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ORIGINAL ARTICLE

Identification of small circular DNA viruses in coyote fecal samples from Arizona (USA)

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Abstract

Coyotes (*Canis latrans*) have a broad geographic distribution across North and Central America. Despite their widespread presence in urban environments in the USA, there is limited information regarding viruses associated with coyotes in the USA and in particular the state of Arizona. To explore viruses associated with coyotes, particularly small DNA viruses, 44 scat samples were collected (April–June 2021 and November 2021–January 2022) along the Salt River near Phoenix, Arizona (USA), along 43 transects (500 m). From these samples, we identified 11 viral genomes: two novel circoviruses, six unclassified cressdnaviruses, and two anelloviruses. One of the circoviruses is most closely related to a circovirus sequence identified from an aerosolized dust sample in Arizona, USA. The second circovirus is most closely related to a rodent-associated circovirus and canine circovirus. Of the unclassified cressdnaviruses, three encode replication-associated proteins that are similar to those found in protists (*Histomonas meleagridis* and *Monocercomonoides exilis*), implying an evolutionary relationship with or a connection to similar unidentified protist hosts. The two anelloviruses are most closely related to those found in rodents, and this suggests a diet-related identification.

Introduction

Coyotes (*Canis latrans*) are mid-sized mesocarnivores in the family Canidae that have an expansive range across the gradient of urbanization, ranging from rural to urban areas throughout North America and much of Central America [1, 2]. They are born into small packs consisting of their parents and siblings [3]. Notably, these animals are physiologically similar to domestic dogs (*Canis lupus familiaris*) [4]. In general, coyotes tend to eat small animals but have been known to kill larger animals such as white-tailed deer (*Odocoileus virginianus*) [5]. In addition, they also consume plant matter, such as fruits and seeds [5, 6]. Their presence in human-occupied environments has led to a long history of human-coyote conflict due to coyote predation of domesticated animals, such as sheep and pets [5–7]. Due to their generalist habitat requirements, social interactions, and omnivorous diet, coyotes are exposed to a suite of pathogens, which also can be transmitted to other wildlife and humans. However, the suite of pathogens that coyotes carry is unknown in many ecosystems, especially those influenced by urbanization.

Over the last six decades, there have been a significant number of viruses identified infecting coyotes. These include viruses in the families *Adenoviridae*, *Orthoherpesviridae*, and *Papillomaviridae* with double-stranded DNA genomes, *Coronaviridae*, *Flaviviridae*, *Picornaviridae*, and *Togaviridae* with single-stranded, positive-sense RNA genomes, and *Orthomyxoviridae*, *Paramyxoviridae*, *Peribunyaviridae*, *Phenuiviridae*, and *Rhabdoviridae* with single-stranded, negative-sense RNA genomes. Due to concerns of viral spillover between coyotes and domestic animals, there has been a significant amount of surveillance work associated with coyotes infected with or exposed to rabies virus [8–13],

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canine morbillivirus [14–16], and canine parvovirus [14, 17–20]. This is not surprising, due to the close encounters of coyotes with humans and other canids (both wild and domestic) [21]. Nonetheless, the majority of the work on identification of virus infections in coyotes has been done through serological assays. To date, 151 viral sequences in GenBank associated with coyotes belong to members of the following species: *Varicellovirus canidalpha1* (family *Orthoherpesviridae*; *n* = 2), *Morbillivirus canis* (family *Paramyxoviridae*; *n* = 20), *Chaphamaparvovirus carnivoran1* (family *Parvoviridae*; *n* = 4), *Protoparvovirus carnivoran1* (family *Parvoviridae*; *n* = 34), and *Lyssavirus rabies* (family *Rhabdoviridae*; *n* = 93). Of these, there are only seven complete genome sequences: two from *Chaphamaparvovirus carnivoran1*, three from *Protoparvovirus carnivoran1*, and two from *Lyssavirus rabies* (Table 1).

Given the limited knowledge of circular small DNA viruses associated with coyotes in urbanized areas, we undertook a pilot project to see if we can detect these in a non-invasive manner via scat sampling. We sampled scat along the Salt River near Phoenix, Arizona, United States (USA), and amplified circular DNA viruses from these samples using a viral metagenomics protocol coupled with PCR amplification and cloning of viral genomes, resulting in our discovery of 11 novel viruses. Here, we describe our identification of two new anelloviruses, three new circoviruses, and six new viruses in the phylum *Cressdnaviricota* that cannot be assigned to currently established families.

Materials and methods

Sample collection

Coyote scat samples were collected along the Salt River within the Phoenix Metropolitan Area and Tonto National Forest, Arizona, USA, between April and June 2021 and between November 2021 and January 2022. Scats were collected monthly during the study duration along 43 (500 m) transects, which were recorded using the Gaia GPS phone application (https://gaiagps.com/). All scats were cleared from each transect one month prior to the first collection date at each site. Sites were then visited monthly for two to three months to collect coyote scats. Scats were identified morphologically [22] and collected in plastic bags with associated spatial locations and confidence in species identification (50%-100%). Scats were then stored in a -80°C freezer. Scats with less than 100% confidence in species ID were later swabbed for remnant epithelial DNA and sent for microsatellite marker genetic testing to confirm they were deposited by a coyote (https://www.wildlifegenetics.ca/). Any scats not deposited by a coyote were excluded from analysis, as well as any scats unable to be swabbed that had less than 100% confidence in species ID. A random subset of coyote scats with 100% confidence in morphological ID were also swabbed for DNA to confirm the success of our methodology. A subset of the collected scats (n = 44) was then used for viral analysis. Scats were selected based on a representative sample of location and season (fall-winter or spring-summer) collected, and ~2 g of the fecal material was used downstream for viral work (Fig. 1).

For diet assessments, we conducted hard-parts analysis of prey remains in the coyote scats. We first sanitized the scats by baking them in an oven for 24 h at 60°C [22]. We then soaked scats in soapy water for 24–48 h and rinsed out any remaining fecal material using cheesecloth to isolate the dietary remains left in the scats for identification. Scat contents were then left to air-dry for a subsequent 24–48 h before storing in envelopes. We classified dietary items as mammal (to the lowest taxonomic classification possible), bird, snake, fish, arthropod (including insects, scorpions, and crayfish), vegetation (to family or genus, when possible), trash, eggshell, and unidentifiable (including within each mammal category and overall). To identify mammals, five random samples of hair per scat were examined under a microscope (AmScope B120, United Scope, LLC, Irvine, CA) [23] and photographed to compare with (1) reference slides of hair using specimens from Arizona State University's Natural History Collections, (2) identification keys [24–26], and expert solicitation. We also identified teeth remains found in scats using reference images of teeth from specimens at Arizona State University's Natural History Collections, as well as expert solicitation. In addition to hair, domestic cat (*Felis catus*) remains were also identified via the presence of claws. Domestic cats were further distinguished from bobcats (*Lynx rufus*) via hair color and texture, as well as location, when compared with where bobcats were detected on camera traps. Similarly, domestic dogs (*Canis familiaris*) were distinguished from coyotes based on hair color and texture, as well as expert review. Lagomorph hairs were classified as desert cottontail rabbit (*Sylvilagus audubonii*) or unidentifiable lagomorph based on the number of rows of medulla within a sample being either greater than four (i.e., cottontail rabbit) or less than or equal to four (i.e., to distinguish between cottontail rabbit and black-tailed jackrabbit, *Lepus californicus* [24]). Any sample with at least one lagomorph hair greater than four medulla cells wide was identified as being cottontail rabbit—and often corroborated by lagomorph teeth of a smaller size more likely representative of a cottontail rabbit—while lagomorph samples with only thinner hairs in terms of medullar width were identified as unidentifiable lagomorph. Neotominae species (i.e., *Neotoma* or *Peromyscus* spp.) were grouped together due to the similarity in their hair structure. Bird remains were identified via feathers or hollow bones present in the scats, while reptiles and fish were identified via scales and

