Articles

Detection and genetic characterization of Densvirus from Oriental Pied hornbill in Guangxi, China

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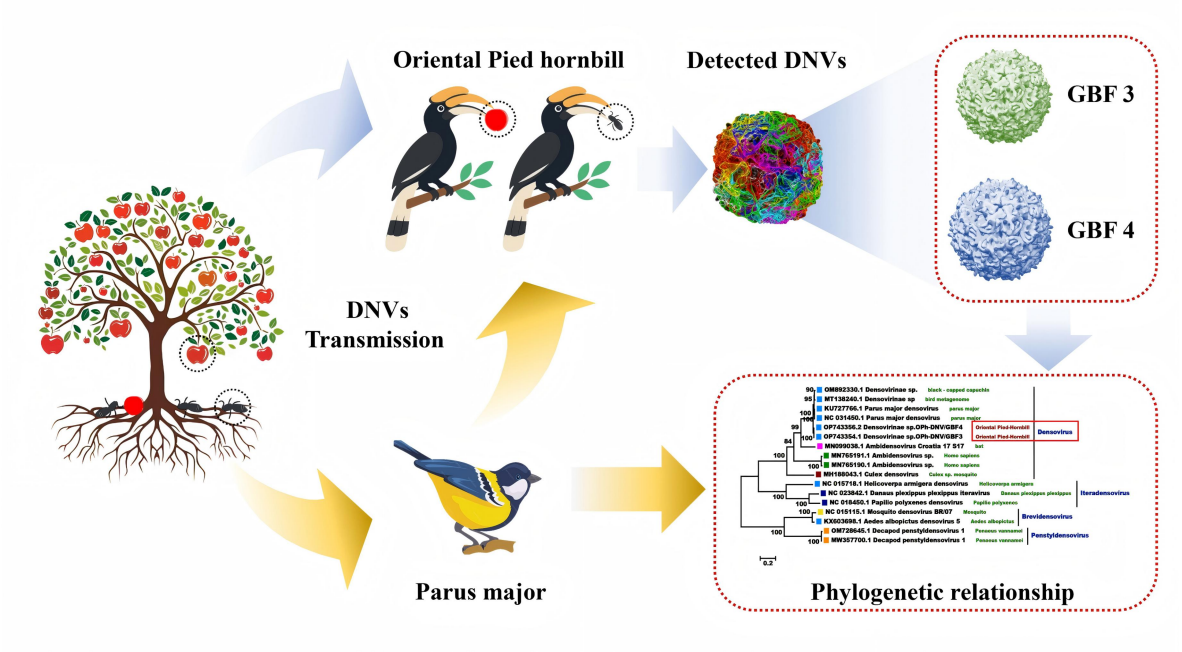
Densoviruses (DNVs), members of the family Parvoviridae, infect a broad range of invertebrate and vertebrate hosts, yet their presence in birds remains poorly understood. Densoviruses have been increasingly recognized for their ecological roles in controlling insect populations and potentially affecting vertebrate hosts through trophic interactions. In this study, we investigated DNVs in the Oriental Pied hornbill, a widespread species in Southeast Asia and Southern China. Thirteen fecal samples were collected and analyzed using viral metagenomics and molecular techniques. PCR screening detected DNVs in two samples, and complete genomes of two strains (GBF3 and GBF4) were successfully obtained. Phylogenetic analysis of conserved NS1 and VP1 regions showed that both strains belong to the genus Densovirus (subfamily Densovirinae) and cluster closely with PmDNV-JL previously identified in Parus major. These findings represent the first genomic evidence of DNVs in Oriental Pied hornbills, emphasizing the potential for cross-species transmission and the importance of birds as ecological reservoirs of arthropod-associated viruses. Our study also provides insights into viral diversity, host adaptation, and the evolutionary dynamics of DNVs in natural ecosystems. Future research should explore the replication capacity and pathogenic potential of DNVs in avian hosts to assess their epidemiological significance.



