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Novel polyomaviruses identified in fecal samples from four carnivore species

Simona Kraberger[1] · Laurel E. K. Serieys[2,3] · Seth P. D. Riley[3] · Kara Schmidlin[1] · Eric S. Newkirk[4] · John R. Squires[5] · Christopher B. Buck[6] · Arvind Varsani[1,7]

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Abstract

Polyomaviruses are oncogenic viruses that are generally thought to have co-evolved with their hosts. While primate and rodent polyomaviruses are increasingly well-studied, less is known about polyomaviruses that infect other mammals. In an effort to gain insight into polyomaviruses associated with carnivores, we surveyed fecal samples collected in the USA from bobcats (*Lynx rufus*), pumas (*Puma concolor*), Canada lynxes (*Lynx canadensis*), and grizzly bears (*Ursus arctos*). Using a viral metagenomic approach, we identified six novel polyomavirus genomes. Surprisingly, four of the six genomes showed a phylogenetic relationship to polyomaviruses found in prey animals. These included a putative rabbit polyomavirus from a bobcat fecal sample and two possible deer-trophic polyomaviruses from Canada lynx feces. One polyomavirus found in a grizzly bear sample was found to be phylogenetically distant from previously identified polyomaviruses. Further analysis of the grizzly bear fecal sample showed that it contained anelloviruses that are known to infect pigs, suggesting that the bear might have preyed on a wild or domestic pig. Interestingly, a polyomavirus genome identified in a puma fecal sample was found to be closely related both to raccoon polyomavirus 1 and to Lyon-IARC polyomavirus, the latter of which was originally identified in human saliva and skin swab specimens but has since been found in samples from domestic cats (*Felis catus*).

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✉ Simona Kraberger
simona.kraberger@asu.edu

✉ Arvind Varsani
arvind.varsani@asu.edu

[1] The Biodesign Center for Fundamental and Applied Microbiomics, Center for Evolution and Medicine and School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA

[2] Panthera, 8 W 40th St, 18th Floor, New York, NY 10018, USA

[3] Santa Monica Mountains National Recreation Area, National Park Service, Thousand Oaks, CA 91360, USA

[4] Speedgoat Wildlife Solutions, Missoula, MT 59801, USA

[5] U.S. Forest Service, Rocky Mountain Research Station, 800 East Beckwith Avenue, Missoula, MT 59801, USA

[6] Lab of Cellular Oncology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

[7] Structural Biology Research Unit, Department of Integrative Biomedical Sciences, University of Cape Town, Cape Town 7925, South Africa

Obtaining blood and/or tissue samples from large mammals can be difficult, as they can be highly elusive and logistically challenging to capture and handle [2]. Non-invasive methods, such as fecal sampling, have thus become increasingly popular among wildlife researchers, conservation biologists, and wildlife managers [14]. Analysis of fecal material can help address a wide range of questions, from host population genetics within a conservation framework identifying prey and investigating gut microbiota. Through metagenomics and bioinformatic analysis of conserved RNA-dependent RNA polymerase sequences [9], at least 18 families of RNA viruses whose members infect mammals have been identified. On the other hand, fewer studies have examined DNA viruses. To date, the DNA viruses identified in mammals include double-stranded DNA viruses of the families *Adenoviridae*, *Papillomaviridae*, *Poxviridae*, and *Polyomaviridae* [11, 12, 17, 23] and single-stranded DNA viruses of the families *Anelloviridae*, *Circoviridae*, *Genomoviridae*, *Parvoviridae*, and *Smacoviridae* [5, 29, 30, 35, 37, 39].

Polyomaviruses (family *Polyomaviridae*) are oncogenic double-stranded circular DNA viruses with broad tissue tropism [24]. They have been extensively studied in humans and other mammals due to the diseases they can cause,

such as kidney damage [22] and cancer [41]. Over the past decade, divergent arachnid, avian, and fish polyomaviruses have also been characterized [8, 44, 50, 51]. Polyomavirus genomes have been identified in the fecal samples of avian [52], bat [48], felid [17], rodent [55], and primate [45] species.