Table 1 Summary of sequences of viruses associated with coyotes available in the GenBank database prior to this study

Genome type	Family	Genus	Species	Virus	Accession no.	Country	Genome sequences		References
							Partial	**Complete**	
dsDNA	*Orthoherpesviridae*	*Varicellovirus*	*Varicellovirus canidalpha1*	Canid alphaherpesvirus 1	KX062143, KX062144	Canada	2	-	[93]
ssRNA(-)	*Paramyxoviridae*	*Morbillivirus*	*Morbillivirus canis*	Canine distemper virus	OK666919, OK666920, OK666921, OK666922, OK666923, OK666924, OK666925, OK666926, OK666927, OK666928, OK666929, OK666930, OK666931, OK666932, OK666933, OK666934, OK666935, OK666936, OK666937, KT387222	Canada, Mexico	20	-	[94]
ssDNA(+/-)	*Parvoviridae*	*Chaphamaparvovirus*	*Chaphamaparvovirus carnivoran1*	Cachavirus	OM640108, OM640109, OM640110, OM640111	Canada (Newfoundland)	2	2	[18]
ssDNA(+/-)	*Parvoviridae*	*Protoparvovirus*	*Protoparvovirus carnivoran1*	Canine parvovirus	MH480648, MG425949, MG425950, KJ813866, KJ813867, KJ813868, KJ813872, KJ813874, KJ813875, KJ813876, KJ813883, KJ813884, KJ813885, KJ813886, KJ813887, KJ813888, KJ813889, KJ813891, KJ813892, JX475238, JX475239, JX475269, JX475273, JX475274, JX475275, JX475276, JX475277, JX475278	Canada (Newfoundland), Mexico, USA (Alabama, Alaska, Arkansas, Georgia, Montana, Nebraska, North Dakota, Oklahoma)	31	3	[17–19, 95]

Table 1 (continued)

Genome type	Family	Genus	Species	Virus	Accession no.	Country	Genome sequences		References
							Partial	**Complete**	
ssRNA(-)	*Rhabdoviridae*	*Lyssavirus*	*Lyssavirus rabies*	Rabies virus	OM203023, OM203045, MW566991, KU963491, KU963495, KJ174683, KC791818, KC791823, KC791961, KC792102, JQ685917, JQ685973, JQ685994, FJ228520, FJ228526, FJ228529, FJ228530, FJ228536, FJ228551, FJ228552, FJ228557, FJ228563, FJ228564, FJ228567, FJ228568, FJ228569, FJ228571, FJ228575, FJ228576, FJ228577, FJ228580, FJ228584, FJ228585, FJ228587, FJ228588, FJ228589, FJ228591, FJ228592, FJ228594, FJ228604, FJ228605, FJ228606, FJ228607, FJ228608, FJ228612, FJ228613, FJ228614, FJ228616, FJ228617, FJ228618, FJ228619, FJ228620, FJ228622, FJ228623, FJ228624, FJ228625, FJ228626, FJ228627, FJ228629, FJ228632, FJ228633, FJ228634, FJ228636, FJ228637, FJ228639, FJ228640, FJ228641, FJ228644, FJ228645, FJ228648, FJ228650, FJ228651, FJ228652, FJ228653, FJ228654, FJ228655, FJ228656, FJ228658, FJ228660, FJ228662, FJ228663, FJ228664, FJ228665, FJ228670, FJ228674, FJ228676, FJ228683, FJ228685, DQ118248, AY561764, AF369289, AF369308, AF369337	Canada (Ontario), Mexico (Coahuila, Texas US-Mexico border), US (Arizona, California, New Mexico. Texas)	91	2	[13, 96–103]

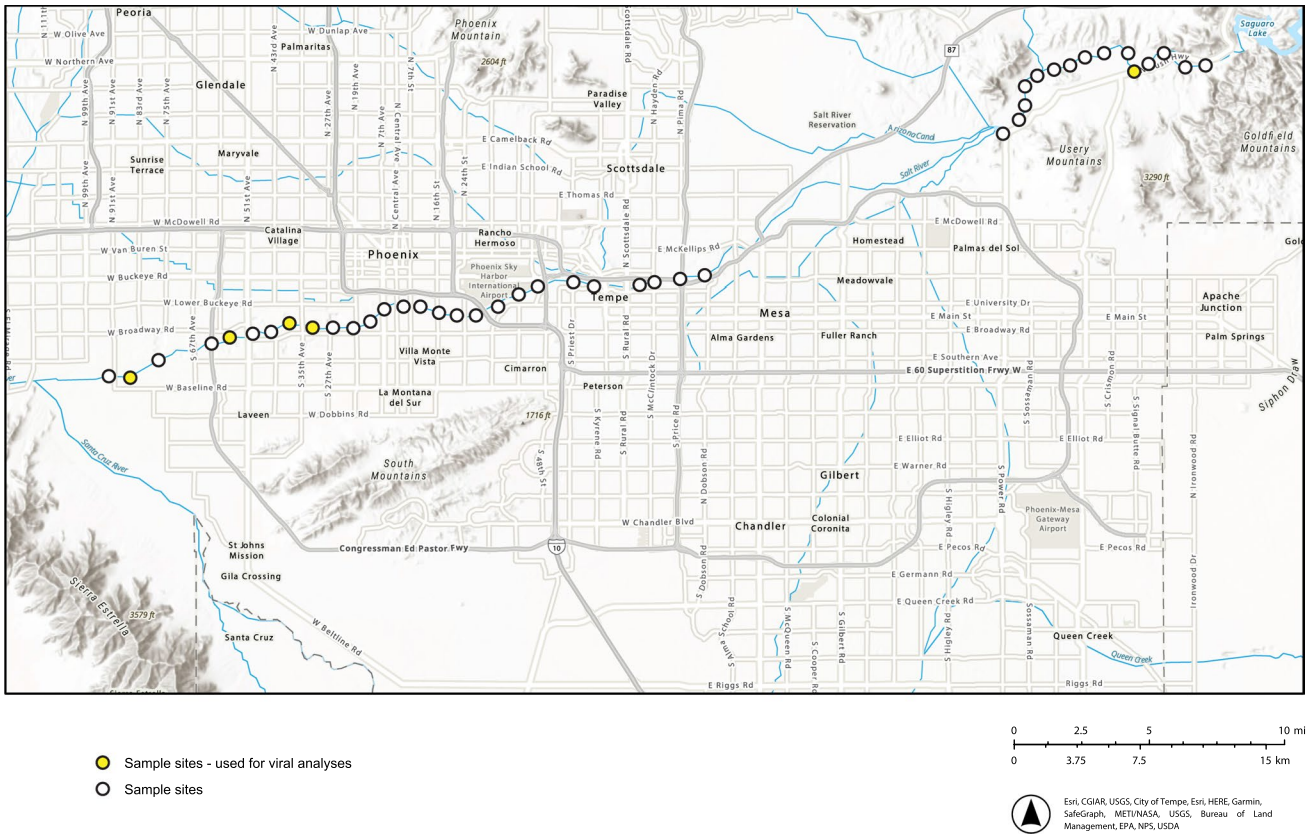


Fig. 1 Map of study sites along the Salt River Corridor in the Phoenix Metropolitan Area, Arizona, USA. Black circles indicate study sites where scats were collected for analysis but were not used due to an inability to detect, amplify, and clone the viruses from these samples. Sites in yellow indicate areas where coyote scats were analyzed for viruses, including (from left to right) samples V16_S08, N10_S01, N07_S03, N06_S02, and T05_2F02.

confirmed via expert review. Due to desiccation, crayfish remains were only identifiable when washing the scats prior to drying. Vegetation, particularly seeds, was identified via expert solicitation, and eggshells and trash were identified by sight. Diet remains that contributed to <1% of the volume of a dried scat were not considered in analyses, as these items were expected to be incidental.