Introduction

Densoviruses (DNVs), also known as densonucleosis viruses, are 18–22 nm non-enveloped icosahedral viruses in the family Parvoviridae. DNVs replicate in the nuclei of invertebrate hosts, forming large cuboidal or circular inclusions[1]. Although originally considered specific to invertebrates, recent studies have revealed their broader ecological roles and potential interactions with vertebrates through indirect pathways such as predation.

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DNVs infect humans and a broad range of animals, from mammals to crustaceans, and are generally associated with a variety of acute and chronic diseases. Most DNVs cause serious diseases in their hosts and have been considered for the biocontrol of significant insect pests owing to their high virulence and ease of transmission[2,3]. Recent molecular investigations have shown that DNVs can persist in insect populations without causing immediate mortality, suggesting complex dynamics between virus and host that may influence ecological networks.

DNVs have been identified in both aquatic and terrestrial ecosystems. They are distributed among arthropods ranging from shrimp to mosquitoes, occupying diverse ecological niches across wide geographic regions. Their ubiquity suggests a potentially strong, yet insufficiently explored, influence on host populations. Until 2013, a total of 33 DNV genomes had been sequenced, exhibiting broad variation in genome structure and organization and classified into six genera: Densovirus, Brevidensvirus, Iteradensvirus, Miniambidensovirus, Penstyldensovirus, and Hepandensovirus, according to the International Committee on Taxonomy of Viruses (ICTV)[4,5]. This genetic

diversity highlights the need for comprehensive surveillance in non-traditional hosts, including birds, to better understand viral evolution and cross-species transmission.

DNVs primarily infect insects. DNVs are mainly found in the fat body of insects and could be transmitted both horizontally and vertically. Previous research has shown that specific orders of insects (Diptera, Lepidoptera, Dictyoptera, Orthoptera, Odon-ata, and Hemiptera), decapod crustaceans can harbor DNVs[6-10].Their predators, particularly insectivorous birds and bats, may act as mechanical carriers, creating a pathway for viruses to enter vertebrate food webs.

Recent research continues to expand the known diversity and ecology of DNVs, including the identification of new strains in Spodoptera frugiperda[11], the application of AgDNV in mosquito vector studies[12], and reports of DNV like sequences in insectivorous bat feces[13]. These findings emphasize the potential for indirect transmission to vertebrates, reinforcing the importance of monitoring wild birds that regularly prey on insects. Despite the extensive knowledge on DNVs in insects, information regarding their presence, diversity, or genetic characteristics in birds remains extremely limited, and reports of DNV detection in avian hosts are very rare, highlighting a significant gap in current research. This gap is particularly important because wild birds regularly prey on insects and could potentially act as passive carriers or incidental hosts.

Wild birds are important hosts to study infectious diseases in public health because they carry emerging zoonotic pathogens, either as a reservoir host or by dispersing infected arthropod vectors[14]. For example, birds are central to the epidemiology of West Nile virus and can spread pathogens across large geographic areas through migration, underlining the ecological relevance of understanding virus-host interactions in avian species.The Oriental Pied hornbills (Anthracoceros albirostris) belong to the genus Anthracoceros of the family Bucerotidae [15]. They are considered to be among the smallest and most common of the Asian hornbills, with the most distribution in the genus, and are found in the Indian Subcontinent and Southeast Asia, including Southern China. These birds frequently encounter diverse insect populations, potentially exposing them to a variety of insect-associated viruses and positioning them as important ecological indicators for viral circulation. The primary objectives of this study were to investigate the viral metagenome of fecal samples from Oriental Pied hornbills in Guangxi Province, China, and to analyze the genetic characteristics of the DNVs detected. By doing so, we aim to provide novel insights into the diversity, evolution, and host interactions of DNVs in avian species.

