Research article

# Viral metagenomic analysis reveals diverse viruses and a novel bocaparvovirus in the enteric virome of snow leopard (*Panthera uncia*)

Kingsley Ikechukwu Chukwudozie [a,b,1], Haoning Wang [c,d,1], Xiaolong Wang [e], Chunying Lu [a], Jiaxin Xue [a], Wen Zhang [a,*], Tongling Shan [f,**]

[a] *Department of Laboratory Medicine, School of Medicine, Jiangsu University, Zhenjiang. Zip code: 212300, PR China*
[b] *Department of Microbiology, University of Nigeria, Zip code: 410001, PR China*
[c] *Heilongjiang cold Region Wetland Ecology and Environment Research key laboratory, school of geography and tourism, Harbin university, 109 zhongxing Road, Harbin, 150086, Heilongjiang province, PR China*
[d] *School of Geography and Tourism, Harbin University, Harbin 150086, Heilongjiang province, PR China*
[e] *The Key Laboratory of Wildlife Diseases and Biosecurity Management of Heilongjiang Province. Zip code: 154100, PR China*
[f] *Department of Swine Infectious Disease, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, PR China*

## ARTICLE INFO

## ABSTRACT

The enteric virome, comprising a complex community of viruses inhabiting the gastrointestinal tract, plays a significant role in health and disease dynamics. In this study, the fecal sample of a wild snow leopard was subjected to viral metagenomic analysis using a double barcode Illumina MiSeq platform. The resulting reads were *de novo* assembled into contigs with SOAPdenovo2 version r240. Additional bioinformatic analysis of the assembled genome and genome annotation was done using the Geneious prime software (version 2022.0.2). Following viral metagenomic analysis and bioinformatic analysis, a total of 7 viral families and a novel specie of bocaparvovirus tentatively named *Panthera uncia* bocaparvovirus (PuBOV) with GenBank accession number OQ627713 were identified. The complete genome of PuBOV was predicted to contain 3 open reading frames (ORFs), contains 5433 nucleotides and has a G + C content of 47.40 %. BLASTx analysis and pairwise sequence comparison indicated the novel virus genome was a new species in the genus *Bocaparvovirus* based on the species demarcation criteria of the International Committee on the Taxonomy of Viruses. This study provides valuable insights into the diversity and composition of the enteric virome in wild endangered snow leopards. The identification and characterization of viruses in wildlife is crucial for developing effective strategies to manage and mitigate potential zoonotic and other viral disease threats to human and animal health.

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* Corresponding author.
** Corresponding author.
*E-mail addresses:* zhangwen@ujs.edu.cn (W. Zhang), shantongling@shvri.ac.cn (T. Shan).
[1] Shared first author.

## 1. Introduction

The gut virome of wild animals are considered reservoirs for emerging and reemerging viruses [1], and the surveillance of viral pathogens in wildlife is critical for the prevention and control of emerging and reemerging viral infectious diseases in humans and animals [2,3]. Due to this, there has been a growing effort to employ metagenomic approaches to document the broad spectrum of viral diversity of wildlife species [2]. One the commonly encountered viruses in the enteric virome of wild animals is Parvoviruses.

Parvoviruses belonging to the *Parvoviridae* family are small non-enveloped viruses with icosahedral symmetry, and carrying single-strand negative DNA of 4–6 kilobases (kb) as genetic material [4]. At present, the *Parvoviridae* family is divided into three subfamilies: *Densovirinae* and *Parvovirinae*, which infect arthropods and vertebrates respectively, and the newly discovered subfamily *Hamaparvovirinae*, which can cause infections in both [5,6]. The *Parvovirinae* subfamily is subdivided into ten genera: *Tetraparvovirus, Protoparvovirus, Amdoparvovirus, Bocaparvovirus, Artiparvovirus, Erythroparvovirus, Aveparvovirus, Dependoparvovirus, Loriparvovirus, and Copiparvovirus* [6]. Bocaparvovirus (BOVs) and other parvoviruses share a number of distinguishing characteristics, but unlike most parvoviruses, BOVs has three open reading frames (ORFs) in their genome [7]. Bocaparvoviruses are important diseases causing agents that have a broad host range [8]. The fecal-oral route is a common way for bocaparvoviruses to infect animals. Young animals and humans can develop respiratory and gastrointestinal symptoms from bocaparvoviruses [9,10], although adults are frequently asymptomatic [5]. The virus can spread horizontally to other people when it is discharged from the respiratory or digestive system of an infected host. So far, bocaparvoviruses have been identified from a variety of animal hosts; cats [8,11], pigs [12], California sea lions [13], gorillas [14], dogs [15] bats [16] and rats [17] which suggested that they have a very broad host range. BOVs are known to undergo a high incidence of genetic recombination [18,19], and can be disseminated across species to new hosts [20]. Feline bocaparvovirus was initially discovered in samples collected from stray cats in Hong Kong [21]. Since then, utilizing high-throughput sequencing technologies, they have been found in cats in various nations.