Mammalian polyomaviruses typically have two major oncogenes on the virion strand, encoding the large and small T antigens [31]. All polyomavirus genomes encode the major and minor capsid proteins VP1 and VP2 on the complementary strand. Among mammals, polyomaviruses appear to be host-species-specific, although incongruencies in the phylogenesis of the polyomaviruses and their hosts indicate a complex evolutionary history, which likely included ancient recombination events and possible rare instances of switching between closely related host animals [8, 47]. In this study, we expand the current knowledge of polyomaviruses in mammalian species by identifying and characterizing the genomes of six novel polyomaviruses from fecal samples from members of four carnivore groups in North America.

Two fecal samples were collected from bobcats (*Lynx rufus*) and one from a puma (*Puma concolor*) in California, two from Canada lynx (*Lynx canadensis*) in Montana, and one from a grizzly bear (*Ursus arctos*) in Wyoming. Fresh samples were collected and stored at -20 °C. These were then processed according to a previously reported protocol [28]. Briefly, viral DNA was isolated using a High Pure Viral Nucleic Acid Kit (Roche, USA), and circular DNA was selectively amplified by rolling-circle amplification (RCA), using a TempliPhi 2000 kit (GE Healthcare, USA). The RCA products were used to generate Illumina sequencing libraries using a TrueSeq Nano Library Prep Kit and sequenced on a HiSeq 4000 System at Macrogen Inc. (Korea). Reads were quality trimmed using Trimmomatic [6] and assembled *de novo* using metaSPAdes v3.12.0 [4] or Megahit v1.2.9 [32], and the resulting contigs were screened for similarities to polyomaviruses using a BLASTx [3] search of a viral protein database downloaded from GenBank. Inverse PCR primers were designed and used to amplify circular polyomavirus genomes. PCR amplification was performed using 10 µl of Kapa HiFi DNA polymerase (Roche Diagnostics, USA), 8 µl of sterile distilled water, 1 µl of RCA DNA as template, and 0.5 µl of specific forward and reverse primers (Fig. 1A). Cycling was performed according to the manufacturer's recommendations with a primer annealing temperature of 55 °C. The amplified genomes were resolved on a 0.7% agarose gel and cloned into the pJET1.2 plasmid vector (Thermo Fisher Scientific, USA). Purified recombinant plasmids were sequenced at Macrogen Inc. (Korea) by primer walking. Sanger sequence reads were trimmed and assembled using Geneious Prime v2022.0.2 (Biomatters Ltd., New Zealand).

The six recovered polyomavirus genomes varied in size, ranging from 4.9 to 5.4 kb (Fig. 1). These are referred to as follows: Canada lynx fecal polyomavirus 1 (Lyfec PyV1-MAF4; OM338643), Canada lynx fecal polyomavirus 2 (Lyfec PyV2-MAF12; OM338644), grizzly bear fecal polyomavirus 1 (Grifec PyV1-GB3; OM338642), puma fecal polyomavirus 1 (Pumfec PyV1-LSF128; OM338645), bobcat fecal polyomavirus 1 (Bofec PyV1-LSF72; OM338640), and bobcat fecal polyomavirus 2 (Bofec PyV2-LSFL22; OM338641). Open reading frames encoding the capsid proteins (VP1 and VP2), the large T antigen (LT), and the small T antigen (ST) were identified in all six of these polyomavirus genomes (Fig. 1).