Materials and Methods

Sample collection

Thirteen fecal samples were collected from Oriental Pied hornbills at the Terrestrial Wildlife Rescue Research and Epidemic Disease Monitoring Center of Guangxi Zhuang Autonomous Region, China. The samples were immediately placed in cryopreservation tubes and stored at −80 °C in EP tubes containing virus preservation medium. The collected samples were then divided into two pools. Care was taken to preserve the total bird population by returning the birds to their natural habitat promptly after sample collection. This study was approved by the Experimental Animal Ethics Committee of Guangxi Medical University (20210183).

Viral genome isolation and sequencing

To analyze the genetic characteristics of the virus, total viral RNA and DNA were extracted from the 13 bird samples using the Axygen AxyPrep Stool RNA column extraction kit (Beijing Baiolaibo) and the E.Z.N.A Mag-Bind Soil DNA Kit (OMEGA, M4015–00), respectively. RNA reverse transcription was performed using the Thermo Scientific RevertAid First-strand cDNA Synthesis Kit (Thermo Scientific, K1622).

cDNA libraries were prepared according to the QIAGEN QIAseq FX DNA Library Kit protocol (QIAGEN, 180475), and DNA libraries were prepared following the Hieff NGS MaxUp II DNA Library Prep Kit protocol (YEASEN, 12200ES08). All libraries were subsequently amplified, quantified, and sequenced using an Illumina Novaseq 6000 platform.

Virus sequence gap filling by PCR

The sequence reads were refined with CUTadapt[16], and compared with the viral nr database using Burrows-Wheeler Aligner (BWA)[17]. Format conversion and coverage calculations were performed using SAMtools. To verify the samples that contain DNV, a pair of polymerase chain reaction (PCR) amplification primers was designed based on the PmDNV-JL sequence from National Center for Biotechnology Information (NCBI) Reference Sequence NC_031450.1 (Table 1) and were synthesized by Sangon Biotech Co., Ltd. The PCR conditions of the target gene amplification step were 94°C for 3 minutes; 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute for 35 cycles; and 72°C for 5 minutes, then held at 4oC. After completing the PCR steps above, the PCR products were then detected by electrophoresis, 1.0% agarose gel, 120 V, and electrophoresed for 30 min. The amplified DNA product was sent to Guangzhou Qingke Biotechnology Co., Ltd. for Sanger sequencing to fill the gap. Sanger sequencing was performed using an ABI 3730 genetic analyzer (Sangon Biotech (Shanghai) Co., Ltd.).

Table 1. Primers used for detection and sequence amplification of GBF3&GBF4-DNVs

Primer	sequence (5′–3 ′)	Position[a]	Length of fragment (bp)
Forward	TTCGAAGTAG	2658-267	679
	CCTTGTGCGT	7	
Reverse	ACCTACGGGT	3336-331	
	TTCGCAACAT	7	

Phylogenetic analysis

The full-length genome nucleotide sequences of GBF3 and GBF4 were deposited in GenBank under accession numbers (OP743354 and OP743356). The nucleotide sequences were compared to other virus sequences from GenBank using the Basic Local Alignment Search Tool (BLASTN). Seventeen different DNVs sequences were downloaded from GenBank and mapped to our sequence. Their VP1 and NS1 amino acid sequences were also obtained. Sequence similarity analysis of nucleotide and amino acid sequences was conducted by BLASTn. The phylogenetic trees were constructed by the Maximum Likelihood method (bootstrap replications:1000) with the

Jones-Taylor-Thornton model of the program MEGA software 7.0 [18]. Open reading frames (ORFs) were determined using a translated BLAST search[19].

Results

Two samples were confirmed to contain DNVs (GBF3 and GBF4) by PCR amplification with specific primers (Figure 1). After electrophoresis analysis, the amplicons have a size of 679 bp on the gel, corresponding to the product size from the primer design, showing that the DNA sequences of GBF3 and GBF4 were successfully amplified.

