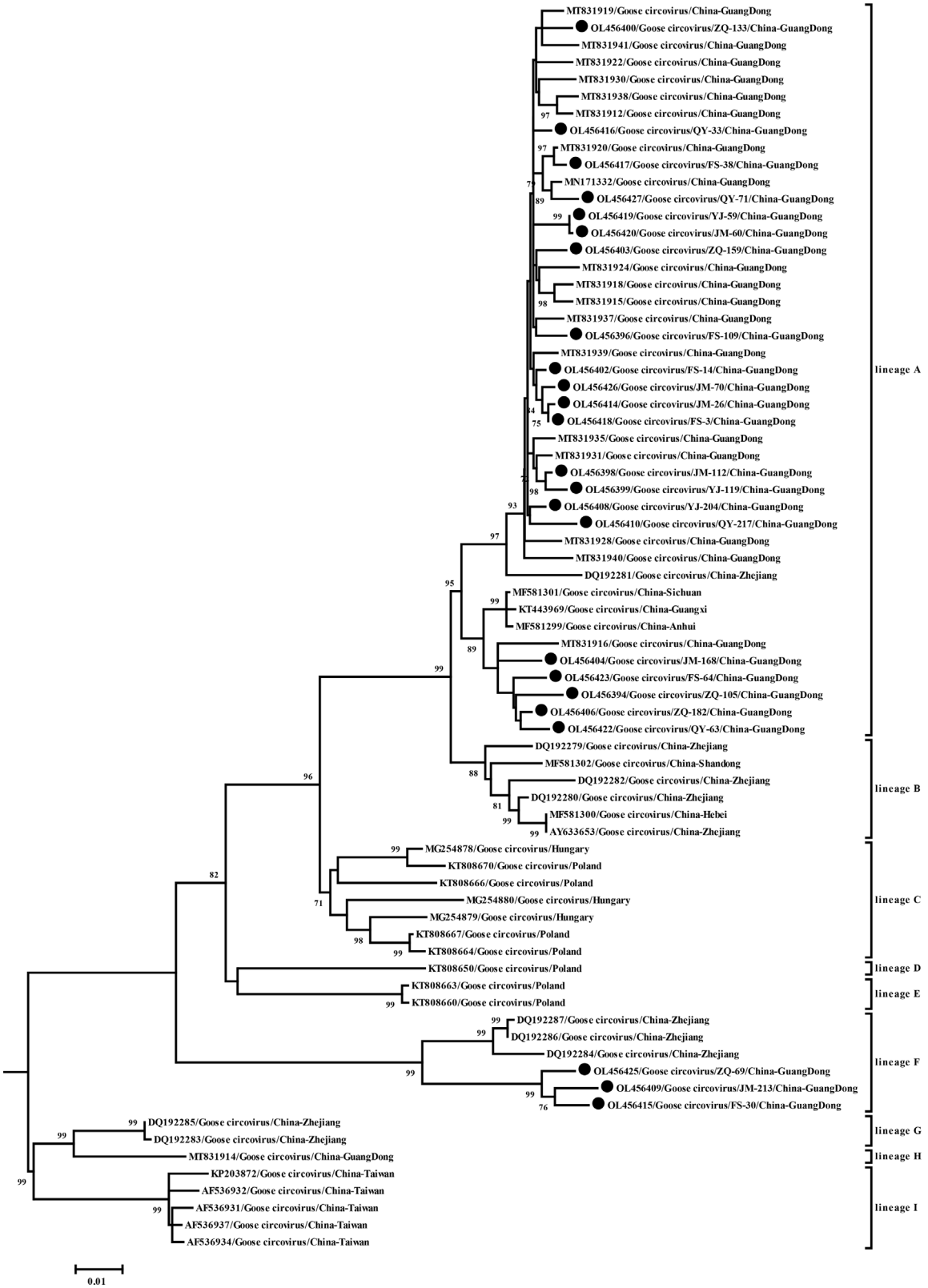

Figure 3. Recombination free Maximum-likelihood phylogenetic tree based on the complete genomic sequences of GoCVs. Bootstrap values reconstructed by using the maximum likelihood (ML) method available within the MEGA version 7.0 under GTR+Γ+I model with 1,000 replicates of the alignment, and only bootstrap values >70% are shown at appropriate nodes. Black dot indicates the sequences determined in this study. Abbreviation: GoCVs, goose circovirus.

