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Identification of a novel polyomavirus in wild Sonoran Desert rodents of the family Heteromyidae

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Abstract

Rodents are the largest and most diverse group of mammals. Covering a wide range of structural and functional adaptations, rodents successfully occupy virtually every terrestrial habitat, and they are often found in close association with humans, domestic animals, and wildlife. Although a significant amount of research has focused on rodents' prominence as known reservoirs of zoonotic viruses, there has been less emphasis on the viral ecology of rodents in general. Here, we utilized a viral metagenomics approach to investigate polyomaviruses in wild rodents from the Baja California peninsula, Mexico, using fecal samples. We identified a novel polyomavirus in fecal samples from two rodent species, a spiny pocket mouse (*Chaetodipus spinatus*) and a Dulzura kangaroo rat (*Dipodomys simulans*). These two polyomaviruses represent a new species in the genus *Betapolyomavirus*. Sequences of this polyomavirus cluster phylogenetically with those of other rodent polyomaviruses and two other non-rodent polyomaviruses (WU and KI) that have been identified in the human respiratory tract. Through our continued work on seven species of rodents, we endeavor to explore the viral diversity associated with wild rodents on the Baja California peninsula and expand on current knowledge of rodent viral ecology and evolution.

With an estimated number of >2,500 species, rodents are the largest and most diverse group of mammals [1]. Due to the adaptability of rodents, they occupy terrestrial habitats globally (except Antarctica) in both rural and urban environments [2, 3]. Rodents are vectors or reservoir hosts of some human and livestock pathogens. An examination of 2,277 rodent species revealed that approximately 10% of them served as reservoirs of up to 11 zoonoses. Furthermore, among the 138 species carrying a single zoonotic pathogen, viruses accounted for 41% of the infectious agents [4]. One severe and life-threatening disease-causing RNA virus carried by

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rodents belongs to the family *Hantaviridae* and causes hantavirus pulmonary syndrome (HPS). In southwestern North America in the late 1990s, deer mice (*Peromyscus maniculatus*) were identified as the primary reservoir for members of the viral species *Orthohantavirus sinnombreense* (previous species name *Sin Nombre orthohantavirus*), which become airborne from rodent urine, droppings, or saliva [5, 6]. In desert regions in North America, heteromyids are the most abundant and speciose rodent guild. Most species in this rodent family are nocturnal and granivorous and live in underground burrows [7]. While rodent ecology has been extensively studied, there remains a gap in our understanding of the viruses that rodents harbor.

Towards filling fundamental gaps in our understanding of the diversity of DNA viruses associated with wild rodents – in this case, small double-stranded DNA viruses – we utilized a viral metagenomics approach to analyze 99 rodent fecal samples collected during the northern hemisphere Fall 2021 and Spring 2022 across the Baja California peninsula, Mexico, in accordance with institutional guidelines under ASU-IACUC # 22-1940R, and samples were transported to the U.S. under CDC permit #20220221-0658A. Fecal samples were collected fresh (within 8 hours of being deposited) from little desert pocket mouse (*Chaetodipus arenarius, n* = 17), Bailey's pocket mouse (*Chaetodipus baileyi, n* = 6), spiny pocket mouse (*Chaetodipus spinatus, n* = 10), Merriam's kangaroo rat (*Dipodomys merriam*i, *n* = 18), Dulzura kangaroo rat (*Dipodomys simulans, n* = 13), Bryant's woodrat (*Neotoma bryanti, n* = 7), and eastern deer mouse (*Peromyscus maniculatus, n* = 28). The fecal pellets were collected inside Sherman traps used to temporarily capture the rodents or while momentarily handling individuals for tissue sampling. Pellets were stored in 0.5 ml tubes, kept cold on wet ice for up to three weeks in the field, and then stored at -20°C in the laboratory until processing for viral DNA and downstream analysis. For nucleic acid extraction, 2 to 5 fecal pellets were transferred to a 1.5-ml screw-cap tube containing stainless steel beads (Next-Advance, Inc., USA), and 800 µl of SM buffer (0.1 M NaCl, 50 mM Tris-HCl [pH 7.4], and 10 mM $MgSO_4$) was added. Pellets were homogenized using a Bullet Blender® Storm 24 (Next-Advance, Inc., USA) for 3 min at a speed setting of 10. The homogenate was centrifuged on a benchtop centrifuge (Eppendorf, Germany) for 1 min at 5,000 × *g*, and the supernatant was filtered sequentially through 0.45-µm and 0.2-µm syringe filters (Cole-Parmer LLC., USA). Then, 200 µl of filtrate was used to extract viral DNA using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics, USA), following the manufacturer's protocol. To preferentially target circular DNA, 2 µl of viral DNA extract from each sample was used for rolling-circle amplification (RCA) using a TempliPhi Kit (GE Healthcare, USA). RCA amplicons were used to generate Illumina sequencing libraries using an Illumina® DNA Prep Kit with IDT® Illumina Nextera DNA Unique Dual Indexes. Libraries were sequenced on an Illumina HiSeq 2500 system at Psomagen, Inc. (USA), resulting in approximately 250 million reads.