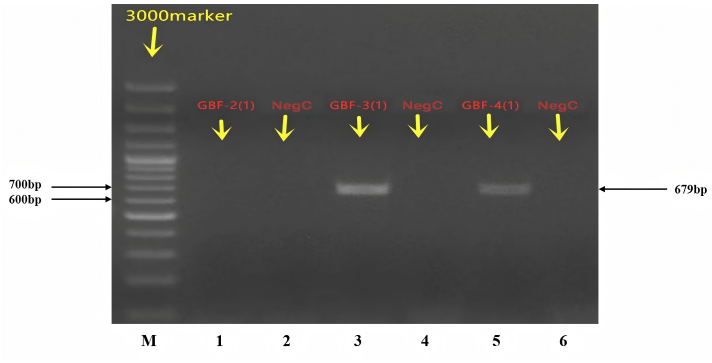

Figure 1. Electrophoretic analysis of PCR amplification products of viral nucleic acid isolated from stool samples.

The whole genome sequences of two DNVs from the H18 group were obtained, named GBF3 and GBF4, both have the length of 5164 bp.

After obtaining the complete genome sequence of the two samples, GBF3 and GBF4, the consensus sequences in this study were deposited in GenBank under accession numbers GBF3 (ID: OP743354) and GBF4 (ID: OP743356). After performing BLAST comparison of the NCBI nucleic acid library[19], the nucleotide sequences of GBF3 and GBF4 showed 74.65%–99.42% and 74.61%–99.19% similarity, respectively, to the first 34 DNV sequences. Both GBF3 and GBF4 belong to the genus DNVs, subfamily Densovirinae, family Parvoviridae. Then, 8 complete sequences of DNVs from different regions and different species in NCBI's Nucleotide data, and 7 sequences of the genus Brevivirus, Iteravirus, and Penstyldensovirus, together with 2 sequences GBF3, GBF4 of the Oriental Pied-hornbill obtained from the laboratory were integrated into a FASTA file (complete genome). Those sequences were compared with each other, and also a phylogenetic tree was constructed (shown in Figure 2). Information on the 17 strains is mentioned in Table 2.The complete genome phylogenetic trees were constructed with 17 DNV strain's complete genome sequences that share the most similarities to the nucleotide sequence of GBF3 and GBF4 (Figure 2). The result showed that the sequence of GBF3 and GBF4 was cluttered, and they were closest to the sequence of PmDNV-JL (KU727766.1) with 97.54% similarity.

