Original Article
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Genomic analysis of Sheldrake origin goose hemorrhagic polyomavirus, China

Chunhe Wan*, Cuiteng Chen, Longfei Cheng, Rongchang Liu, Guanghua Fu, Shaohua Shi, Hongmei Chen, Qiuling Fu, Yu Huang

Fujian Provincial Key Laboratory for Avian Diseases Control and Prevention, Fujian Animal Diseases Control Technology Development Center, Institute of Animal Husbandry and Veterinary Medicine of Fujian Academy of Agricultural Sciences, Fuzhou 350013, China

Goose hemorrhagic polyomavirus (GHPV) is not a naturally occurring infection in geese in China; however, GHPV infection has been identified in Pekin ducks, a domestic duck species. Herein, we investigated the prevalence of GHPV in five domestic duck species (Liancheng white ducks, Putian black ducks, Shan Sheldrake, Shaoxing duck, and Jinyun Sheldrake) in China. We determined that the Jinyun Sheldrake duck species could be infected by GHPV with no clinical signs, whereas no infection was identified in the other four duck species. We sequenced the complete genome of the Jinyun Sheldrake origin GHPV. Genomic data comparison suggested that GHPVs share a conserved genomic structure, regardless of the host (duck or geese) or region (Asia or Europe). Jinyun Sheldrake origin GHPV genomic characterization and epidemiological studies will increase our understanding of potential heterologous reservoirs of GHPV.

Keywords: Sheldrake, genome, goose hemorrhagic polyomavirus

Introduction

Goose hemorrhagic polyomavirus (GHPV) is a non-enveloped, double-stranded (ds) DNA virus that belongs to the genus *Gammapolyomavirus* in the family *Polyomaviridae*. Members of *Gammapolyomavirus* can infect birds and some can cause fatal diseases, but without an oncogenicity syndrome observed [11]. The circular GHPV genome is approximately 5200 bp in size, ranging from 5252 to 5256 bp in length [3,5,8,9]. The GHPV genome contains five main open reading frames (ORFs): three structural proteins (namely, VP1, VP2, and VP3) and two tumor antigena (namely, large and small tumor antigena, LTAg and STAg, respectively). The LTAg coding sequences, which are used as criteria for definition and creation of polyomavirus species, are based on the observed LTAg protein distances. Additionally, ORF-X is a unique protein that was recently characterized in the GHPV genome [5,9]. ORF-X is similar to that in avian polyomaviruses and is involved in transcription and virus maturation.

GHPV has been characterized as the causative agent of fatal illnesses in geese, including hemorrhagic nephritis enteritis of geese (HNEG) in Europe [1,3,4,7,10,12]. HNEG was initially described in Hungary in 1969 [2]; subsequently, the disease was detected in Germany, France, and Belgium. Geese infected with HNEG had high mortality and morbidity rates of up to 43% and 30% in Germany and Poland, respectively [6,16]. Based on field epidemiological investigation, geese (*Anser anser*) infected with HNEG are generally aged from 3 to 13 weeks [3,4,6,7,12]. Necropsy of infected animals has revealed nephritis, hemorrhagic enteritis, edema, and gelatinous ascites of subcutaneous tissues. Moreover, *A. anser* infected with HNEG exhibit an immunosuppressive effect via B-cell depletion in bursa of *Fabricius*. Birds that recovered from GHPV infection were persistently infected [10].

Initially, GHPV was identified in Muscovy ducks (age, 8−12 weeks) and Mule ducks (age, 3−4 weeks) in France, with no typical signs observed, suggesting that ducks may be a potential reservoir of GHPV [3,13]. Subsequently, GHPV was detected in Pekin ducks (aged within 50 weeks) in China [8]. Due to a lack of evidence of GHPV infections occurring naturally in geese in China, the source of the virus in Pekin ducks (a domestic duck species in China) was unclear. In the present study, we used the polymerase chain reaction (PCR) method to assess the prevalence of GHPV in five domestic duck species in southern China. We observed that Jinyun Sheldrake (a domestic duck species in China) were positive for GHPV infection.

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*Corresponding author: Tel/Fax: +86-591-87574801; E-mail: chunhewan@126.com
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Whole genome sequences of the Jinyun Sheldrake GHPVs suggested that GHPVs share a conserved genomic structure, regardless of host (duck or goose) or region (Asia or Europe). The study data will help us gain a deeper insight into the genetic diversity of GHPVs circulating in waterfowl, in particular, in domestic ducks in China.