Raw paired-end reads (2×150 nt) were trimmed using Trimmomatic v.0.39 [8] and subsequently assembled *de novo* using MEGAHIT v.1.2.9 [9, 10]. Circular contigs were identified based on terminal redundancy, using a custom Python script. All contigs >1,000 nt were analyzed using Diamond BLASTx v.2.1.5 [11] against a local viral RefSeq protein database (release 210, March 2022). Contigs were screened using Cenote-Taker2 v.2.1.5 [12] for preliminary virus discovery and genome annotation. Following *de novo* assembly and annotation, we identified polyomavirus (PyV) sequences from a spiny pocket mouse (*Chaetodipus spinatus*) sample from the town of San Javier and a Dulzura kangaroo rat (*Dipodomys simulans*) sample from the town of Santa Rita, both located in Baja California Sur, Mexico. These *de novo*-assembled sequences were ~5450 nt in length with terminal redundancy and thus circular contigs with a length of 5311 nt. These contigs were 99.9% identical to each other and, based on BLASTn analysis, shared 68.15% identity with 21% coverage (e-value, 3E-29) with porcine polyomavirus strain PP214 (MH824513). A total of 2,744,509 (average depth, 78,474.1) and 347 (average depth, 9.9) raw reads from the spiny pocket mouse and Dulzura kangaroo rat samples, respectively, mapped to each of these contigs. Based on the *de novo*-assembled PyV sequence, we designed abutting primers (F: 5'-CGTGTCTGCAGCAAACCCTATATACATGC-3'; R: 5'-CAGGGATGTAAAATGTGTGATGATAAAGCACTCC-3') to amplify the complete viral genome by polymerase chain reaction (PCR). Then, PCR reactions were run with 10 µl of Kapa HiFi DNA Polymerase (Roche Diagnostics, USA), 8 µl of sterile distilled water, 1 µl of RCA DNA as template, and 1 µl of forward and reverse primers. The PCR conditions were according to the manufacturer's protocol, with an annealing temperature of 55℃ and an extension time of 5 min. Amplicons were resolved by electrophoresis in a 0.7% agarose gel. A MEGAquick-spin Plus Fragment DNA Purification Kit (iNtRON Biotechnology, South Korea) was used to purify the excised 5-kb amplicons, which were then cloned into the pJET1.2 cloning plasmid (Thermo Fisher Scientific, USA), and recombinant plasmids were introduced into competent *Escherichia coli* XL1-blue cells by transformation. Recombinant plasmids were purified and sequenced by the Sanger method at Macrogen, Inc. (Korea) by primer walking. Open reading frames were identified using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) coupled with visual inspection. The sequences were deposited in the GenBank database with the accession numbers OQ799311 and OQ799312.

Polyomaviruses (PyVs) are small (~5,000 nt) double-stranded circular DNA viruses with capsids that have