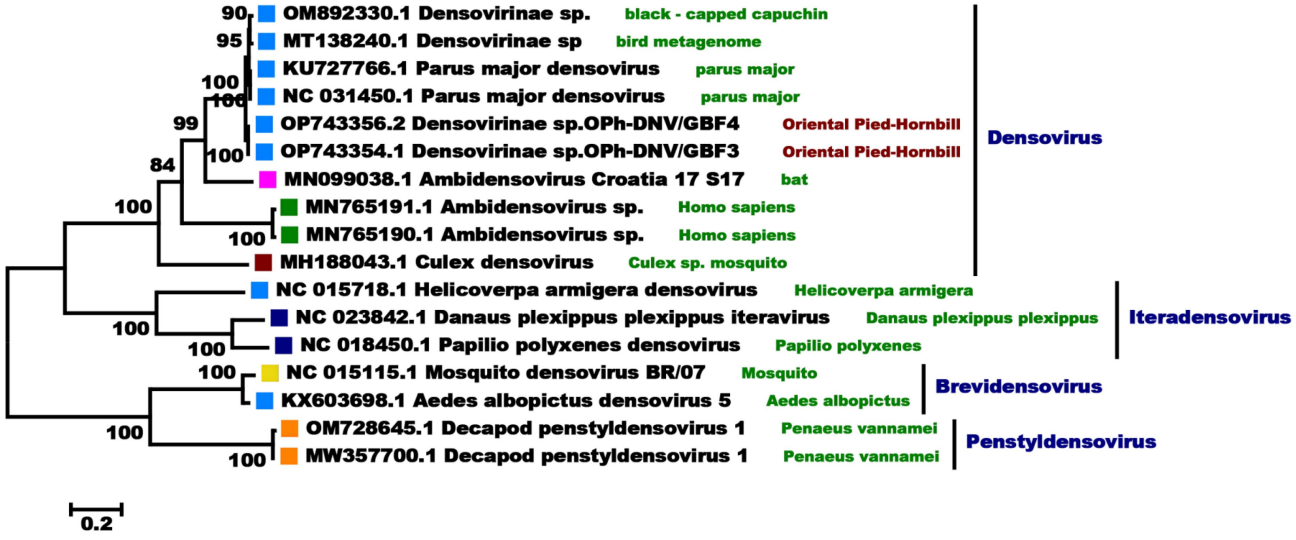

Figure 2. Maximum-likelihood phylogenetic trees of the full nucleotide sequence of Densoviruses. Bootstrap proportions are shown for each node. Two viruses detect from this study are marked with red squares.

Table 2. List of information of Densoviruses that share highest similar to GBF3 and GBF4 nucleotide sequence according to NCBI.

NC	Virus Name	ID	Host	Location	Family	Subfamily	Genus
1	Mosquito densovirus BR/07	NC_015115.1	Mosquito	Brazil	Parvoviridae	Densovirinae	Brevidensovirus
2	Danaus plexippus plexippus iteravirus	NC_023842.1	Danaus plexippus plexippus	Canada	Parvoviridae	Densovirinae	Iteradensovirus
3	Papilio polyxenes densovirus/IAF	NC_018450.1	Papilio polyxenes;	Canada	Parvoviridae	Densovirinae	Iteradensovirus
4	Helicoverpa armigera densovirus	NC_015718.1	Helicoverpa armigera	China	Parvoviridae	Densovirinae	Iteradensovirus
5	Aedes albopictus densovirus 5/GZ05	KX603698.1	Aedes albopictus	China	Parvoviridae	Densovirinae	Brevidensovirus
6	Decapod	OM728645.	Penaeus vannamei	Peru	Parvovirida	Densovirina	Penstyldensoviru

NC	Virus Name	ID	Host	Location	Family	Subfamily	Genus
	penstyldensovirus 1	1			e	e	s
7	Decapod penstyldensovirus 1	MW357700	Penaeus vannamei; postlarvae	Peru	Parvoviridae	Densovirinae	Penstyldensovirus
8	Densovirinae sp GBF-3	OP743354	Oriental Pied-Hornbill	China	Parvoviridae	Densovirinae	unclassified Densovirinae
9	Densovirinae sp GBF-4	OP743356	Oriental Pied-Hornbill	China	Parvoviridae	Densovirinae	unclassified Densovirinae
10	Densovirinae sp	MT138240	bird metagenome	China	Parvoviridae	Densovirinae	unclassified Densovirinae
11	Parus major densovirus	KU727766	Parus major	China	Parvoviridae	Densovirinae	Densovirus
12	Culex densovirus	MH188043	Culex sp. mosquito	USA	Parvoviridae	Densovirinae	Densovirus
13	Ambidensovirus Croatia 17_S17	MN099038	bat	Croatia	Parvoviridae	Densovirinae	Densovirus
14	Ambidensovirus sp	MN765191	Homo sapiens	Tanzania	Parvoviridae	Densovirinae	Densovirus
15	Ambidensovirus sp	MN765190	Homo sapiens	Tanzania	Parvoviridae	Densovirinae	Densovirus
16	Densovirinae sp 155Un-Den	OM892330	black-capped capuchin	China	Parvoviridae	Densovirinae	Densovirus
17	Parus major densovirus PmDNV-JL	NC031450	Parus major	China	Parvoviridae	Densovirinae	Densovirus

The BLAST tools were used to evaluate the open reading frames (ORFs). These two strains' genomes contained five ORFs with positions analogous to those of the PmDNV-JL, disseminated by the Parus major bird[8]. Further analysis showed that ORF3, ORF4, and ORF5 encode NS proteins on one DNA strand, and ORF1 and ORF2 encode VP proteins on the other complementary strand (Table 3).