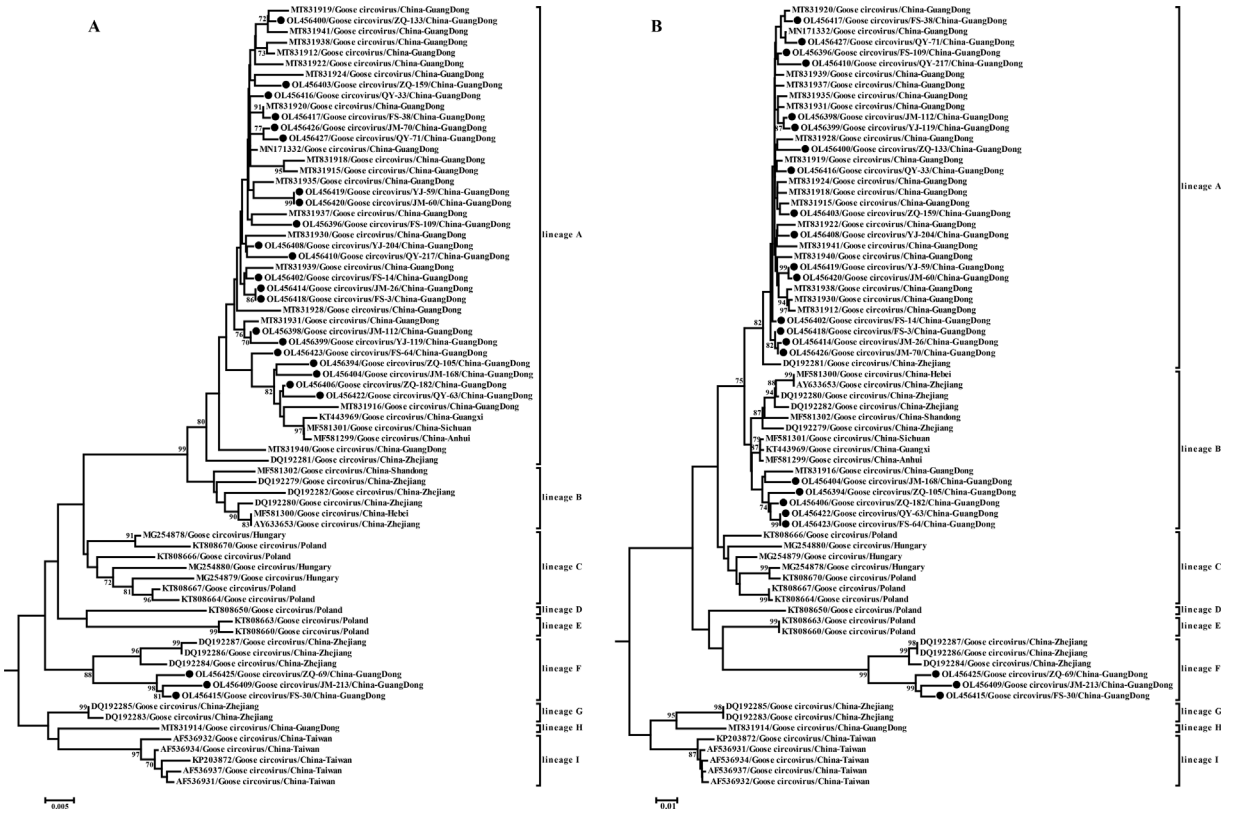

Figure 4. Phylogenetic trees reconstructed based on (A) ORF V1 and (B) ORF C1 gene of GoCVs with the removal of detected recombination evens. Bootstrap values reconstructed by using the maximum likelihood (ML) method available within the MEGA version 7.0 under GTR+Γ+I model with 1,000 replicates of the alignment, and only bootstrap values >70% are shown at appropriate nodes. Black dot indicate the sequences determined in this study.Abbreviation: GoCVs, goose circovirus.