Materials and Methods

Ethics statement

The animal protocol used in this study was approved by the Research Ethics Committee of the College of Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agriculture Sciences (permission No. FAAS-AHVM2017-16). All of the ducks were handled in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of China.

Sample collection

A previous study revealed that GHPV could be found in Pekin duck (a domestic duck species) in China [8]. In order to assess the prevalence of GHPV infection in other local domestic duck species in China, five domestic duck species, namely, Liancheng White ducks (Liancheng, Fujian Province), Putian Black ducks (Putian, Fujian Province), Shan Sheldrakes (Zhangzhou, Fujian Province), Jinyun Sheldrakes (Lishui, Zhejiang Province), and Shaoxing ducks (Shaoxing, Zhejiang Province) were collected for analysis. Samples from GHPV-infected ducks were stratified based on the age of ducks (four groups, n = 5 per group): Group A (less than 4 weeks old), Group B (5–8 weeks old), Group C (9–12 weeks old), and Group D (older than 13 weeks old). Twenty liver samples were collected from each duck species and each age group (Groups A–D), for a total of 100 samples, which were stored at −80°C until use. The samples were collected from at least two different local commercial waterfowl farms randomly located within the regions studied (Fujian and Zhejiang Provinces of China) between January 2015 and September 2017. The sampled ducks showed no typical clinical manifestations, such as diarrhea, ascites, flabby heart, or gout.

DNA extraction

A total of 100 samples were used for nucleic acid extraction individually. Each sample was homogenized in phosphate-buffered saline and supplemented with streptomycin (100 μg/mL) and penicillin (100 U/mL). All of the suspensions were subjected to three freeze-thaw cycles and then centrifuged at 8,000 × g at 4°C for 30 min. The supernatants were collected and stored at −80°C until virus DNA isolation. Viral nucleic acids were extracted using the EasyPure Viral DNA/RNA Kit (TransGen Biotech, China), following the manufacturer's recommendations and were then used for virus detection and isolation.

Molecular analysis

The GHPV-specific diagnostic PCR primers (PolyVP1-F and PolyVP1-R) used were previously described by Guerin *et al*. [7]. The optimized PCR reactions were prepared at a final reaction volume of 50 μL, which consisted of 25 μL Premix Taq (Ex Taq version 2.0 plus dye; TaKaRa, China), 1 μL each of PolyVP1-F and PolyVP1-R (20 μM for each), 1 μL of the isolated template DNA, and 22 μL of nuclease-free water. The PCR reaction conditions were one cycle of 94°C for 5 min and 35 cycles of 94°C for 50 sec, 54°C for 35 sec, and 72°C for 45 sec, with one cycle of 72°C for 10 min for final extension. Then the PCR product was subjected to electrophoresis on 1.0% agarose gels to observe the results. Since GHPV-positive ducklings are almost always positive for duck circovirus (DuCV) as well, we also assessed the prevalence of DuCV infection in the GHPV-positive ducks by performing real-time quantitative PCR as previously described [15].

Virus isolation

The supernatant of each GHPV-positive sample was filtered first through 0.45 μm filters (Merck KGaA, Germany) then through 0.22 μm filters, and the final filtrate was used for virus isolation as previously described [7]. Each sample used five specific pathogen-free (SPF) chicken embryos (9-day) and SPF Shaoxing Sheldrake duck embryos (10-day) for virus isolation. Each inoculated embryo was incubated at 37°C for one week, with candling of the inoculated embryos every 12 h.

Genome sequencing

Each GHPV-positive sample was used for genome sequencing by PCR according to the strategy described by Fehér *et al*. [5] and confirmed by Jiang and Zhang [8], with overlapped fragments encompassing the whole GHPV genome. The target PCR product for each fragment was purified with Gel Extraction Kit (Omega Bio-Tek, USA), and then T-A cloned using the pMD18-T vector (TaKaRa). The vector was used to transform competent *Escherichia coli* strain DH5α (TaKaRa). After identification, positive transformants were submitted to a commercial company (Sangon, China) for nucleotide sequence determination. For each PCR product, three colonies were selected for Sanger sequencing in both directions performed using an ABI model 3730 automatic DNA sequencer (ABI, USA). The full-length GHPV genome for each sample was assembled using the Lasergene package (v7.1; DNASTAR, USA), and then submitted to GenBank (National Center for Biotechnology Information, USA).