Table 3. Organization of VP and NS coding sequences

ORF	Proteins	Position (nt)	Numbers of amino acid (aa)
ORF3	NS1	871–2472	533
ORF4	NS2	878–1675	265
ORF5	NS3	325–837	170
ORF1	VP1	2473–4254	593
ORF2	VP2	4194–5105	303

The phylogenetic trees were constructed with other DNV strains amino acid sequences to analyze the amino acid sequences of NS1 (non-structural protein 1) and VP1 (viral protein 1). The result also indicated that the NS1 sequences of GBF3 (WCH76306.1) and GBF4 (WCH76311.2) are clustered and they were closest to those sequences of PmDNV-JL (YP_009310053.1) with about 99.44% similartity. (Figure 3).

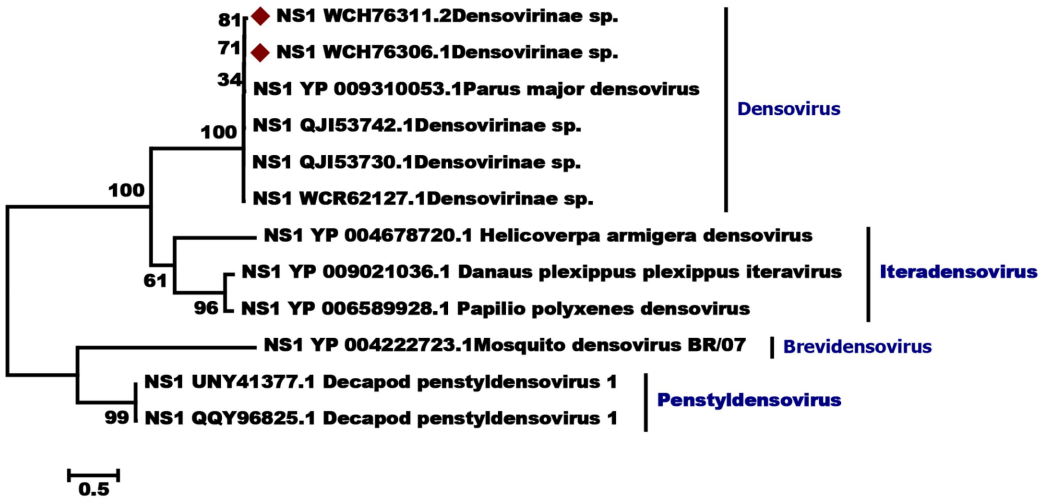

Figure 3. Maximum likelihood phylogenetic tree of the entire NS1 amino acid sequence of Densovirus.

The VP1 sequences of GBF3 (WCH76304.1) and GBF4 (ID: WCH76309.1) are clustered, and they are closest to those sequences of Densovirinae.sp par081par3 (ID: QJI53744.1) with 97.81% similarity and with Ambidensovirus Croatia 17_S17(ID: QHY93494.1) with 64.31% similarity. VP1 sequences of GBF3 and GBF4 were evolved as an independent branch (Figure 4).