relationship with those sampled from Guangdong province, while 3 sequences (JM-213, FS-30, and ZQ-69) formed a sister lineage with the viral sequences identified from Zhejiang province. Phylogenetic tree also shown that all viral sequences used in this analysis were clearly segregated into nine well-separated lineages (A–I). Among them, viruses clustered into lineage A, B, F, G, H, and I were detected in China, while lineage C, D, and E detected in Europe (Poland and Hungary), suggesting the clustering of GoCV are associated with their geographic origins. Moreover, lineages A, F, and H were detected in Guangdong province, indicating that genetic diversified GoCV is co-circulating in Guangdong.

Additionally, the ML tree estimated based on ORF V1 gene showed the similar topology with that of complete genome sequences. However, there are several differences between the topologies of ORF C1 and ORF V1 trees (Figure 4). Some viral sequences that fell into lineage A of the ORF V1 tree were clustered together with the sequences in lineage B of the ORF C1 tree, and the branching order and branch length of the lineage A differed between the phylogenies based on these 2 genes. These results indicating a higher genetic diversity of ORF C1 gene of GoCV than that of ORF V1 gene.

Selection Pressures on the Genome of GoCVs

The 139 complete genome sequences, including 102 retrieved from GenBank and 37 generated in this study, were used to analysis the selection pressures acting on the evolution of GoCV genomes. Negatively selected sites ($dN - dS < 0$) were observed predominantly across the Rep and Cap protein coding region (Figure 5A), and the mean dN/dS ratios estimated in the coding sequence of Rep and Cap protein were lower than 1 (0.07 and 0.12, respectively), reflecting the predominance of purifying selection in the genomic evolution of GoCV. Although 3 and 2 putatively positive selection sites in the coding region of Rep and Cap protein were simultaneously predicted by three methods (FEL, FUBAR, and MEME; Table 3), the functional significance of these sites putatively under positive selection need further experimentally investigation.

Moreover, amino acid residues entropy that indicating the genetic complexity was calculated (Figure 5B). The entropy values of amino acids of Rep and Cap protein were low (0–0.81 and 0–1.1 for Rep and Cap, respectively), and the majority of amino acid residues entropy of Rep and Cap was approximately ≤0.1, indicating that amino acid sequences of Rep and Cap possessed relatively low genetic complexity. Additionally, the entropy values of amino acids of Cap protein were generally higher than that of Rep protein, suggesting Cap protein of GoCV showed high genetic diversity when compared to Rep protein.

DISCUSSION

Goose circovirus infection in domesticated geese have been confirmed in Germany (Soike et al., 1999), Poland