Pairwise comparisons of full polyomavirus genome sequences using SDT v1.2 [36] showed that five of the six genomes shared 50-59% identity with previously reported polyomavirus sequences, qualifying them as members of new species [24, 34, 40]. The one exception is Bofec PyV2, which shares 81% identity with a polyomavirus recently identified in an Iberian hare (*Lepus granatensis*) (Iberian hare polyomavirus; MN994868) [1]. Further comparison of the VP1, VP2, LT, and ST sequences to those of closely related polyomaviruses was undertaken using clinker v0.0.23 [19]. The analysis showed a high level of similarity across the major proteins, with Bofec PyV2 and the Iberian hare polyomavirus sharing 86-94% identity (Fig. 1B). Similarly, Lyfec PyV2 shares 65-81% sequence identity in the major proteins with a polyomavirus whose genome sequence was assembled from a shotgun genomics dataset (SRR6878776) from a steenbok antelope (*Raphicerus campestris*) [10] (Fig. 1C). The other four polyomaviruses Grifec PyV1, Lyfec PyV1, Pumfec PyV1, and Bofec PyV1, share low levels of amino acid sequence similarity in their major proteins with those of the most closely related polyomaviruses (Fig. 1C and D).

In light of the detection of a prey-associated polyomavirus in bobcat and Canada lynx fecal samples, we used phylogenetic analysis to infer likely hosts of origin for the other four fecal polyomavirus sequences. The approach rests on the assumption that polyomaviruses generally cospeciate with their mammalian hosts [8, 47]. A dataset was built from representative large T-antigen protein sequences from each polyomavirus species or grouping available in the GenBank database (downloaded 01 March 2022). This large T-antigen protein sequence dataset was aligned using MUSCLE [15], and model tests were performed using ProtTest 3 [13]. The substitution model rtREV+I+G+F was used to construct maximum-likelihood phylogenies using PhyML 3 [21] implemented in Seaview [20]. The phylogenetic tree was rooted with fish and invertebrate polyomavirus sequences, and branches with less than 0.8 aLRT support were collapsed using TreeGraph2 [46].