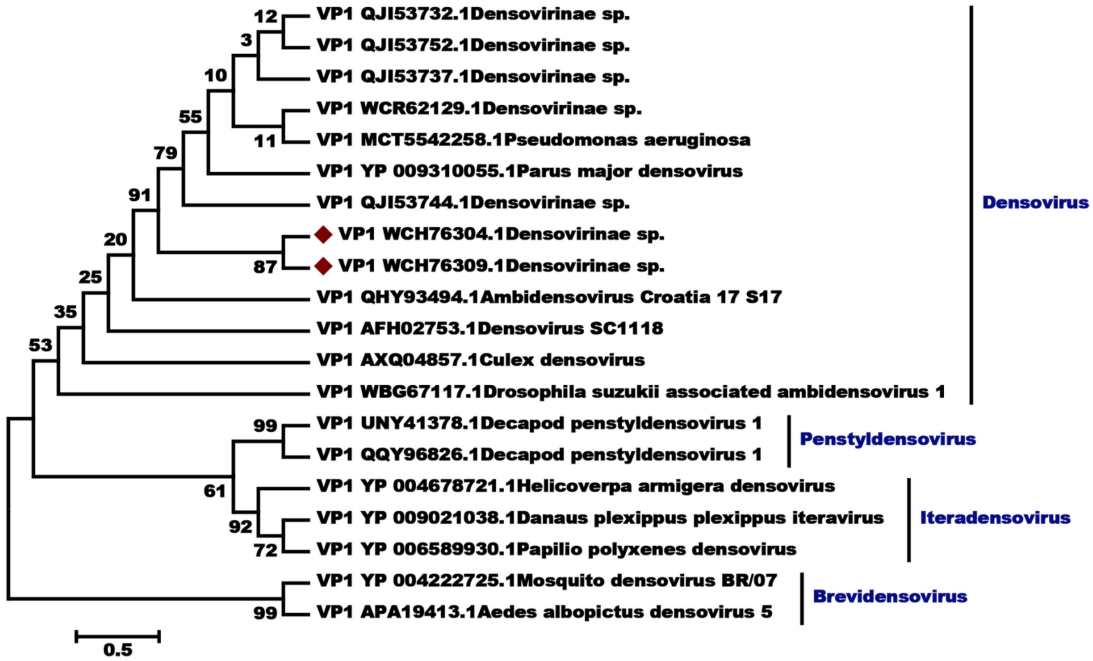

Figure 4. Maximum likelihood phylogenetic tree of the entire VP1 amino acid sequence of Densovirus.

Discussion

The first described DNV was discovered as a pathogen of wax moth (Galleria mellonella) caterpillars in 1964, although infections previously described in mosquito larvae from California and Louisiana were likely caused by DNVs and not cytoplasmic polyhedrosis viruses as attributed at the time[20,21]. DNVs have since been identified in numerous invertebrate species, including crustaceans and members of at least five insect orders[22,23]. Despite extensive knowledge of DNVs in insects, information regarding their presence, diversity, or genetic characteristics in birds remains extremely scarce, representing a significant research gap[8,24]. From 13 fecal samples of Oriental Pied hornbills (Anthracoceros albirostris) belonging to two groups, 18H and 19H, two samples from 18H tested positive for DNVs (GBF3 and GBF4) using PCR amplification with specific primers. Complete genome sequences were obtained (OP74335 4 and OP743356), each 5164 bp in length, designated as GBF3 and GBF4 strains. Phylogenetic analysis showed that GBF3 and GBF4 cluster within the same major clade as the previously reported PmDNV-JL detected in Parus major[8], yet they form an independent branch, indicating potential local evolution or host-specific adaptation. Combining the species information in (Table 4) , the species infected or carrying Densovirus in this group are mainly birds, Bat, black-capped capuchin, Homo sapiens, and Culex sp. Mosquito. While the Brevidensovirus genus group are mainly Mosquito, Aedes albopictus, the Iteradensovirus genus group is mainly Danaus plexippus plexippus, Papilio polyxenes, and Helicoverpa armigera.and the Penstyldensovirus genus group are mainly Penaeus vannamei, Penaeus vannamei postlarvae. GBF3 and GBF4 has a high degree of homology with Parus major densovirus PmDNV-JL sequence (ID: KU727766.1). The results of the whole sequence phylogenetic analysis indicated that the Oriental Pied-hornbills, the hosts of GBF3 and GBF4 strains, may have come from the above-mentioned different areas, and may have been infected with DNVs from the natural foci of the above-mentioned areas, and there may be a certain species relationship.

Phylogenetic trees were constructed using amino acid sequences of NS1 and VP1. The NS1 sequences of GBF3 (WCH76306.1) and GBF4 (WCH76311.2) clustered closely with PmDNV-JL (YP_009310053.1) at 99.62% similarity. NS1 protein is the largest of the non coding proteins. The traditionally designated NS1 is a multi-domain protein that contains a highly conserved domain which are essential for viral replication. The stable status of the NS1 sequence is the main reason that the phylogenetic analysis of NS1 revealed a close relationship between densovirus family members.