The snow leopard (*Panthera uncia*) is commonly found in the snow mountain ranges of South and central Asia, and has a distribution range that covers 1.2–1.6 million $km^2$, spanning over 12 countries, and has long been one of the least studied, and hence poorly understood, of the large cats [22]. The International Union for the Conservation of Nature's (IUCN) classified it as vulnerable in their red list of threatened species. The majority of infectious illnesses that are known to infect domestic cats are probably contagious in snow leopards [23]. The most frequent disease transmission methods in snow leopards are probably direct channels, which involve both intraspecies contact (mating and socializing) and interspecies interaction with wild and domestic prey, other carnivores, and scavengers [24]. Disease transmission may also occur through indirect channels such drinking water, animal carcasses, and human activity [25].

There is a dearth of published information on infectious diseases affecting free-ranging snow leopards, owing to their remote and inaccessible habitat, in addition to the species' elusive nature. Due to this, there is very limited information regarding the prevalence and thus potential threat of infectious diseases to which wild snow leopards are susceptible to, as well as the microorganisms that are most frequently identified in the species. This study is therefore aimed at evaluating the fecal virome of a wild snow leopard using metagenomic and bioinformatic tools to determine the viral composition, with a view to identify potential viral pathogens that can affect this felid.

## 2. Materials and methods

### 2.1. Sample collection and viral nucleic acid extraction

The fecal sample of the snow leopard was collected at the mountain range of Heilongjiang province in NorthEast China by wildlife experts using sterile disposable containers and transported to the laboratory on dry ice. About one gram of the fecal sample was re-suspended in 2 mL of phosphate-buffered saline (PBS), vigorously vortexed for 5 min, and then centrifuged for 10 min at 15000 g. To eliminate bacterial and eukaryotic cell-sized particles, the supernatant from centrifugation was collected and filtered through a 0.45-m filter (Merck Millipore, MA, USA). The viral-enriched filtrate was collected and treated with nuclease enzymes (Qiagen) at 37 °C for 60 min to digest unprotected nucleic acids. Viral nucleic acids were extracted by using the QIAamp MinElute Virus Spin Kit (Qiagen) according to the manufacturer's protocol.

### 2.2. Library construction and bioinformatics analysis

cDNA of viral RNA was synthesized by reverse transcription, then Klenow Fragment DNA polymerase (New England Biolabs, USA) was used to generate the complementary chain of cDNA. A Nextera XT DNA Sample Preparation Kit (Illumina) was then used to create a 250-bp paired-end cDNA library, and the sample was sequenced using a double barcode Illumina MiSeq platform. The generated paired-end reads were debarcoded using vendor software from Illumina, and the adaptors removed using VecScreen's default settings. The Phred quality score of 10 was used as the threshold to remove tails of low sequencing quality. Bacterial reads were removed by mapping to the bacterial nucleotide sequences from the BLAST NT database using Bowtie2 v2.2.4. The cleaned reads were *de novo* assembled into contigs with SOAPdenovo2 version r240 using Kmer size of 63 [26]. The assembled contigs were matched to an in-house viral proteome database using BLASTx with an E-value cutoff of $<10^{-5}$ [27]. To eliminate false-positive viral reads, the putative viral reads were then aligned to a custom-made non-virus non-redundant (NVNR) protein database [28]. For obtaining the full genome sequence, each viral contig was used as a reference for mapping to the raw data using the Low Sensitivity/Fastest setting in Geneious prime v11.1.2. Prediction of the open reading frames (ORF) was done using the ORF prediction function in Geneious prime

version 2022.0.2 with default parameters [29].