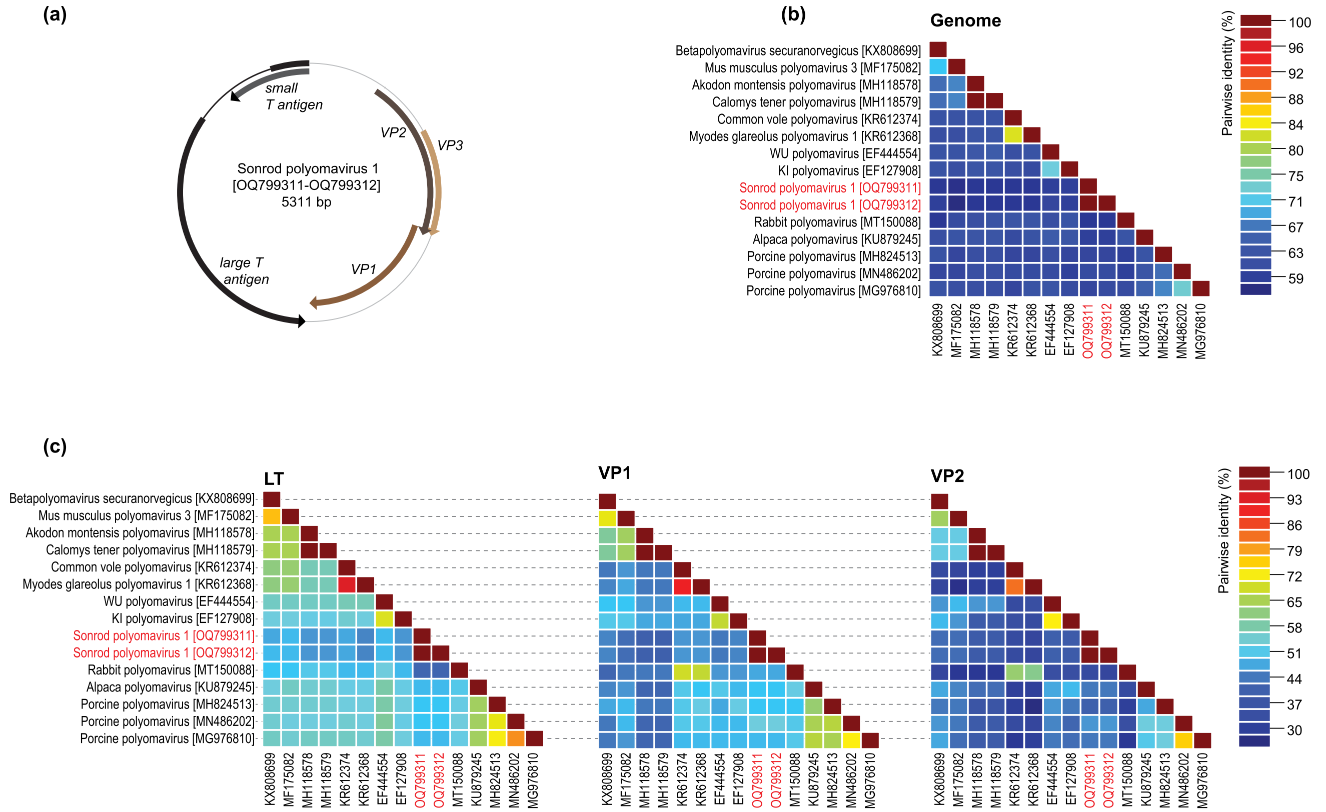

Fig. 1 (a) Genome organization of sonrod polyomavirus 1. GenBank accession numbers are shown in brackets, and the genome size in base pairs is indicated. (b) Pairwise identity matrix generated with SDT v1.24 [34] showing the percentage of pairwise similarity between nucleotide sequences of PyVs based on (b) the full genome and (c) the large tumor antigen (LT) and the capsid proteins VP1 and VP2. The PyV from this study is indicated in red.

icosahedral symmetry [13]. PyV genomes have a noncoding control region (NCCR), an early region, and a late region. The early and late encoding regions are transcribed in opposite directions and are separated by the NCCR [14]. The NCCR is a key regulatory region of approximately 400 nt containing the origin of viral DNA replication and sequences required for early and late transcription [15]. The early region encodes the large T antigen (LT) and the small T antigen (ST). Additional proteins such as the middle T antigen are present in some PyVs that infect rodents. The late region encodes at least two viral capsid proteins – VP1 and VP2 – although an ORF for a third structural protein (VP3) is found in most PyVs, including murine polyomavirus [15–17]. Additional proteins, such as ALTO and agnoprotein, have been described in some mammalian polyomaviruses, although their functions are still unknown [18, 19].

Polyomaviruses are known to infect an array of hosts, including mammals [20–22], birds [23–25], fish [18, 26–29], and arthropods [18, 30]. Murine polyomavirus (MPyV), or mouse polyomavirus, was the first member of the family *Polyomaviridae* confirmed in rodents. It was identified in 1953 by Dr. Ludwik Gross at the Veterans Administration Hospital Cancer Research Unit in New York City, USA [31, 32]. The discovery of MPyV provided deep insights related to experimental cancer research and the study of genes implicated in many mouse and human cancers. In humans, JC polyomavirus and BK polyomavirus were the first PyVs to be identified. Although most human polyomavirus infections are asymptomatic, some PyVs, including JC and BK, can cause severe disease in immunosuppressed individuals. JCPyV can cause progressive multifocal leukoencephalopathy and has been implicated in the development of various human neoplasms. BKPyV is commonly associated with ureteral stenosis, hemorrhagic cystitis, and nephropathy [14]. PyVs have also been associated with malignant brain tumors in free-ranging raccoons in the western United States [33]. The family *Polyomaviridae* is currently composed of 117 species, most of which have been assigned to eight genera: *Alphapolyomavirus*, *Betapolyomavirus*, *Deltapolyomavirus*, *Epsilonpolyomavirus*, *Gammapolyomavirus, Zetapolyomavirus*, *Etapolyomavirus*, and *Thetapolyomavirus* [13, 21].