VP1 sequences of GBF3 (WCH76304.1) and GBF4 (ID: WCH76309.1) are clustered, and they are closest to those sequences of Densovirinae.sp par081par3 (ID: QJI53744.1) with 97.81% similarity. with Ambidensovirus Croatia 17_S17(ID: QHY93494.1) with 64.31% similarity. According to NCBI, the Densovirinae.sp par081par3 strains were isolated from the wild and zoo birds in Jiangsu, China, and Ambidensovirus Croatia 17_S17 strains were isolated from bats in Croatia. Those hosts shared some common features with the Oriental Pied-hornbill in terms of flight ability and food source. Wild-bird virome studies have revealed the presence of diverse parvovirus-like sequences in cloacal swabs of both insectivorous and frugivorous species (25)(26) Furthermore, recent metagenomic investigations have identified highly divergent parvovirus related genomes in wild birds and in the intestinal viromes of insectivorous bats (26)(27). Together, these findings support the ecological likelihood that flying, insect and fruit eating birds such as hornbills may function as mechanical carriers or transient hosts for insect derived viruses, further reinforcing their potential role in the transmission and dissemination of these viruses across ecological systems.

Birds are one of the essential hosts for viruses pathogens. Some

viruses replicate in the digestive tract of wild birds and are then excreted in high titers in the feces. The presence of DNVs, arthropod viruses, in the bird feces implies that Oriental Pied hornbills may serve both as predators and as disseminators of viruses among insects. Moreover, various researchers have shown that DNV-infected insects can be transmitted to the host. In 2016, Yang et al. isolated DNVs from the lung tissue of Parus major (PmDNV-JL) in the Jilin province of China. The Parus major birds mainly depend on insects for their food (8). A study by Ge et al. in 2012 also spotted DNVs in the insectivorous bat's excreta in China (24). In addition, a recent study of Tian et al. 2022 indicated that asymptomatic pangolins can harbor a range of DNVs through mixed infections (28). Further studies are needed to determine whether DNVs can replicate in bird hosts or are only transmitted mechanically. In 2016, a DNV genome was found in a human cerebrospinal fluid sample from an unexplained episode of encephalitis, which was verified by metagenomics and PCR (29). In recent studies, DNVs were also confirmed in the human plasma of Brazillian adults in 2020 (30) and Tanzanian children in 2021 suffering from fever (31). However, the replication and pathogenicity of DNVs in vertebrates remain unclear, highlighting the need for experimental studies.

In our fecal samples, only DNVs were detected in the hornbill specimens. However, viral co-infections are common in natural ecosystems; for example, co-infection of DNV and picornavirus has been reported in Spodoptera littoralis larvae (32). Therefore, future studies are needed to evaluate the potential effects of DNVs when occurring alongside other viruses and to determine how such interactions may influence viral replication, host susceptibility, and transmission dynamics.

Conclusion

This study provides the first evidence of Densoviruses (DNVs) in Oriental Pied hornbills from Guangxi, China, with two complete genomes (GBF3 and GBF4) showing 97 – 99% similarity to PmDNV-JL from Parus major. The findings highlight the potential role of hornbills as ecological reservoirs or mechanical carriers of insect-associated viruses, likely linked to their diverse diet. While the capacity of DNVs to replicate in avian hosts remains unclear, this work establishes a foundation for future investigations into viral replication, coinfections, cross-species transmission, and the ecological and epidemiological significance of DNVs in wild birds. Overall, our results expand current knowledge of DNV diversity, host range, and the role of birds in shaping viral ecology.

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Nucleotide sequence accession number:

The complete genome sequence of GBF3 and GBF4 with annotation was deposited in the NCBI nucleotide database under the accession numbers GBF3 (OP743354) and GBF4 (OP743356).

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Not applicable.

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Conflicts of Interest:

The authors declare no conflict of interest.