Genomic analysis and phylogenetic analysis

Genomic comparative research was undertaken by using the ClustalW method for pairwise comparison. Previously reported GHPV genomes were retrieved from GenBank, and the information for each of the GHPVs is listed in Table 1. To

Table 1. Information of the goose hemorrhagic polyomaviruses strains used in this study

GenBank accession No.	Strain	Host	Length (bp)	Area	Reference
AY140894	Germany 2001	Goose	5256	Germany	[9]
HQ681902	Toulouse Goose 2008	Goose	5254	France	[3]
HQ681903	Toulouse Goose 2000	Goose	5252	France	[3]
HQ681904	Toulouse Muscovy Duck 2008	Muscovy duck	5254	France	[3]
HQ681905	Toulouse Mule Duck 2008	Mule duck	5253	France	[3]
JF304775	106	Pekin duck	5254	China	[8]
KJ452212	14234	Goose	5252	Hungary	[5]
MG190356	JY140Ma	Sheldrake	5254	China	This study
MG670535	JY141Ma	Sheldrake	5254	China	This study

determine the relationships among these viruses, genome and LTAg protein sequences were aligned in ClustalW, as implemented in MEGA6.0 [14]. Phylogenies were reconstructed in MEGA6.0 using the maximum likelihood and neighbor-joining methods with 1,000 bootstrap pseudo-replicates. Another four viruses within the *Polyomaviridae* family were used as the out-group, which included the Budgerigar fledgling disease virus-1 (Aves polyomavirus 1, GenBank accession No. AF241168), the respective species of the *Gammapolyomavirus* genus; Mus musculus polyomavirus 1 (GenBank accession No. AF442959), the respective species of the *Alphapolyomavirus* genus; Macaca mulatta polyomavirus 1 (GenBank accession No. J02400), the respective species of the *Betapolyomavirus* genus; and Human polyomavirus 6 (GenBank accession No. HM011560), the respective species of the *Deltapolyomavirus* genus.

Results

Prevalence of GHPV based on PCR

No positive result was obtained in the samples collected from Liancheng white ducks, Putian black ducks, Shan Sheldrakes, or Shaoxing ducks that were tested via PCR assay for GHPV epidemiological determination. Only two Jinyun Sheldrakes (designated as strain JY140 and JY141) tested positive with a GHPV-positive rate of 10% (2/20). Real-time quantitative PCR revealed no DuCV positive signals from these two Sheldrake genomic DNA samples. The supernatants JY140 and JY141 were then used for virus isolation. No virus was isolated from either the SPF chicken or duck embryos, and no deaths or pathological changes were observed after five passages. Each embryo was homogenized and confirmed to be GHPV-negative by applying the PCR method.

Genomic organization

After assembly with the Lasergene package, the Sheldrake origin GHPV (strains JY140 and JY141) genomes were obtained and submitted to the GenBank under accession Nos. MG190356 (strain JY140) and MG670535 (strain JY141). Genomic comparison revealed that these two virus genomes shared 100% sequence identities.

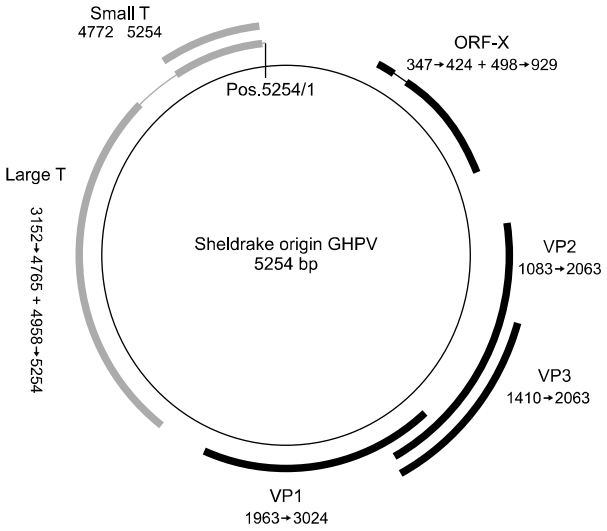

Fig. 1. Schematic organization of Sheldrake origin goose hemorrhagic polyomavirus (GHPV). The LTAg and STAg coding regions were complementary. Pos, positions; ORF, open reading frames.