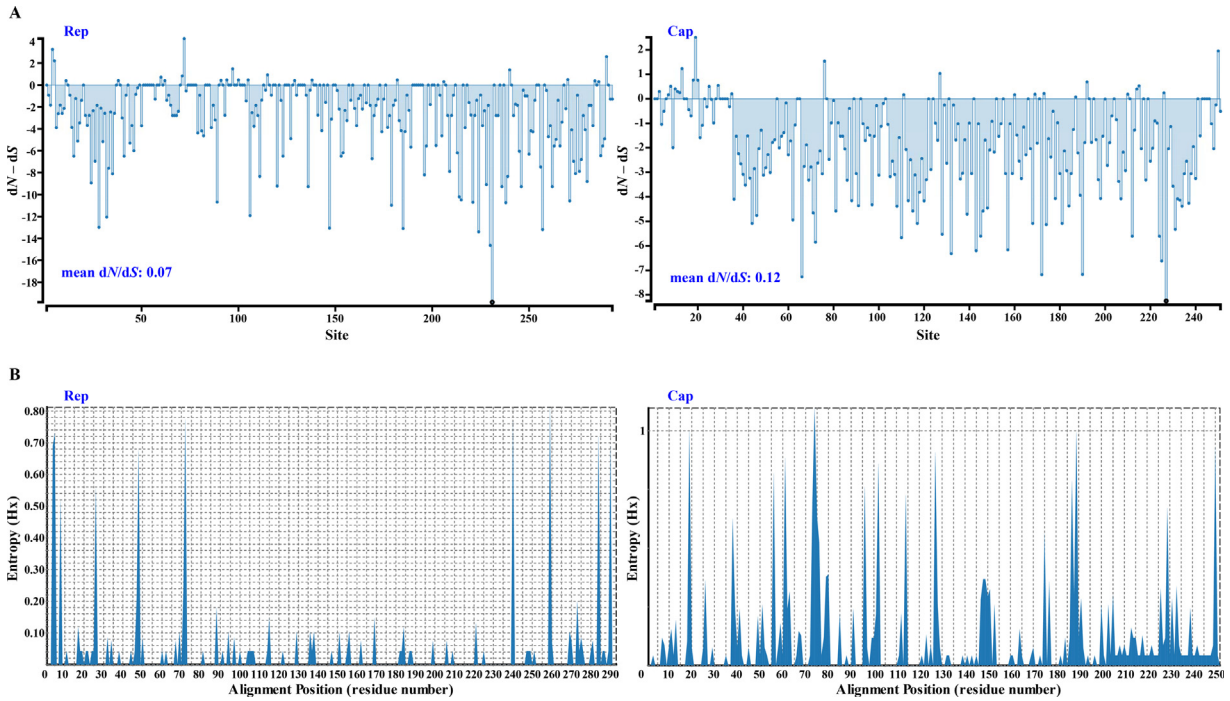

Figure 5. Selection pressures analysis based on the Rep and Cap proteins coding region of GoCVs. (A) The difference between non-synonymous and synonymous rates ($dN - dS$) plot for the Rep and Cap proteins coding region. (B) Amino acid entropy rates for Rep and Cap protein. Abbreviation: GoCVs, goose circovirus.

(Stenzel et al., 2018), Hungary (Cságola et al., 2012), and China (Chen et al., 2003; Yu et al., 2007; Niu et al., 2018). According to the sequence data available from GenBank, the majority of genome sequences of GoCV were determined in China, including Taiwan, Zhejiang, Guangxi, Shandong, Anhui, Sichuan, Hebei, and Guangdong provinces, indicating the wide geographical distribution of GoCV in China. In addition, the overall prevalence of GoCV detected in this study was 35.5%, which was higher than that determined in mainland China previously (Niu et al., 2018), but consistent with the prevalence in other countries based on published data (Stenzel et al., 2018). Our data, and information provided by previous studies conducted in mainland China collectively suggested GoCV was widely distributed in China with a high infection rate in goose population.

In the present study, genetic analysis revealed that all GoCV sequences recovered herein shared the highest genome-wide pairwise identity with those generated from geese in China. Phylogenetic analysis based on recombination-free complete genome sequences also showed that these GoCV strains present the closest evolutionary relationship with those identified in China, suggesting the clustering of GoCV displayed geographical pattern. Noticeably, 3 viral strains (JM-213, FS-30, and ZQ-69) generated in this study showed the closest relationship with viruses previously identified in Zhejiang province, which might be resulted from frequent poultry trade and breeds introduction between Guangdong and Zhejiang provinces. This hypothesis is reasonable since Guangdong province is the largest goose breeding and consumption area in China. Previous study has also provided evidence of geographical structure of the clustering of GoCV (Stenzel et al., 2018). Moreover, the recombination-free phylogenetic tree showed that all GoCV sequences used in this analysis could be divided into 6 well-separated lineages according to their geographic origins (Figures 3 and 4). More importantly, 4 of the 6 lineages were detected in China (A, B, E, and F), and lineages A, E, and F were detected in Guangdong province. These results demonstrated the geographical pattern of GoCVs clustering, and also indicated the genetic diversity of GoCV circulating in China, and genetic diversified GoCVs were co-circulating in Guangdong province.