Sample processing and viral metagenomics

The 44 scat samples were resuspended separately and homogenized in 3 ml of SM buffer. The homogenate was centrifuged at 10,000×*g* and passed through a 0.22-µm syringe filter. Two hundred microliters of the filtrate was used for DNA extraction using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics, USA). A TempliPhi 2000 kit (GE Healthcare, USA) was used to amplify circular DNA in the viral extracts, using rolling-circle amplification (RCA). The RCA products were combined into three pools, and these were used to generate Illumina sequencing libraries using Illumina DNA Prep, (M) Tagmentation (96 Samples, IPB) Kit (Illumina, USA) and sequenced on an Illumina HiSeq2500 instrument at Psomagen Inc. (Maryland, USA). The raw reads (2 × 150 nt) were trimmed using Trimmomatic v0.39 [27] and assembled *de novo* using MEGAHIT v1.2.9 [28]. Contigs of >1000 nt were compared, using BLASTx [29], to a viral RefSeq protein sequence database (release 207) for viral-like sequences.

Recovery of circular viral genomes

Based on the *de novo*-assembled contigs with similarities to small circular DNA viruses, abutting primer pairs were designed and used to amplify complete viral genomes (Supplementary Table S1). The primers were used in a polymerase chain reaction (PCR) with KAPA HiFi HotStart DNA Polymerase (Roche Diagnostics, USA), using the following thermal cycling conditions to screen and amplify the genome with the specific primers and the RCA product from the 44 scat samples as templates: initial denaturation at 98°C for 3 min, followed by 25 cycles of 98°C for 15 s, 60°C for 15 s, and 72°C for 3 min, and a final extension at 72°C for 3 min. The PCR amplicons were resolved on a 0.7% agarose gel, and bands corresponding to the expected nucleotide length

were excised and gel-purified using a MEGAquick-spin Plus Fragment DNA Purification Kit (iNtRON Biotechnology, South Korea). The purified product was ligated into the pJet 1.2 plasmid vector (Thermo Fisher Scientific, USA), and the construct was used to transform competent *E. coli* DH5 cells. Bacterial colonies were grown overnight, and recombinant plasmids were extracted using a Fast DNA-spin Plasmid DNA Purification Kit (iNtRON Biotechnology, South Korea) and then sequenced at Macrogen Inc. (South Korea) by primer walking. The Sanger sequences were assembled using Geneious Prime (Biomatters Ltd., New Zealand). The genomes were annotated using ORFfinder (ncbi.nlm.nih.gov/orffinder/) and manually checked.

Viral genome analysis

For the cressdnaviruses, a dataset of replication-associated protein (Rep) sequences was assembled from ~ 4000 sequences, which were extracted from genome sequences of representative members of established virus families as well as currently unclassified viruses with sequences available in the GenBank database, and those from this study. This Rep protein sequence dataset was used to cluster new sequences at the family level based on sequence similarity network (SSN) analysis, using EST-EFI [30] with a threshold of 60. The SSN was visualized using Cytoscape v3.8.2 [31] with an organic layout. The clusters containing the Rep sequences from this study were extracted for downstream analysis. Representative classified cressdnavirus and CRESSV1-6 group Rep sequences were aligned with the Rep sequences from this study, together with the other sequences from their clusters (clusters 1–4), using MAFFT [32]. The alignment was trimmed using TrimAL [33], and this was then used to construct a maximum-likelihood phylogenetic tree using IQ-Tree 2 [34] with the Q.pfam+F+G4 substitution model, and the tree was visualized in iTOL v6 [35]. For each cluster, outgroup Rep sequences were included and aligned using MAFFT [32].

For the anelloviruses, ORF1 is the most highly conserved coding region. Therefore, a representative dataset of ORF1 protein sequences from GenBank was compiled, and the sequences were aligned with those from this study, using MAFFT [32]. The coyote anelloviruses clustered with members of the genus *Wawtorquevirus.* Therefore, a maximum-likelihood phylogenetic tree based on ORF1 sequences of viruses of the genus *Wawtorquevirus* was constructed, using ORF1 sequences of thetatorqueviruses as an outgroup.

The best amino acid substitution model for each phylogeny was determined using ProtTest 3 [36]. The phylogenetic trees were inferred using PhyML 3 [37] with aLRT branch support. Branches with <0.8 posterior support were collapsed using TreeGraph2 [38]. All pairwise identities (genome-wide and amino acid) were determined using SDT v1.2 [39].

Results and discussion

Among the *de novo* assembled contigs, we identified 192 viral contigs (>1000 nt). Of these, six were identified to be related to anelloviruses, 39 to cressdnaviruses, and 147 to bacteriophages (microviruses and caudoviruses). We selected the largest contigs (>1500 nt) that had similarities to anelloviruses ($n=2$) and cressdnaviruses ($n=8$) and designed abutting primer pairs (Table 2) to screen and amplify the genomes from the 44 scat samples.

We were able to detect, amplify, and clone the viral genomes from five of the 44 samples, i.e., sample ID T05_2F02 ($n=6$), N10_S01 ($n=1$), N07_S03 ($n=1$), N06_S02 ($n=1$), V16_S08 ($n=2$) (see Table 3 for summary and Fig. 1). Of the 11 viral genomes that were detected, nine were from members of the phylum *Cressdnaviricota* (three from the family *Circoviridae* and six from unclassified

Table 2 List of viruses identified with their GenBank accession numbers and the coyote scat sample information

Scat sample ID	Latitude	Longitude	Sampling date	Family/group	Virus name	GenBank accession no.
T05_2F02	33.54753	− 111.602	17 Dec 2021	*Anelloviridae*	Banfec anellovirus 1	OQ599920
				Anelloviridae	Banfec anellovirus 2	OQ599921
				Cluster 2	Banfec virus 2	OQ599926
				Cluster 3	Banfec virus 1	OQ599925
				Cluster 4	Banfec virus 4	OQ599928
				Cluster 4	Banfec virus 5	OQ599929
N10_S01	33.40255	− 112.178	24 Apr 2021	*Circoviridae*	Banfec circovirus 1	OQ599922
N07_S03	33.41004	− 112.142	23 Apr 2021	*Circoviridae*	Banfec circovirus 1	OQ599923
N06_S02	33.41114	− 112.129	23 Apr 2021	*Circoviridae*	Banfec circovirus 2	OQ599924
V16_S08	33.38929	− 112.245	26 Apr 2021	CRESSV1	Banfec virus 3	OQ599927
				Cluster 1	Banfec virus 6	OQ599930

Table 3 Summary of the rolling-circle replication (RCR) endonuclease motifs (motifs I, II, and III) and the superfamily 3 (SF3) helicase motifs (Walker A, Walker B, and Motif C) of the cressdnaviruses