The genome of Sheldrake origin GHPV (both JY140 and JY141 strains) were found to be 5254 nucleotides in length. The genome length of duck origin GHPV in China was 5254 bp, whereas the strains from France and Germany had a genome length range of 5252 to 5256 bp, respectively (Table 1). All of the GHPVs had the same coding regions (six ORFs) in length, including the ORF-X, VP2, VP3, VP1, LTAg, and STAg. The putative ORF-X protein encodes 169 amino acid (aa; positions 347–424 plus 498–929); VP2 encodes 326 aa (position 1083–2063); VP3 shares the same terminal as VP2 and encodes 217 aa (position 1410–2063); LTAg encodes 636 aa (positions 3152–4765 plus 4958–5254); and STAg encodes 160 aa (position 4772–5254). The locations of the protein coding regions are shown in Fig. 1.

Sequence comparison and phylogenetic analysis

The genome of duck origin GHPV displayed a conserved structure when compared with seven other GHPVs retrieved from GenBank, and they shared 99.8% to 99.9% genomic nucleotide sequence identities. Compared with the reference GHPV strain (GenBank accession No. AY140894), the Sheldrake origin GHPV had two sequence deletions at C (position 138) and T (position 280), and five variations with T/C (position 2076), T/G (position 2057), A/G (position 2807), T/A (position 5060), and G/A (position 5062). The homologies of LTAg among the GHPVs were higher than 99.7% (nucleotide level) and 99.8% (aa level).

Phylogenetic analysis based on the genome (panel A in Fig. 2) and the LTAg protein (panel B in Fig. 2) indicated that GHPVs share a very close evolutionary relationship with each other, similar to the results of the phylogenetic analysis based on the other five coding regions (ORF-X, VP2, VP3, VP1, and STAg; data not shown). The data pertaining to the GHPV phylogenetic relationship indicate similarities in GHPV evolution regardless of host (geese or ducks) or geographic region (Asia or Europe).

Discussion

GHPV, the causative agent for HNEG in geese, was identified as a novel polyomavirus 31 years after an initial report suggested grouping it into the proposed subgenus *Avipolyomavirus* after its full-length genome was sequenced [7,9]. Previous field investigation revealed that GHPV infected geese between the ages of 3 and 13 weeks. Ducklings were typically considered clinically refractory to GHPV inoculation. GHPV infection in