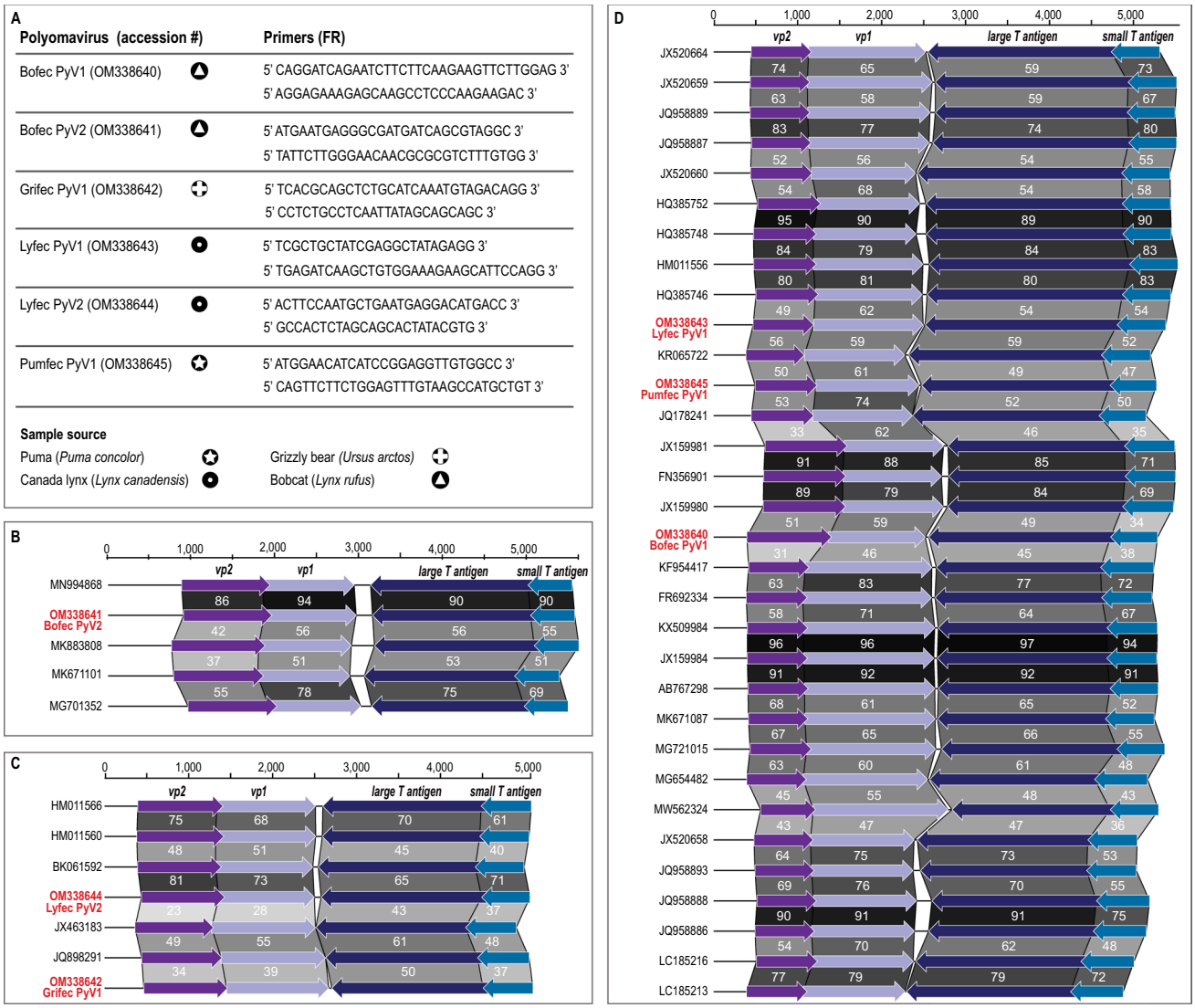
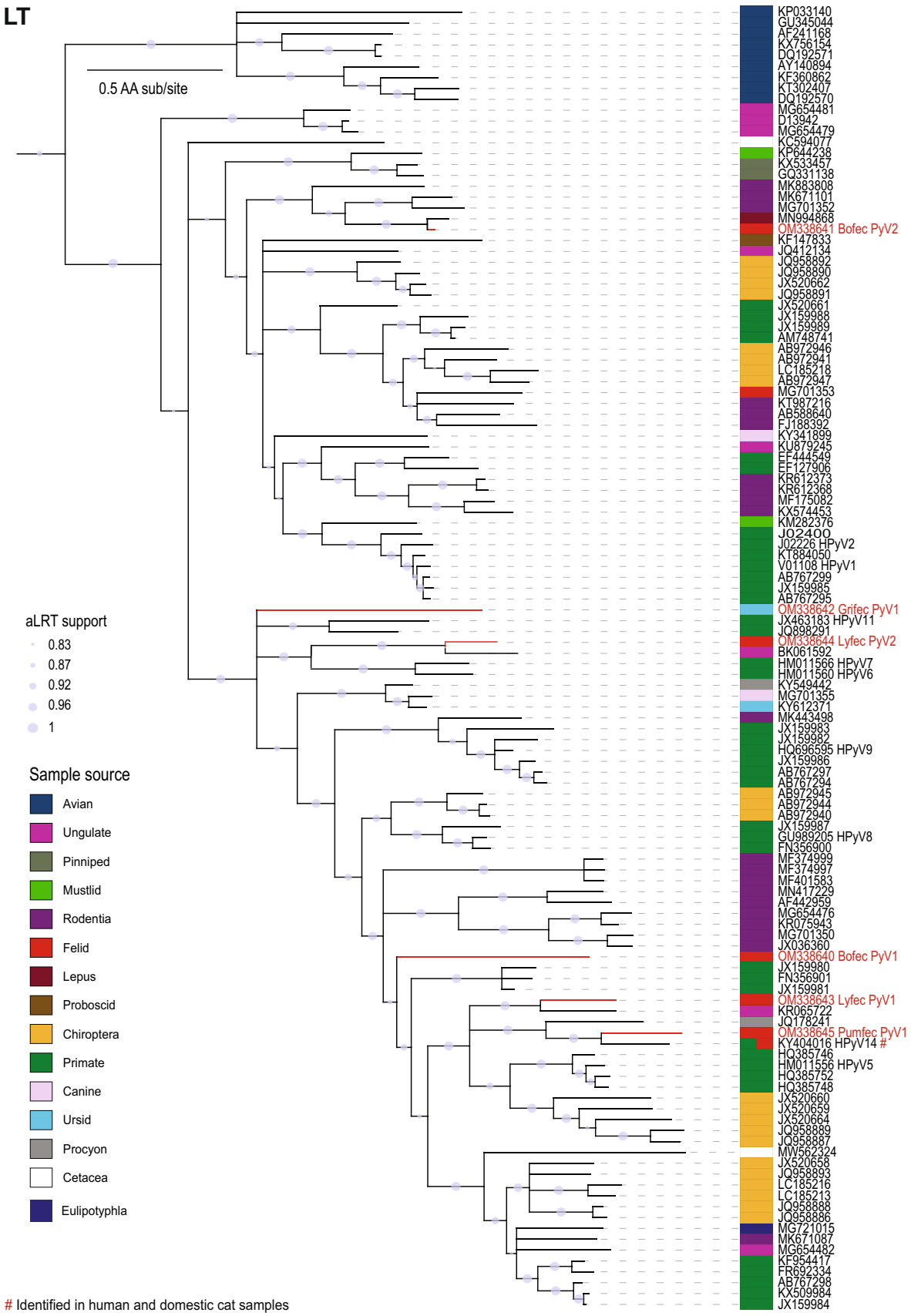