Family/group	Virus	Accession no.	Motif I	Motif II	Motif III	Walker A	Walker B	Motif C
Circoviridae	Banfec circovirus 1	OQ599922	CFTVNN	PHLQG	QNQAYCQK	GPPGVGKTRYAV	ILDDF	ITSN
	Banfec circovirus 1	OQ599923	CFTVNN	PHLQG	QNQAYCQK	GPPGVGKTRYAV	ILDDF	ITSN
	Banfec circovirus 2	OQ599924	CFTINN	PHLQG	SNRDYCSK	GPPGCGKSRLCF	ILDDF	ITSN
Unclassified CRESSV1	Banfec virus 3	OQ599927	CVTLFK	IHWHL	EAIAYAKK	GAAGSGKSHHCW	WFDEF	ISTV
Unclassified cluster 1	Banfec virus 6	OQ599930	CFTLNN	PHLQG	QASNYCKK	GKSGVGKTRFAV	IIDDF	ITCE
Unclassified cluster 2	Banfec virus 2	OQ599926	VITWNN	HHIQG	DCILYCRK	GRPGTGKSRWAR	IIDDF	VTSN
Unclassified cluster 3	Banfec virus 1	OQ599925	QFTLNQ	KHMHI	QNIAYIEK	GDTGCGKTYNAY	VIEEF	ICSI
Unclassified cluster 4	Banfec virus 4	OQ599928	AFTLNN	PHLQG	QNKKYCSK	GPARKGKTNKAM	VFDDF	FTSI
	Banfec virus 5	OQ599929	AFTVNN	AHLQG	QNKKYCTK	GPARKGKTRRAM	IFDDF	FTSI

cressdnaviruses), and two were from members of the family *Anelloviridae*. It is noteworthy that we amplified two circovirus isolates sharing 97.4% identity using the same primer pair from two samples (N10_S01 and N07_S03) collected a day apart.

We were able to use the primer pairs on only a subset of the samples because some of the scat samples had been degraded due to prolonged exposure to the dry and hot climate in Phoenix prior to sampling. Notably, six viruses were identified from sample ID T05_2F02, which was noted for being particularly wet at the time of collection compared to the other scat samples used for analysis, suggesting that recoverability of viral signatures may be a function of the time since deposition, or at least the moisture of the sample. A diet analysis showed that sample T05_2F02 contained evidence of Neotominae species (likely *Neotoma* or *Peromyscus*), N10_S01 had desert cottontail rabbit (*Sylvilagus audubonii*), Neotominae, and avian species, N07_S03 had desert cottontail rabbit and Chaetodipus species, and N06_S02 and V16_S08 primarily had desert cottontail rabbit.

The viral sequences identified here have been deposited in the GenBank database under the accession numbers OQ599920-OQ599930, and their names are derived from the term “ban” for the O’odham word for coyote and “fec” as an abbreviation for "feces" (Table 2).

Cressdnaviricota

Cressdnaviruses (phylum *Cressdnaviricota*) are circular Rep-encoding single-stranded DNA viruses that infect a broad range of eukaryotic species [40]. The Rep protein has a HUH endonuclease domain and a superfamily 3 helicase domain, which are essential for initiating rolling-circle replication [41–44]. Within the phylum *Cressdnaviricota*, there are 12 established families (*Amesuviridae*, *Bacilladnaviridae*, *Circoviridae*, *Geminiviridae*, *Genomoviridae*, *Metaxyviridae*, *Nanoviridae*, *Naryaviridae*, *Nenyaviridae*, *Redondoviridae*, *Smacoviridae*, and *Vilyaviridae*) with six additional informal groupings of viruses labeled CRESSV1-6 [40, 45, 46] (Fig. 2). Many cressdnaviruses have been identified but not yet classified [47, 48].

Because the Rep protein is the most conserved protein among cressdnaviruses, SSN analysis has been used in the past to roughly group Rep sequences of new viruses at the family level with a network threshold of 60 [47, 49–51]. Based on SSN analysis using EFI [30], the nine Rep sequences of the cressdnaviruses identified in this study fall into clusters with those of members of the family *Circoviridae* (n = 3) and the clusters CRESSV1 ($n = 1$), cluster 1 ($n = 1$), cluster 2 ($n = 1$), cluster 3 ($n = 1$), and cluster 4 ($n = 2$) (Fig. 2). The CRESSV1 group has been described previously and mentioned in literature but is currently not classified [40, 45, 46]. In the Rep sequences of these nine cressdnaviruses, we identified the three HUH endonuclease domains and the three SF3 helicase domains (Table 3).

Circoviridae

Circoviruses (family *Circoviridae*) are classified into two genera: *Circovirus* and *Cyclovirus* [52, 53]. Members of the genus *Circovirus* have been found to be associated with various diseases in avian and mammal species, e.g., psittacine beak and feather disease caused by beak and feather disease virus [54] and postweaning multisystemic wasting syndrome caused by porcine circovirus 2 [55]. Approximately a decade ago, canine circovirus 1 was identified and associated with hemorrhagic enteritis in canines, and it appears to have become widespread globally [56–61]. In general, circoviruses have small circular ssDNA genomes of ~1.7-2.2 kb encoding a capsid protein (CP) and a Rep protein [52]. The species demarcation threshold for circoviruses is 80% pairwise identity [43].

From the scat (sample IDs N10_S01, N07_S03, and N06_S02), we identified three circovirus genomes (two banfec circovirus 1 genomes [OQ599922 and OQ599923] and one banfec circovirus 2 genome [OQ599924]). The two