## 2.3. Phylogenetic analysis

To evaluate the evolutionary relationship between PuBOV and other bocaparvoviruses, the reference genomes of 20 bocaparvo-virus species were extracted from the GenBank database, with reference to the closest viral relatives determined by the best BLASTx hit and representative members of related viral species or genera.From these genome sequences, the nonstructural protein 1 (NS1) and viral capsid protein (VP1) nucleotide sequence were translated into amino acid sequences using the Geneious Prime software. Sequence alignment of the NS1 and VP1 protein sequences was done with Muscle implemented in MEGA-X using the default settings [30]. Phylogenetic trees of both NS1 and VP1 protein sequences were then generated using MrBayes software (version 3.1.2) [31] with the parameters "lset nst = 6 rates = invgamma". This setting applied the GTR substitution model with gamma-distributed rate vari-ation across sites and used a proportion of invariable sites ("GTR + I + Γ"). Additionally, "prset aamodelpr = mixed" was employed to enable the program to use the ten built-in amino acid models. The maximum number of generations was set to be ten million, and sampling occurred at every 50 generations, with the first 25 % of Markov chain Monte Carlo (mcmc) samples being discarded as burn-in. Convergence was confirmed when the standard deviation of split frequencies was below 0.01 [32]. To visually represent the phylogenetic tree, it was visualized and edited by Figtree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and Adobe Illustrator 2020 v26.0.1.

## 2.4. Prediction of spatial structure of PuBOV structural protein VP1

The three-dimensional spatial structure of the viral structural protein of PuBOV was predicted using ColabFold [33]. In order to predict and compare the similarity between the spatial structure of PuBOV structural protein VP1 and the structure encoded by the currently known sequences, the sequence with the highest degree of identity to PuBOV structural protein VP1, feline bocaparvovirus type 3 VP1 protein sequence was downloaded from the GenBank database and converted into the three-dimensional spatial structure using ColabFold. The resulting spatial structures were imported into PyMOL software v2.0 in PDB format and subjected to pairwise comparisons.

## 2.5. Confirmation of the fecal sample

To determine that the fecal sample used in this study is from snow leopard, we downloaded the mitochondrial genome of snow leopard and mapped to reference with the raw reads of our metagenomic sequencing using the map to reference function on Geneious prime software. This was done so as to search for any snow leopard mitochondrial contigs using our metagenomic sequencing reads as reference. The presence of snow leopard mitochondrial contigs in the metagenomic sequencing reads will confirm that the feces sample is from snow leopard.



**Fig. 1.** A pie chart showing the relative abundance and percentage composition of the enteric virome detected in the snow leopard.

## 3. Results

A total of 323,62400 reads was generated after sequencing, with an average GC content of 48.80 %, and the average coverage of the sequencing is 57.326. After bioinformatic analysis, 7 viral families were detected. (Fig. 1). The result showed that the most abundant virus family was *Parvoviridae* (92.25 % of the total analyzed virus reads), followed by the virus families of *Genomoviridae* (3.03 %), *Smacoviridae* (1.18 %), *Circoviridae* (0.79 %), *Picobinaviridae* (0.56 %), *Anelloviridae* (0.39 %), and *Adenoviridae* (0.06 %). A novel bocaparvovirus sequence tentatively designated as *Panthera uncia* bocaparvovirus (PuBOV) was also identified. The complete genome of PuBOV was 5433 nt long (Fig. 2a), with a G + C content of 47.40 %. Based on BLASTx analysis, the full nucleotide sequence of PuBOV was most similar to feline bocaparvovirus 3 (accession number: ON595887) with which it shares 81.58 % sequence identity. The genome organization of PuBOV was predicted to contain 3 open reading frames (ORFs) flanked by 5′ and 3′ untranslated regions (UTRs). ORF1 (nt 269-2666) encodes a putative nonstructural protein, NS1 with 798 amino acids. The NS1 in PuBOV had less than 85 % amino acid identity with the NS1's of other known members of *Parvoviridae*. ORF2 encodes a putative structural protein, VP1, of 1811 amino acids. The capsid protein VP1 had less than 85 % amino acid identity with that of other known members of *Parvoviridae*. ORF3 encodes a highly phosphorylated nonstructural protein (NP1) of 231 aa and which shares a short overlapping sequence with NS1 and VP1.