Fig. 1 (A) Summary of the abutting primers used to recover full polyomavirus genomes in this study. Panels B-D show schematic diagrams of polyomavirus genomes and pairwise amino acid sequence similarity of the VP2, VP1, LT, and ST proteins to other polyomavirus proteins that are similar to those from this study, as follows: (B) Bofec PyV2-LSFL22 (OM338641), with its nearest relatives from a hare (MN994868) and a rodent (MK883803, MK671101, MG701352); (C) Lyfec PyV2-MAF12 (OM338644) and Grifec PyV1-GB3 (OM338642), with the most closely related PyVs from humans (HM011566, HM011560, JQ898291, JX463183) and an antelope (BK061592); (D) Lyfec PyV1-MAF4 (OM338643), Pumfec PyV1-LSF128 (OM338645), and Bofec PyV1-LSF72 (OM338640), with the most closely related PyVs from bats (JX520664,JX520659, JQ958889, JQ958887, JX520660, JX520658, JQ958893, JQ958888, JQ958886, LC185216, LC185213), non-human primates (HQ385752, HQ385748, HQ385746, JX159981, FN356901, JX159980, FR692334, KX509984, JX159984, AB767298), humans (HM011556, KF954417), rodents (MK671087, MG721015), a pig (KR065722), a raccoon (JQ178241), an antelope (MG654482), and an orca (MW562324)

As predicted from the similarity analysis, Bofec PyV2 clustered with Iberian hare polyomavirus [1], and Lyfec PyV2 clustered with a virus from a steenbok antelope (*Raphicerus campestris*) (BK061592) [10] (Fig. 2). Interestingly, Lyfec PyV1 (OM338643) is part of a small clade containing porcine polyomavirus 1 (KR065722) [16]. The phylogenetic positions of the two lynx-derived fecal polyomaviruses, together with the fact that artiodactyls such as deer are known to be prey species of Canada lynx [43], suggest that the two viruses are prey-derived.