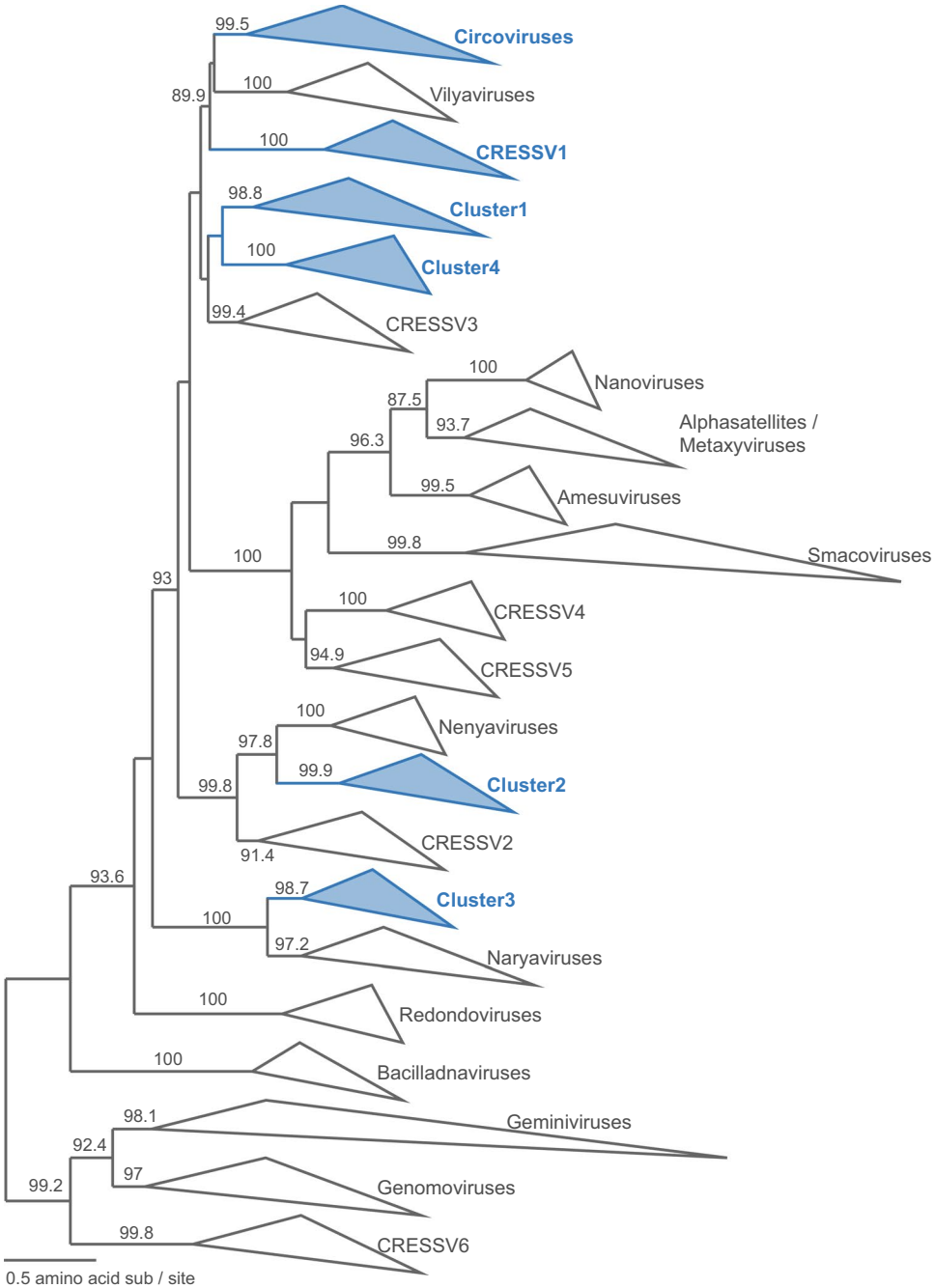


Fig. 2 Maximum-likelihood phylogenetic tree (inferred using the Q.pfam+F+G4 substitution model) based on Rep sequences of representative classified cressdnaviruses, CRESSV1-6, the four clusters of unclassified Rep, and the sequences from this study. The Rep sequences of the cressdnaviruses from this study that fall within the family level clusters are indicated in bold blue font.

banfec circovirus 1 genomes (1680 nt) share 97.4% pairwise nucleotide sequence identity. When compared to other circoviruses, banfec circovirus 1 shares 97.1-97.8% pairwise nucleotide sequence identity with the virus D1_1542 (MW678871), which was identified in aerosolized dust particles in Arizona, USA [62], and 71.3-71.9% with calfel virus LSF45_cir359 (ON596197), which was identified in a fecal sample from a bobcat (*Lynx rufus*) in California, USA [63].

On the other hand, the genome of banfec circovirus 2 (2079 nt) shares 70.8% pairwise nucleotide sequence identity with dipodfec virus UA04Rod_4537 (OM869597) identified in a Merriam's kangaroo rat (*Dipodomys merriami*) fecal sample from Arizona, USA [51], and 69.1% with a canine circovirus (KC241982) identified in the liver of a domestic dog (*Canis lupus familiaris*) [58].

Banfec circoviruses 1 and 2 represent two new species based on the 80% species demarcation criteria outlined for circoviruses [52]. It is also noteworthy that the circoviruses identified in this study are most closely related to other circoviruses from the Southwest of the USA. However, this may be due to a gap in our knowledge of circovirus diversity.

Phylogenetic analysis of the Rep sequences of the circoviruses (Fig. 3) revealed that banfec circovirus 1 forms a clade with viruses from samples of bobcat (*Lynx rufus*) feces collected in California, USA [63] (ON596197), a South China field mouse (*Apodemus draco*) from China (KY370037) [64], a tawny owl (*Strix aluco*) from Italy (OL411978) [65], and a rufous horseshoe bat (*Rhinolophus sinicus*) and an intermediate horseshoe bat (*Rhinolophus affinis*) from China (KJ641723-KJ641724) [66] (Fig. 3). The Rep sequence of banfec circovirus 2 clusters with those of circoviruses identified in samples from a dog (KC241982) [58] in the USA, a bobcat (MT610105) in Mexico [67], and various rodents, i.e., a Merriam's kangaroo rat (OM869597) from Arizona, USA [51], a hoary bamboo rat (*Rhizomys pruinosus*, MF497827) from China [68], and a Siberian jerboa (*Allactaga sibirica*, KY370029), a Chevrier's field mouse