Several conserved domains were identified in the NS1, these includes a replication start off domain (xxHxHxxxxx), located at amino acid 543–552, an SF3 helicase domain with a binding Walker A loop (GxxxxGKT) which plays a critical role in DNA replication [34], and Walker B' loop (KxxxxGxxxxxxxK) (Fig. 2a). VP1 was found to contain a calcium-binding loop (YLGPF) at amino acid position 1057 to 1061 and the phospholipase catalytic residue (DxxAxxHDxxY) of a phospholipase A2 (PLA2) domain (Fig. 2b), which in parvoviruses, is necessary for infectivity and viral particle release [35,36]. The VP1 also contains a glycine-rich (G-rich) at amino acid position 170–181. Conserved polyadenylation signals were absent downstream of the putative NS1 and the VP1 protein-coding



**Fig. 2.** The genome organization of PuBOV. The NS1 ORF is shown in brown, the NP1 ORF is shown in pink, while the VP1 is shown in purple. (A) Identification of the replication initiator domain and SF3 helicase domain in the NS1 protein. (B) Identification of the phospholipase A2 (PLA2) domain in the N-terminal portion of the VP1 protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regions. PuBOV was most closely related to feline bocaparvovirus 3 according to Bayesian phylogenetic trees created based on the amino acid sequences of the NS1 and VP1 proteins (Figs. 3 and 4). In this study, the VP1 protein structure comparison