Pumfec PyV 1 is nested in a clade with raccoon polyomavirus 1 and Lyon-IARC polyomavirus (also known as HPyV14) [17, 18, 33] (Fig. 2). HPyV14 was first detected in 2017 in a single human skin sample, and subsequently in both skin and saliva samples of humans [7, 18, 26, 27, 53]. In 2019, however, nearly identical sequences were identified in fecal material from diarrheic domestic cats



Identified in human and domestic cat samples

◂**Fig. 2** Maximum-likelihood phylogeny based on large T-antigen protein sequences, showing the relationship of the six novel polyomaviruses identified in this study from bobcats (*Lynx rufus*), a puma (*Puma concolor*), Canada lynxes (*Lynx canadensis*), and a grizzly bear (*Ursus arctos*) to other representative polyomaviruses

[17, 33]. Serological studies have found that, in contrast to known human-tropic polyomaviruses, only a handful of blood donors showed detectable immunological responses to HPyV14 [25, 26]. These observations support the view that Pumfec1 PyV1 and HPyV14 are feline-tropic polyomaviruses, although it remains conceivable that HPyV14, in rare instances, also productively infects humans.

Bofec PyV1 and Grifec PyV1 differ from all previously discovered mammalian polyomavirus sequences, making it difficult to infer hosts from this form of analysis. To confirm the possible presence of remnants of particular prey animals in the fecal samples, we searched for the mitochondrial and genomic DNA sequences of non-carnivore mammals. This effort was unsuccessful, likely due to the fact that our metagenomic approach strongly favors the detection of circular DNAs protected within viral capsids. We next used Cenote Taker 2 [49] to search for other viruses that might be specific for particular prey animals. Interestingly, the grizzly bear feces sample was found to contain sequences similar to torque teno sus virus 1, an anellovirus that is widespread in domestic pigs (*Sus scrofa*). Two complete torque teno sus virus genomes were recovered using the back-to-back PCR primer pairs TTVsus2_F (5′-CTT TAC CCA TTA CAA AGT GAT GAC TAC TGG-3′) and TTVsus2_R (GTA CAG TTT ACA GCC GAA GTA TCT AAC-3′) and TTVsus3_F (5′-TAA TTA AGG GAT GGT GGC CTG TAA TAC AG-3′) and TTVsus3_R (5′-CTA CAG ATC TAA CTA CTC TGG GAT TCC ATG-3′). PCR products were cloned and sequenced by the Sanger method as described previously [30]. The full genome sequences of these two torque teno viruses (OP129719 and OP129720) are ~60-70% identical to other TTV sus virus 1 genome sequences available in the public database. Although feral pigs have not yet been reported in Wyoming, they are an increasingly problematic invasive species in 39 other states [54]. Given the observation that Grifec PyV1 is distant from other known polyomaviruses in the broader HPyV10 group and the fact that HPyV10-like polyomaviruses have not yet been reported in artiodactyls, we speculate that Grifec PyV1 may be a pig-trophic polyomavirus. This hypothesis would suggest that feral pigs were present in Wyoming in 2018, when the grizzly bear fecal sample was collected, or that a domesticated pig was preyed upon by the grizzly bear.

Prior to this study, only two polyomaviruses associated with felids had been identified, one from African lions (*Panthera leo*) (MG701353, MG701354) [16] and HPyV14, which, as mentioned above, was detected in domestic cats (MK898813) [17]. Furthermore, only two polyomaviruses had been identified from ursid species: one from a giant panda (*Ailuropoda melanoleuca*) (KY612371) [42] and one from a black bear (*Ursus americanus*) (MN989322) [38]. Although a definitive host for the novel polyomaviruses characterized here cannot be determined without further investigation, this study highlights the utility of sampling fecal matter of carnivores to examine the broader diversity of polyomaviruses from mammalian species.

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Data availability The sequences described in this study have been deposited in the NCBI GenBank database under accession numbers OM338640-OM338645, BK061592, OP129719, and OP129720.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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