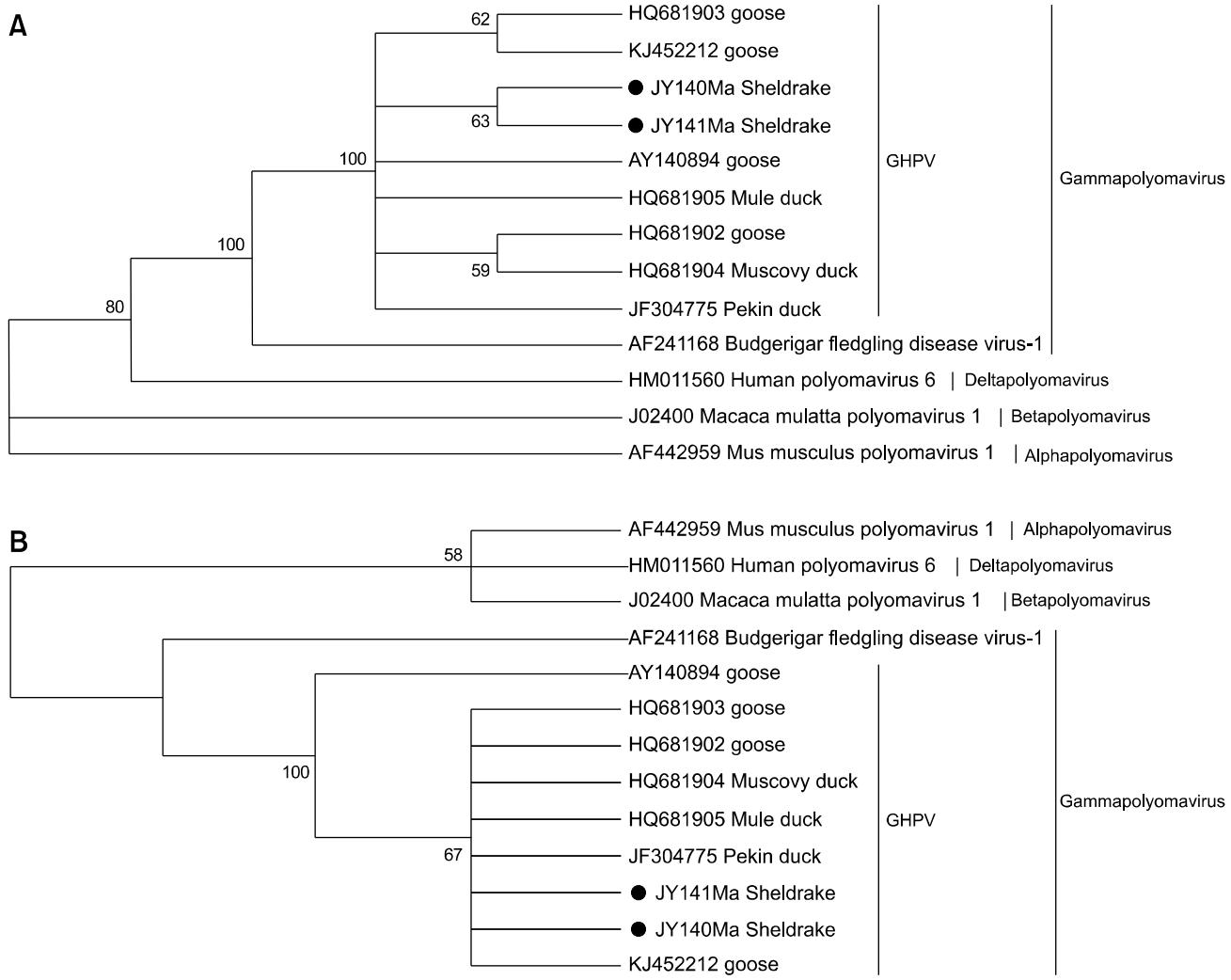

Fig. 2. Phylogenetic relationship of goose hemorrhagic polyomaviruses (GHPVs) in this study, based on the alignment of genome (A) and LTAg protein (B) sequences. The Sheldrake origin GHPVs (JY140Ma and JY141Ma) are indicated with black circles (●). Reference sequences obtained from GenBank are indicated by the accession number. The GHPV host is followed by the GenBank accession number.

ducks was first identified in France in 2008, with GHPV-positive ducklings (Muscovy ducks age, 8−12 weeks; Mule ducks age, 3−4 weeks) also being positive for other viruses, especially DuCV, and were characterized by increased mortality, growth delays, and feathering problems [13]. Curiously, the age of GHPV-infected ducks in China was significantly different: Pekin ducks (age, 50 weeks) exhibit arthritic symptoms [8], whereas, in this study, Sheldrakes (27 weeks for strain JY140 and 34 weeks for strain JY141) had no clinically distinguishing pathogenic characteristics. This suggests that GHPV might be persistently infected in domestic ducks in China, even though no goose GHPV-positivity has been reported in China.

The pathological significance of GHPV infections in ducks should be investigated; however, GHPV isolation methods have varied in previous reports. Guerin *et al*. [7] successfully isolated GHPV, but isolation was unsuccessful in the studies reported by Johne and Müller [9] and Bernáth *et al*. [1]. In addition, Bernáth *et al*. [1] showed that goose embryos experimentally infected with GHPV (with a chorioallantoic membrane infection route) could hatch and shed the virus, followed by a weaker general physical status among the infected geese. That study also indicated the possibility that GHPV might be introduced into uninfected goose flocks via sick birds infected during their embryonic cycle. Jiang and Zhang [8] used goose kidney cell primary cultures, and goose liver cell primary cultures, BHK cells, and Vero cells to isolate the virus, and the results showing negative GHPV infections were obtained from PCR analysis. In this study, we used SPF chicken and duck embryos with a chorioallantoic membrane as described by Bernáth *et al*. [1]; no embryo deaths were observed and GHPV-negativity was observed using PCR. Although ducks are considered potential reservoirs of GHPV, no typical signs were observed. Corrand *et al*. [3] found that duck origin GHPV could produce typical HNEG in 1-day-old goslings, suggesting that duck origin GHPVs are virulent to goslings. Here, we must point out that duck origin GHPV is a real danger to the gosling feed industry in China. While no previous study has verified the presence of GHPV in geese in China, the threat of GHPV infection in domestic goose farms should not be ignored.

In summary, these results indicate a conserved genomic structure and close evolutionary relationship among the GHPVs circulating in Europe and China in both ducks and geese. Ducks are considered potential reservoirs of GHPV, wherein the virus can be replicated with no significant pathological signs. Future research should focus on identifying the pathogenic mechanisms of GHPV infection.

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Conflict of Interest

The authors declare no conflicts of interest.

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