The PyV identified in the two rodents in this study has a genome organization typical of polyomaviruses with two distinct transcription units extending in opposite directions (early and late regions) separated by a non-coding region.

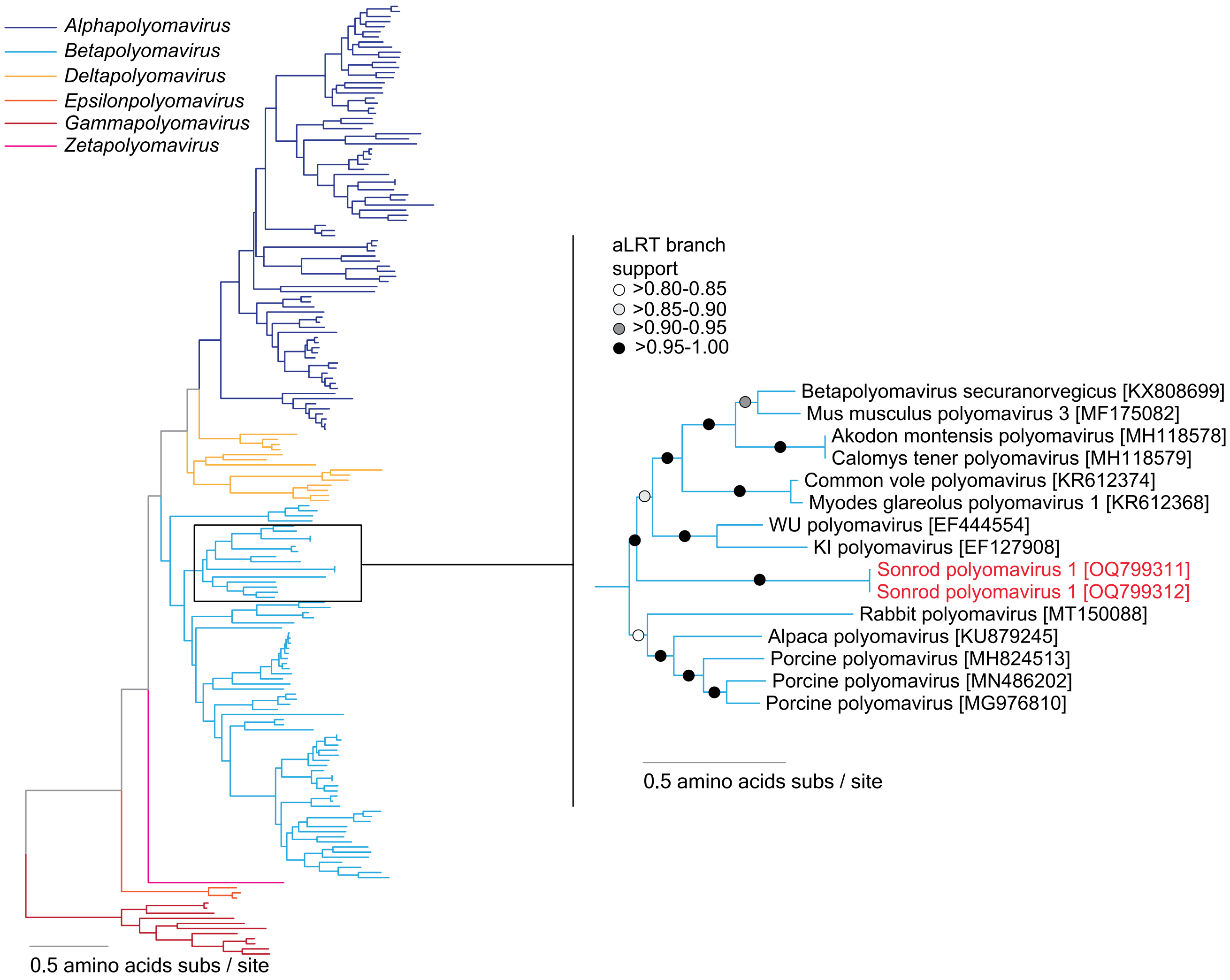

Fig. 2 Maximum-likelihood tree based on large T antigen (LT) protein sequences under the LG+I+G+F model of amino acid sequence evolution. Sonrod polyomavirus 1 sequences cluster with known rodent PyVs and human KI and WU PyVs of the genus *Betapolyomavirus*. Branches with less than 0.8 aLRT were collapsed. Bars indicate an evolutionary distance of 0.05 substitutions per site. The PyV from this study is indicated in red.