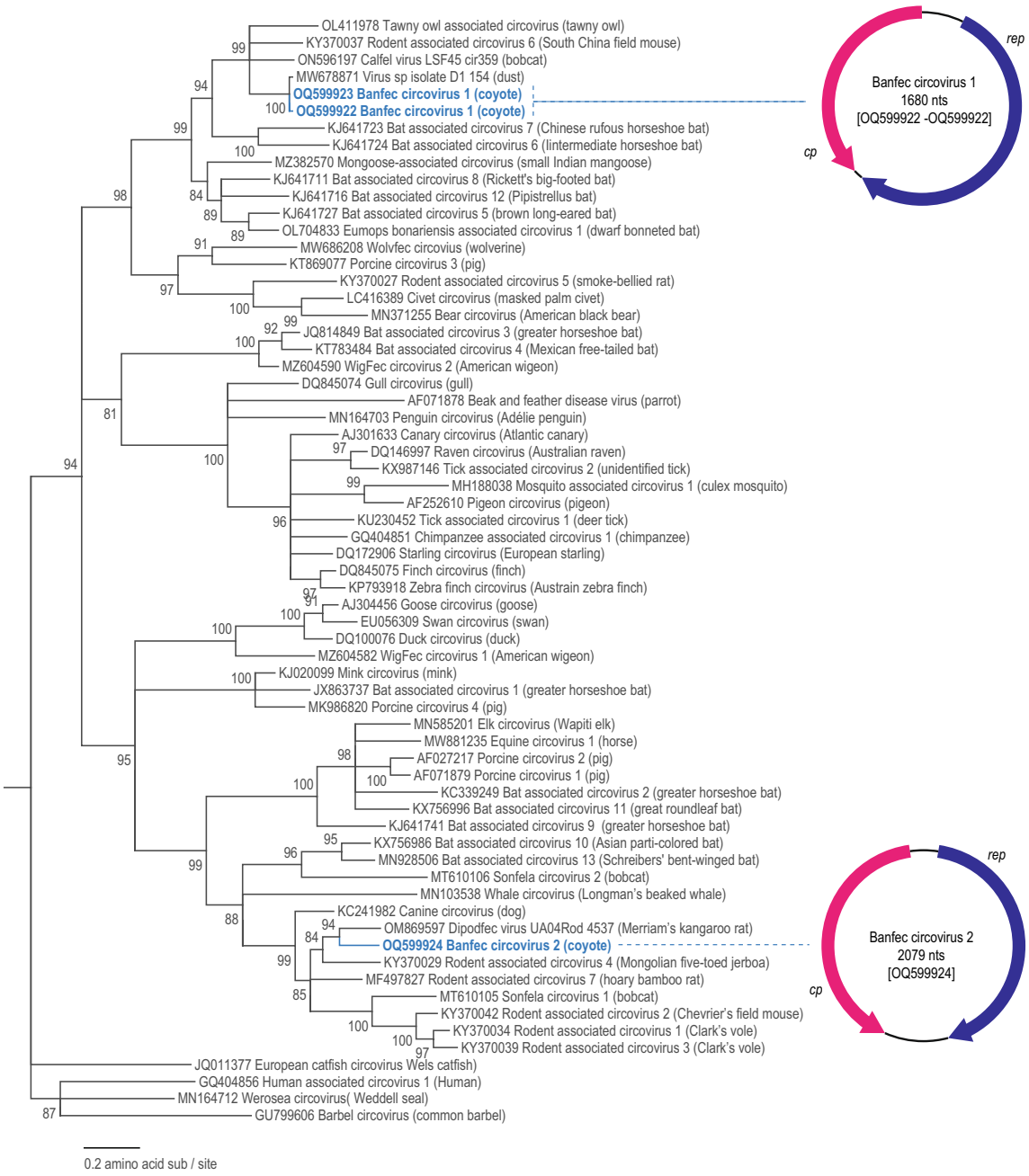
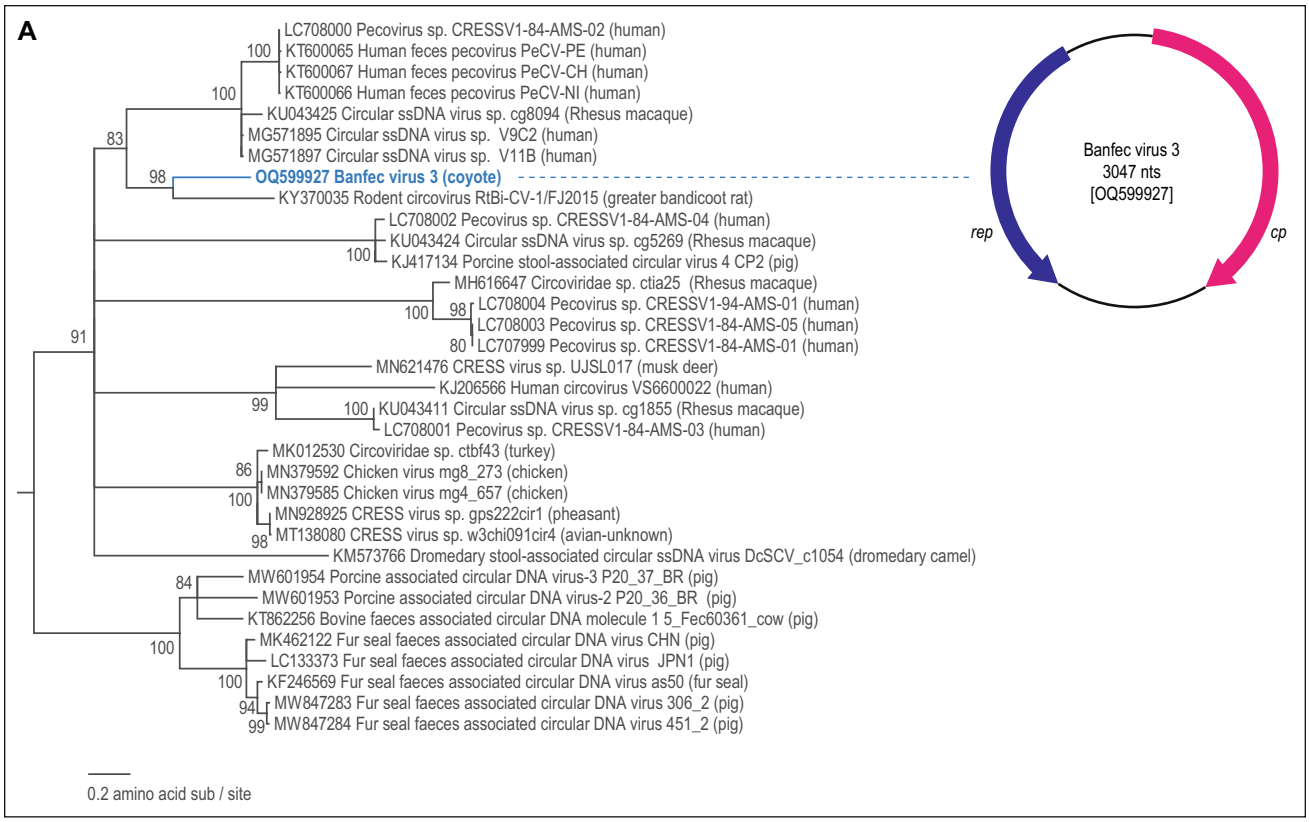
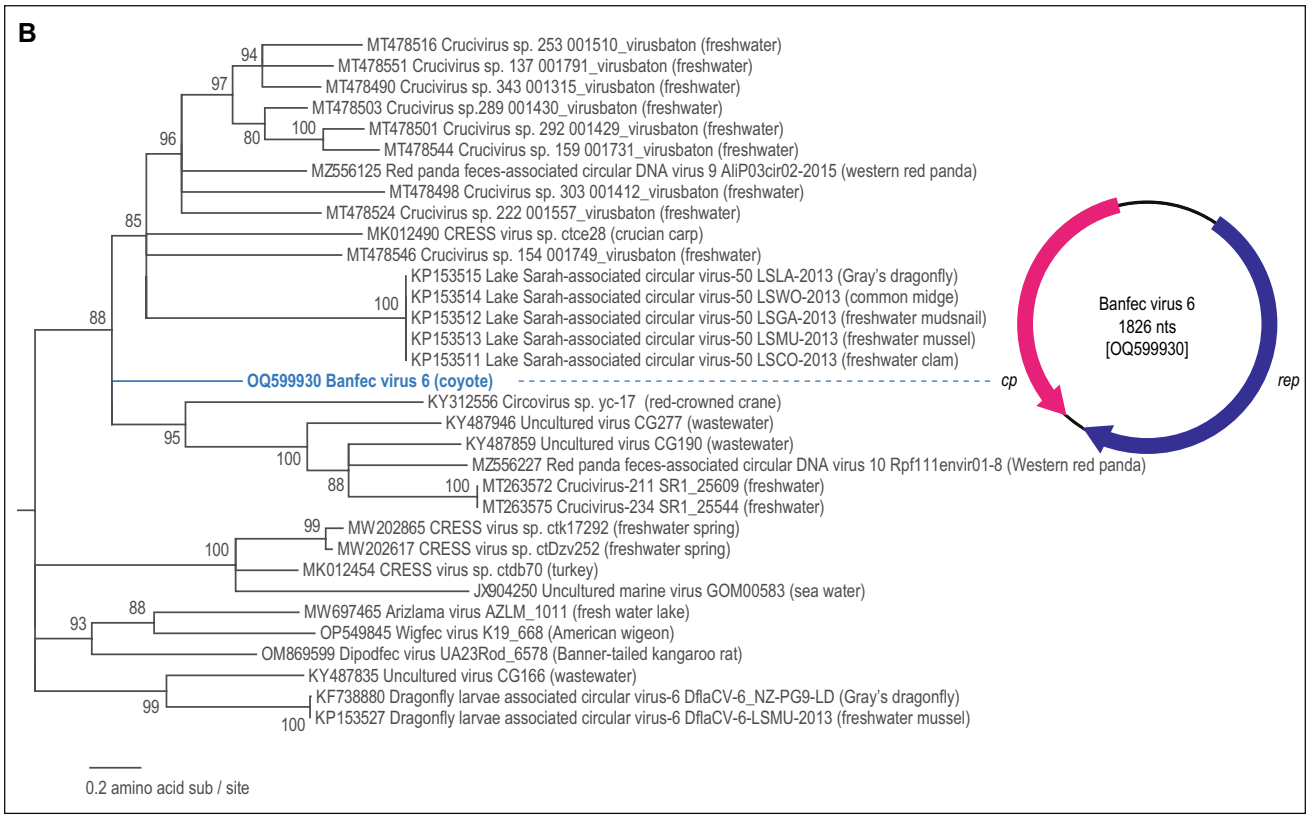


Fig. 3 Maximum-likelihood phylogenetic tree (inferred using the Q.pfam+F+G4 substitution model) based on Rep sequences based on (one per species) of circoviruses and rooted cyclovirus Rep sequences. The sequences from this study are indicated in bold blue font, and the genome organizations of the circoviruses identified in this study are shown to the right of the phylogenetic tree. Common names of the sources/hosts of the viruses are shown in parentheses next to the virus names.