of PuBOV and feline bocaparvovirus 3 gave a root mean square distance (RSMD) of 0.354 (Fig. 5). The result of the bioinformatic analysis for the confirmation of the fecal sample revealed that 134 raw sequence reads can be mapped to the mitochondrial genome, and these 134 sequences reads produced the largest contig with sequence length of 677bp. A BLASTn analysis using this contig as a reference showed that it has the highest sequence identity (98.82 %) to *Panthera uncia* isolate PUN mitochondrion, complete genome accession number (KP202269).

## 4. Discussion

Next-generation sequencing (NGS) technology has been widely applied in virology, including the metagenomic characterization of viruses in humans and animal [37]. Viral Metagenomics sequencing has radically changed our understanding of the diversity, structure and evolution of the animal virome because of its ability to identify multiple viruses simultaneously and detect novel viruses [38]. In this study, we utilized NGS to discover a novel bocaparvovirus, designated as *Panthera uncia* bocaparvovirus (PuBOV) in the fecal sample of a snow leopard living in the mountain range of Heilongjiang province in NorthEast China. BOVs infect a wide range of hosts, and often cause diseases of the gastrointestinal and respiratory tract [39]. In bocaparvoviruses, NS1 is the major nonstructural protein,
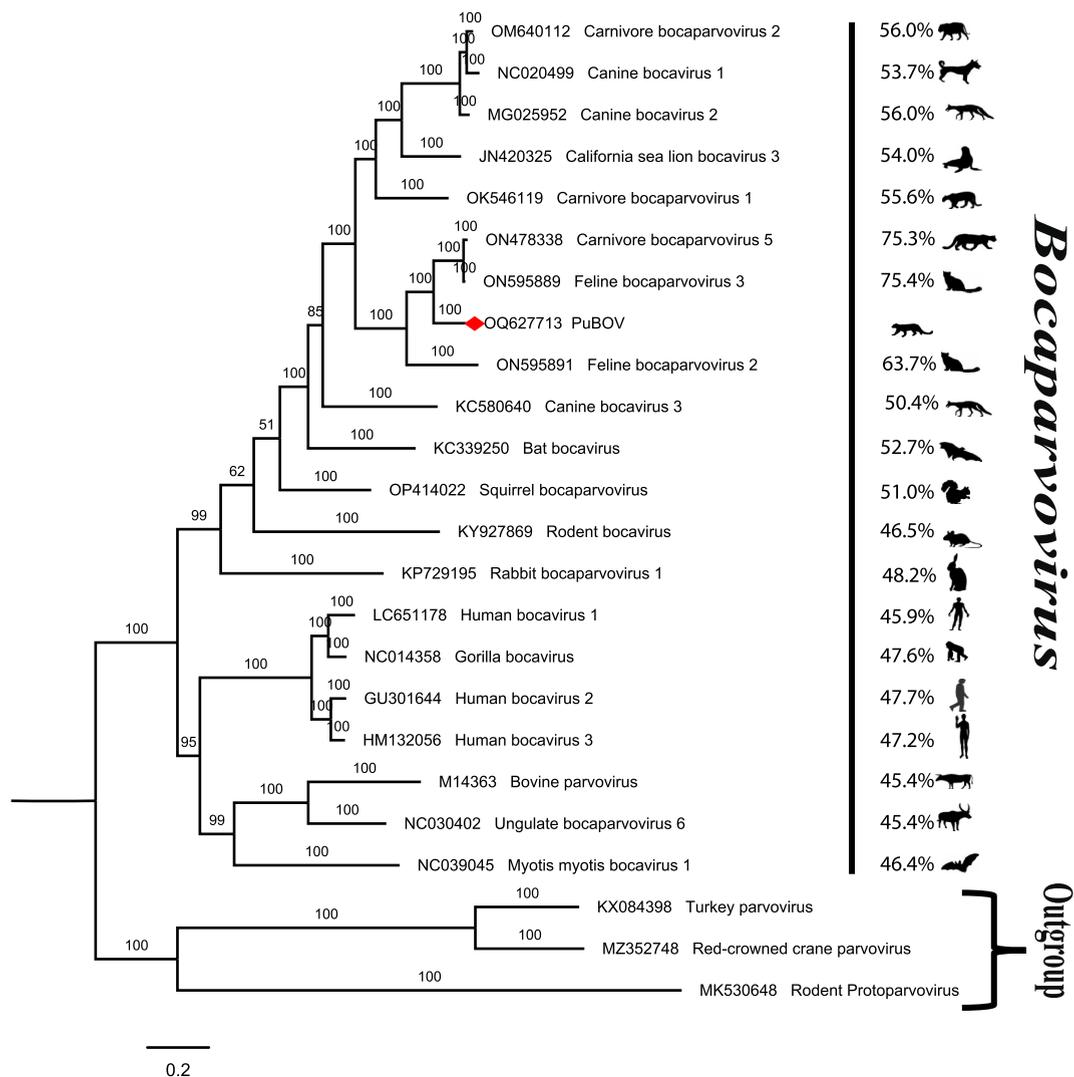