It is noteworthy that various studies have shown that recombination is the primary driver for the diversification of circoviruses in general (Julian et al., 2013; Zhang et al., 2013; Stenzel et al., 2018; Ji et al., 2020). In this study, 9 statistically supported recombination events were detected within 39 of the 139 GoCV complete genome sequences (including 102 retrieved from

Table 3. Prediction of putative positive selection site of hepacivirus genome from different hosts.

Protein	Model	Putative diversifying selection codons	Putative purifying selection codons
Rep	FEL	3 codons: **4, 5, 72**	125 codons
	FUBAR	3 codons: **4, 5, 72**	118 codons
	MEME	5 codons: **4, 5, 72**, 97, 290	–
Cap	FEL	2 codons: **19, 250**	143 codons
	FUBAR	2 codons: **19, 250**	148 codons
	MEME	7 codons: 7, 18, **19**, 20, 127, 150, **250**	–

$P < 0.05$ or posterior probability > 0.95; positions identified as being under positive selection simultaneously predicted by three methods are shown in bold.

GenBank and 37 obtained herein), suggesting the ubiquitous presence of recombination in the evolutionary history of GoCVs (Table 2 and Figure 2). Additionally, 18 of the 69 complete genome sequences of GoCV identified in Guangdong province were identified as recombinant, and the majority of recombination events were determined associate with GoCV sequences identified in Guangdong province, which could explain the genetic diversity of GoCV circulating in Guangdong province. Importantly, sympatric and/or allopatric viruses recombination events were observed in viral sequences determined in Guangdong, where is the intersection of the international migratory bird routes through China. Bird migration has been suggested to the worldwide virus transmission, such as avian influenza virus and West Nile virus, along the flyway (Reed et al., 2003; Tian et al., 2015). Previous study has reported the presence and recombination events of GoCV occurred in migrating wild birds (Stenzel et al., 2018), which suggested that wild birds also play important roles in the worldwide transmission, the gene flow, and the occurrence of genetic recombination events of goose circovirus.

Previous studies have demonstrated the ORF C1 gene that encoding the Cap protein of PiCV, DuCV, and BFDV displayed a high genetic variation, which was believed to cause antigenic differences (Todd et al., 2008; Wang et al., 2011; Julian et al., 2013; Ji et al., 2020). In this study, sequences analysis based on ORF V1 and ORF C1 genes of all the 139 GoCV strains available presently shown that the extent of nucleotide variation of ORF C1 (61.2−100% nucleotide identity) was higher that of ORF V1 (85.4−100% nucleotide identity), also indicating that ORF C1 gene of GoCV exhibited a higher variation rate compared with ORF V1. Additionally, the Rep protein coding region displayed a lower mean dN/dS ratio (0.08) than Cap protein (0.12), and the entropy values of amino acids of Cap protein were generally higher than that of Rep protein in selection pressure analysis (Figure 3), suggesting that Rep protein is evolving under stronger purifying selection and lower genetic variation than Cap protein. Taken together, these results suggesting that the ORF C1 gene shown a higher genetic diversity than ORF V1 gene.

In conclusion, the complete genomes of 37 GoCV strains sampled in Guangdong province were determined and analyzed. Results of this study indicated the presence of high genetic diversified GoCVs co-circulated in goose population in Guangdong province with a high infection rate, and also revealed the ubiquitous presence of recombination in the evolutionary history of GoCVs. These results expand the knowledge about the genetic diversity and evolution of GoCV, and show extensive genetically divergent GoCV strains in China.

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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.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101767.

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