The early coding region transcribes the large T antigen (LT) and the small T antigen (ST), while the late coding region encodes the structural proteins VP1, VP2, and VP3. We identified the signature amino acid motif HPDKGG in the LT antigen (Fig. 1). Pairwise comparisons using SDT v.1.2 [34] revealed that the two genome sequences are 99.9% identical. In these two genomes, there are four polymorphisms, one in the VP2 protein, two in the LT protein, and one in the splicing region of the LT. The LT protein is required for viral DNA replication and plays a central role regulating effective infection [17]. The full genomes share ~60% genome-wide sequence identity with other known PyVs. Analysis of the LT, VP1, and VP2 proteins revealed 45-52%, 43-56%, and 34-49% identity, respectively. The two PyV full genomes, LT, VP1, and VP2 were found to be most closely related to those of porcine polyomaviruses (MN486202 and MH824513), sharing 62%, 52%, 56%, and 49% sequence identity, respectively. The sequence-based criteria for creation of a new PyV species, as established by the Executive Committee of the ICTV, specify that the LT sequence must be less than 85% identical to that of the most closely related virus [35]. Since the sequence identity of the LT to the most closely related PyV is 52%, the two polyomavirus isolates from Sonoran rodents represent a new species. Given that they are from two rodent species, we have named the new polyomavirus sonrod polyomavirus 1 (name derived from **Son**oran desert **rod**ents).

To investigate the phylogenetic relationship of sonrod polyomavirus 1 to other PyVs, we downloaded LT protein sequences of polyomaviruses from https://ccrod.cancer.gov/confluence/display/LCOTF/Polyomavirus and aligned them with those from sonrod polyomavirus

1, using MAFFT v.7.471 [36]. The LT protein alignment was used to construct a maximum-likelihood phylogenetic tree using PhyML v.3.3.2 [37] with the LG+I+G+F amino acid substitution model, as determined by ProtTest3 v.3.4.2 [38], and with the Shimodaira–Hasegawa-like approximate likelihood-ratio test (SH-like aLRT; [37, 39]) for branch support. TreeGraph2 v.2.15.0 [40] was used to collapse branches with <0.8 aLRT support. The LT protein phylogeny placed the new polyomavirus in the genus *Betapolyomavirus*. The LT proteins of sonrod polyomavirus 1 forms a monophyletic clade with those identified in rats (*Rattus norvegicus*), mice (*Mus musculus*), montane akodonts (*Akodon montensis*), vesper mice (*Calomys tener*), common voles (*Microtus arvalis*), bank voles (*Myodes glareolus*), and two human PyVs, KI and WU, identified in children (Fig. 2).

Investigation of the viral diversity found in wild rodents is important for expanding our limited knowledge about DNA viruses and the viral ecology of rodents in general. In this study, we used a viral metagenomics approach to identify polyomaviruses in fecal samples from seven wild rodent species from a wide geographic range on the Baja California peninsula, Mexico. Few viruses from Sonoran Desert rodents have been classified [e.g., 41], and, to our knowledge, this is the first PyV recovered from wild rodents of the family Heteromyidae.

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Author contributions Conceptualization, KLV, MC, AM-V, GAD, AV; methodology, KLV, SK, JMC, ENP, AV; software, KLV, SK, AV; validation, KLV, SK, AV; KLV, SK, AV; investigation, KLV, SK, JMC, ENP, MC, GAD, AV; resources, KLV, MC, AM-V, GAD, AV; data curation, KLV, AV; writing—original draft preparation, KLV, SK, AV; writing—review and editing, KLV, SK, JMC, ENP, MC, AM-V, GAD, AV; visualization, KLV, AV; supervision, MC, GAD, AV; project administration, KLV, MC, GAD, AV; funding acquisition, KLV, GAD, AV. All authors have read and agreed to the published version of the manuscript.

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Data availability The sequences described in this study have been deposited in the GenBank database under the accession numbers OQ799311-OQ799312.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Permits Animal handling and sample collection protocols were approved by the Office of Research Integrity and Assurance at Arizona State University, USA (IACUC # 22-1940R), and U.S. importation was approved under CDC permit # 20220221-0658A issued to G.A.D.

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