**Fig. 3.** Bayesian consensus trees based on the NS1 amino acid sequences of bocaparvoviruses. The novel bocaparvovirus (PuBOV) is marked with a red rhombus. The names of reference sequences, that contain both the GenBank accession number, the virus name and the percentage of sequence identity to PuBOV are shown in black. Bootstrap values for the branches are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
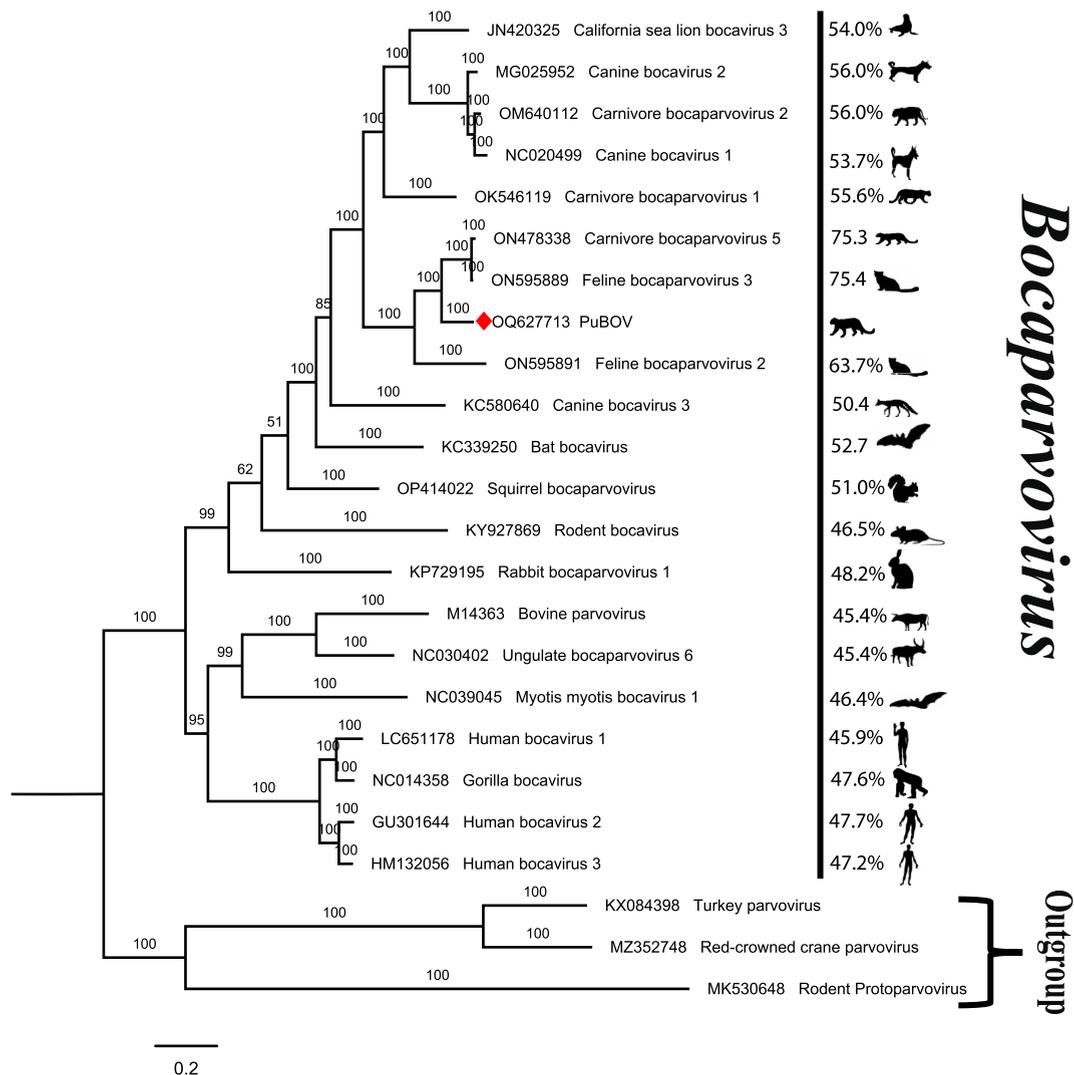
**Fig. 4.** Bayesian consensus trees based on the VP1 amino acid sequences of bocaparvoviruses. The novel bocaparvovirus (PuBOV) is marked with a red rhombus. The names of reference sequences, that contain both the GenBank accession number, the virus name and the percentage of sequence identity to PuBOV are shown in black. Bootstrap values for the branches are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and is essential for DNA replication. As a relatively conserved protein, NS1 divergence is a common standard for species definition. According to the current criteria of the International Committee on Taxonomy of Viruses (ICTV) (https://ictvonline.org/virusTaxonomy.asp), a novel bocaparvovirus specie is defined as one that shares <85.0 % amino acid identity in the NS1 gene with other species. The NS1 gene in PuBOV had less than 85 % amino acid identity with the NS1's of other known members of *Parvoviridae* and Based on these criteria, we propose that PuBOV should be classified as a member of a novel specie in the genus *Bocaparvovirus*.