◂**Fig. 4** Maximum-likelihood trees (inferred using the rtREV+G+I substitution model) based on Rep sequences of members of unclassified cressdnavirus cluster CRESSV1 (**A**) and cluster 1 (**B**) rooted with Reps of vilyaviruses. The sequences from this study are indicated in bold blue font, and the genome organizations of the circoviruses identified in this study are shown to the right of the phylogenetic tree. Common names of the sources/hosts of the viruses are shown in parentheses next to the virus names.

(*Apodemus chevrieri*, KY370042) and a Clarke's vole (*Neodon clarkei*, KY370034, KY370039), all from China [64] (Fig. 1).

The similarity of banfec circovirus 1 to the virus D1_1542 (MW678871) from Arizona suggests that this virus is circulating in animals in Arizona and was inadvertently sampled, likely due to aerosolization of animal fecal matter, by Finn et al. [62]. Furthermore, the similarity to calfel virus LSF45_cir359 (ON596197) from a bobcat [63] provides some support that this may be a virus that is associated with canids, at least in the Southwest of the USA. Given that banfec circovirus 2 is distantly related to known circoviruses, although it clusters with primarily rodent-derived circoviruses, it is likely that this is a rodent circovirus that was present in the prey of the coyote.

Unclassified cressdnaviruses

CRESSV1

The Rep sequence of banfec virus 3 from scat V16_S08 (Table 3) clustered with those of viruses in CRESSV1. Banfec virus 3 (3047 nt; OQ599927) encodes a Rep and a CP (Fig. 4). BLASTn analysis showed that its CP shares 68.51% identity (22% genome coverage; E-value, 2E-61) with the genome of rodent circovirus RtBi-CV-1/FJ2015 (KY370035), derived from a greater bandicoot rat (*Bandicota indica*) in China [64], and its Rep shares 56.9% amino acid sequence identity. The Rep phylogeny showed that it clusters with viruses from human feces from Chile (KT600067) [69], the Netherlands (LC708000) [48], Nicaragua (KT600066) [69], Peru (KT600065) [69], and Venezuela (MG571895, MG571897) [70] and a virus from rhesus monkey feces (*Macaca mulatta*, KU043425) [71] (Fig. 4). Banfec virus 3 would represent a new species in a genus with rodent circovirus RtBi-CV-1/FJ2015 when the viruses in CRESSV1 get assigned to a new family.

Cluster 1

Banfec virus 6 (1826 nt, OQ599930), which encodes a Rep and CP and was identified in scat V16_S08 (Table 3), is part of cluster 1 based on Rep amino acid sequences. A BLASTn analysis revealed that it shares 70.96% sequence identity (16% coverage; E-value, 3E-30) with CRESS virus isolate ctdb70 (MK012454) from turkey (*Meleagris gallopavo*) tissue [47]. The Rep of banfec virus 6 shares <58% pairwise amino acid sequence identity will all other Rep proteins within this cluster. Phylogenetically (Fig. 4), the Rep of banfec virus 6 clusters with those of viruses derived mainly from wastewater in the USA (KY487946, KY487959) [72], water samples from France (MT478490, MT478498, MT478501, MT478503, MT478516, MT478524, MT478544, MT478551, MT478572, MT478575) [73], freshwater invertebrates from Lake Sarah in New Zealand [74], a crucian tissue (*Carassius* sp.) (MK012490) [47], red panda feces in China (*Ailurus fulgens*, MZ556125, MZ556127) [75], and red-crowned crane (*Grus japonensis*, KY3122556) [76].

Cluster 2

Banfec virus 2 (4332 nt, OQ599926) derived from scat T05_2F02 (Table 3) contains three large ORFs in its genome in addition to the Rep and CP genes, and it belongs to cluster 2. The genome of banfec virus 2 shares 68.54% sequence identity (4% coverage; E-value, 2E-06) with human fecal virus Jorvi2 (MF118167) [77]. The Rep of banfec virus 2 shares <44% amino acid sequence identity with those of the other members of this cluster. This cluster consists of Rep-like sequences of circular DNA molecules derived from desert tortoise (*Gopherus* sp.) feces from the USA (MK858259, MK858260) [78], viruses from human feces from Finland (MF118167) [77] and the Netherlands (LC708032, LC708033) [48], striped dwarf hamster (*Cricetulus barabensis*) from China (KY370030, KY370031) [64], and Chevrier's field mouse (*Apodemus chevrieri*), also from China (KY370041) [64] (Fig. 5). A BLASTp analysis of the Rep of banfec virus 2 revealed that it shares 40.89-43.75% amino acid sequence identity with Rep-like sequences (KAH0785549, KAH0792676, KAH0797972, KAH0785997) in the genome of a parasitic protozoan, *Histomonas meleagridis*, derived from turkey (*Meleagris gallopavo*) in Austria (KAH0785549) [79]. *Histomonas meleagridis* is believed to be the cause of black histomoniasis (otherwise known as blackhead disease) in galliforms such as chickens (*Gallus*) [80]. Thus, it is likely that banfec virus 2 may infect a closely related protozoan.

Cluster 3

Based on the Rep analysis, banfec virus 1 (1957 nt, OQ599925) identified in scat ID T05_2F02 (Table 3) clusters with 11 other cressdnaviruses in cluster 3. This cluster includes viruses identified from oral swabs of chickens (*Gallus gallus*) from Georgia, USA (MN379584, MN379586, MN379589, MN379591) [49], a four-spotted chaser (*Libellula quadrimaculata*) from Arizona, USA (KM598400) [81], feces from a Mexican free-tailed bat (*Tadarida brasiliensis*)