Phylogenetic analysis of both the NSI and VP1 amino acid sequence showed that PuBOV had the most evolutionary similarity with Feline bocaparvovirus 3 (Figs. 3 and 4). The major capsid protein VP1 of bocaparvovirus is a protein that is essential for viral capsid assembly and cell attachment during viral infection. Small RMSD values (0–2 Å), shows that the percentage of protein structural similarity is very high [40]. The low RMSD obtained in this study suggests that PuBOV and feline bocaparvovirus 3 may cause similar infections, infect the same or closely related hosts, and share very similar conformational transitions in mediating cell attachment during infectious entry. Feline bocaparvovirus 3 has been frequently detected in cases of Feline panleukopenia, which is a highly contagious, life-threatening infectious disease in cats. characterized by high fever, vomiting and diarrhea [41]. Feline bocaparvovirus 3 can also infect cats sub-clinically [42] and have also been detected in healthy cats [41].

The genome analysis of PuBOV revealed that similar to other bocaparvoviruses, it expresses 3 ORFs namely NS1, NP1 and VP1. The NS1 protein contains an N-terminal origin-binding domain, a helicase domain, and a C-terminal transactive domain, and is essential for effective viral replication and generation of infectious virus [43]. NP1 is a small nonstructural highly phosphorylated protein encoded

RMSD =    0.354



PuBOV vs Feline bocaparvovirus 3 (UZV42144)

■ PuBOV

■ Feline bocaparvovirus 3
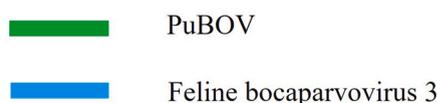
**Fig. 5.** Viral VP1 protein structural model visualization. PDB files were visualized and pairwise aligned using PyMOL v2.0 software.

by an ORF in the center of the viral genome, and is crucial in viral DNA replication and pre-mRNA processing [44]. NP1 is produced from an ORF that coincides with the C-terminus of the NS1 and is conserved among all bocaparvoviruses. Although the amino acid sequence of the NP1 of many bocaparvoviruses differs by just about 48 %, NP1's functions are conserved [44]. The icosahedral capsid protein (VP1) of PuBOV is 80 kDa and consists of 717 aa. The N terminal portion of the VP1 protein includes around 40 amino acids that are conserved in most parvoviruses, but the remaining sequences of VP1 are very variable; this conserved domain has been demonstrated to have secreted phospholipase A2 (sPLA2)-like enzymatic activity [45,46]. This activity has been suggested to be a key for the efficient transfer of the viral genome from late endosomes/lysosomes to the nucleus to commence viral replication in parvoviruses, and amino acid substitution in the active site of the sPLA2 motif would inactivate enzymatic activity, disabling viral infectivity [47,48].

The most striking feature of the fecal sample is the high prevalence (92.25 %) of *Parvoviridae* which are usually associated with feline diarrhea [49]. Feline bocaparvovirus has been frequently detected in cases of Feline panleukopenia, which is a highly contagious, life-threatening infectious disease in cats. characterized by high fever, vomiting and diarrhea [41]. However, the stool sample collected for this study is not a diarrheal stool. This maybe because Feline bocaparvovirus can also infect cats sub-clinically [42], and have also been detected in healthy cats [41]. In this study, the virome was found to include numerous other virus families, such as *Adenoviridea* and *Anelloviridae* that have been reported to affect the health of felids, but whether any of them are associated with disease in the snow leopard was not established. Because of their low relative abundance, we hypothesize that these other viral families detected in the fecal sample may be existing as part of the normal gut flora of the snow leopard, or causing only inapparent or subclinical infections.

The major limitation of the study is the limited sample size, largely due to the sparse population of snow leopards in the wild, and their elusive nature. Nevertheless, this study provides a strong foundation for future efforts to understand the fecal virome of wild snow leopards, which can aid in developing effective strategies to mitigate potential viral disease outbreaks and improve the overall conservation of endangered wild snow leopard populations. Although the novel bocaparvovirus identified in this study have not been conclusively linked to any infections, early detection of such viruses in wild animals provides an opportunity to investigate potential threats to wildlife, domestic animals and humans, and implement preventive measures to reduce spillover events. It is important to continue the sampling in our study area and to expand into other regions of the snow leopard distribution range. Future studies are needed to elucidate the possible etiologic role of PuBOV in feline diseases, prevalence, host range, cross-species transmission potential and epidemiological significance.

## Funding statements

## Data availability statement

The sequencing raw reads analyzed in this study have been uploaded onto the Sequence Read Archive (SRA) at National Center for biotechnology Information (NCBI) under the BioProject accession number PRJNA909234. The complete genome sequence of the novel virus identified in the study is available in the public domain database of NCBI under Accession number OQ627713 at https://www.ncbi.nlm.nih.gov/nuccore/OQ627713.

## CRediT authorship contribution statement

**Kingsley Ikechukwu Chukwudozie:** Writing – original draft, Validation, Investigation, Formal analysis. **Haoning Wang:** Writing – original draft, Validation, Methodology, Investigation, Data curation. **Xiaolong Wang:** Writing – review & editing, Investigation, Data curation. **Chunying Lu:** Writing – review & editing, Investigation, Data curation. **Jiaxin Xue:** Writing – review & editing, Formal analysis, Data curation. **Wen Zhang:** Supervision, Funding acquisition, Conceptualization. **Tongling Shan:** Supervision, Resources, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper..

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