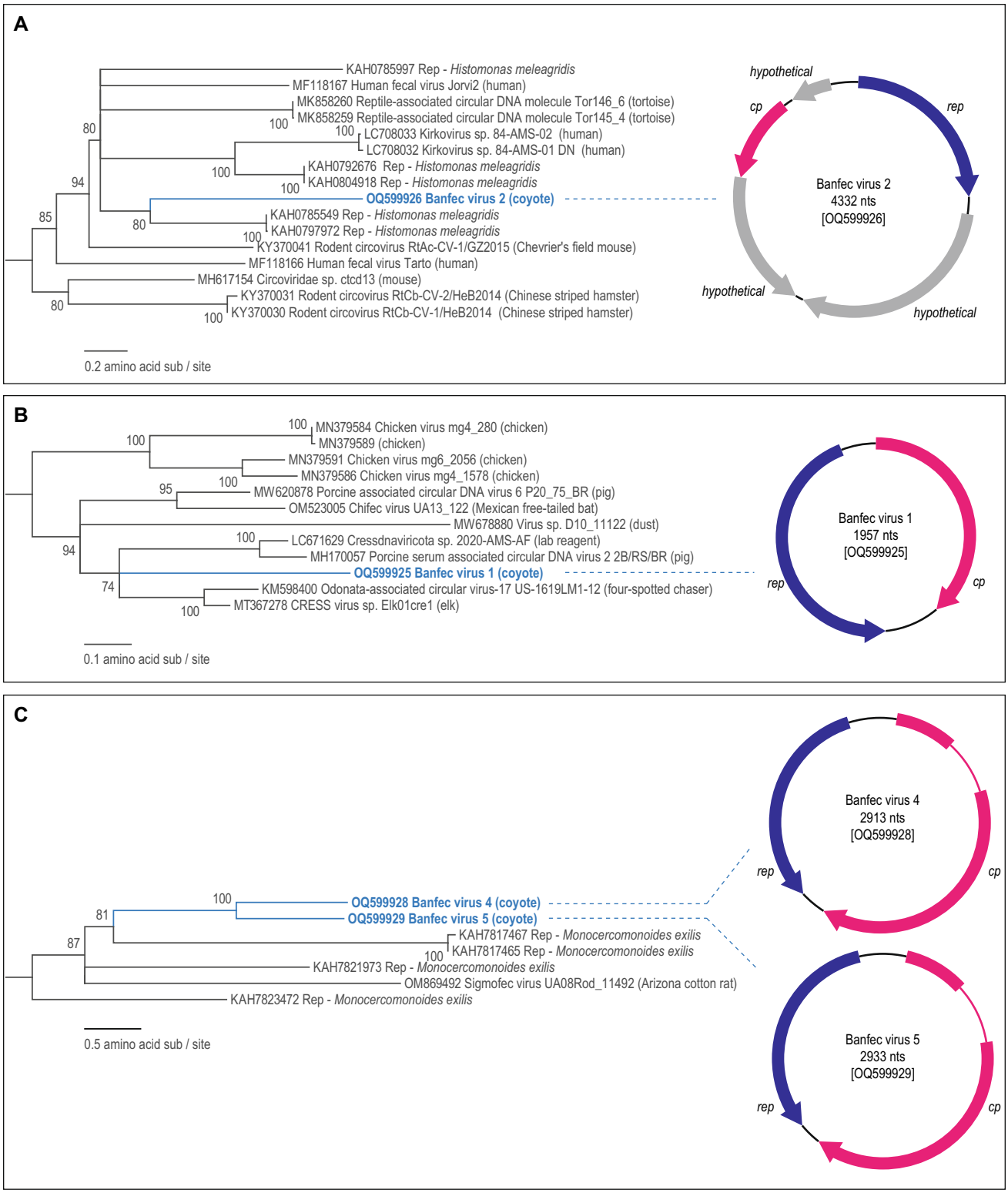


Fig. 5 Maximum-likelihood trees based on Rep sequences of unclassified cressdnavirus in cluster 2, including *Histomonas meleagridis* Rep-like sequences (inferred using the rtREV+G+I substitution model) rooted with vilyaviruses (**A**), cluster 3 (inferred using the rtREV+G+F substitution model) rooted with Rep sequences of narnaviruses (**B**), and cluster 4, including *Monocercomonoides exilis* Rep-like sequences (inferred using the VT+G+I substitution model) rooted with Rep sequences of cycloviruses (**C**). The sequences from this study are indicated in bold blue font, and the genome organizations of the circoviruses identified in this study are shown to the right of the phylogenetic tree. Common names of the sources/hosts of the viruses are shown in parentheses next to the virus names.

in Arizona, USA (OM523005) [50], porcine serum from Brazil (MH170057) [82], dust particles from Arizona, USA (MW678880) [62], and a virus identified as a reagent contamination in a dataset of mycoviruses of a pathogenic fungus found in humans in the Netherlands (LC671629) [83] (Fig. 5). The genome of banfec virus 1 shares 69.25% sequence identity (42% coverage; E-value, 2E-52) with the genome of porcine serum associated circular DNA virus 2 - 2B/RS/BR (MH170057) from porcine serum [82]. The Rep of banfec virus 1 has 57% sequence identity to that of MT367278, a virus identified in elk feces in China (Fig. 5).

Cluster 4

Cluster 4 is composed of three viruses: banfec virus 4 (2913 nt, OQ599928) and banfec virus 5 (2933 nt, OQ599928), derived from scat T05_2F02 (Table 4), and sigmofec virus UA08Rod_11492 (3200 nt, OM869492) from a fecal sample of Arizona cotton rat (*Sigmodon arizonae*) [51]. The genomes of banfec virus 4 and 5 share ~65.7% genome-wide sequence identity, and their CPs appear to have an intron of ~220 nt (Fig. 5). They share 59.3-62.6% sequence identity with the genome of sigmofec virus UA08Rod_11492. A BLASTp analysis of the Rep proteins of all three viruses in this cluster revealed that they share 37.35-43.38% amino acid sequence identity with Rep-like sequences (KAH7821972, KAH7821973, KAH7817465, KAH7817467) and 33.81-54.79% identity with CP-like sequences (KAH7817464, KAH7817468, KAH7817480, KAH7815554, KAH7815557) from the protozoan *Monocercomonoides exilis*, an obligate parasitic protozoan found in mammal, reptile, and insect digestive tracts [84]. *Monocercomonoides exilis* strain PA203 was isolated from a long-tailed chinchilla (*Chinchilla lanigera*) in the Czech Republic [84].

Anelloviridae

Anelloviridae is a family of single-stranded DNA viruses comprising 30 genera [85]. Their genomes range in size from 1.6 to 3.9 kb [86, 87]. Anelloviruses infect a wide variety of animals, including primates, rodents, birds, and mammalian carnivores [85]. Anellovirus genomes contain at least three large open reading frames (ORFs), and the largest of these encodes a capsid protein [87, 88]. ORF1 is the most conserved ORF in anelloviruses and thus is used in their classification. The species demarcation threshold is 69% pairwise identity in ORF1, whereas genera are distinguished based on the ORF1 amino acid sequence phylogeny [85].

The genomes of two anelloviruses, banfec anellovirus 1 (2471 nt, OQ599920) and banfec anellovirus 2 (2479 nt, OQ599921) were identified and amplified from scat sample T05_2F02 and found to share 65.6% nucleotide sequence identity. These two genomes share sufficient identity within the ORF1 region to be considered members of the genus *Wawtorquevirus*. The ORF1 of banfec anellovirus 1 shares 93.5% nucleotide sequence identity with neotofec virus RodL2_5 (OM869618), whereas banfec anellovirus 2 shares 74.5% identity with neotofec virus RodL2_6 (OM869617) (Fig. 6). Both neotofec virus RodL2_5 and RodL2_6 were identified in liver samples of white-throated woodrat (*Neotoma albigula*) from Arizona [51]. Based on the species demarcation criteria for anelloviruses [85], banfec anellovirus 1 and neotofec virus RodL2_5 represent a new species, and banfec anellovirus 2 and neotofec virus RodL2_6 represent another new species, as their ORF1 nucleotide sequences share less than 69% identity with those of any of the other wawtorqueviruses.

All but one of the wawtorqueviruses have been identified in rodent samples, which is a clear indication that banfec anellovirus 1 and 2 were most likely derived from a rodent meal. The exception within this genus, mosquito VEM anellovirus SDRB A (HQ335084) [89], was likely acquired via a blood meal from a rodent (Fig. 6). The two anelloviruses identified here were from sample T05_2F02, in which there was evidence of Neotominae species remains, and this provides further support for the assumption that the source of these two viruses was a rodent that was consumed by the coyote.

Conclusion

From five fecal samples of coyotes, we identified 11 small circular DNA viruses. Of these, we identified three circoviruses representing two new species. The two banfec circovirus 1 genomes share ~97% identity and are most closely related to a circovirus in aerosolized dust from Arizona, suggesting that closely related viruses are circulating in mammals in Arizona. These three circoviruses cluster with other circoviruses from various mammals and an owl. Banfec circovirus 2 clusters with circoviruses from various rodents and carnivores (sonfela circovirus 1 from a felid and canine circovirus from canines). At this stage, we are unable to ascertain whether banfec circovirus 1 or banfec circovirus 2 actually infects coyotes or is merely associated with their diet. Screening of blood or tissue samples would presumably help to identify the tissues and organisms that are infected by these circoviruses. Banfec viruses 1-6 all represent new species of cressdnaviruses in at least five family-level groupings, highlighting the unexplored diversity of cressdnaviruses in these ecosystems. The two anelloviruses that represent two new species in the genus *Wawtorquevirus* are likely derived from rodent prey of the coyote. Furthermore, three of the viruses identified here (banfec virus 1, banfec virus 4, and banfec virus 5), based on amino acid sequence

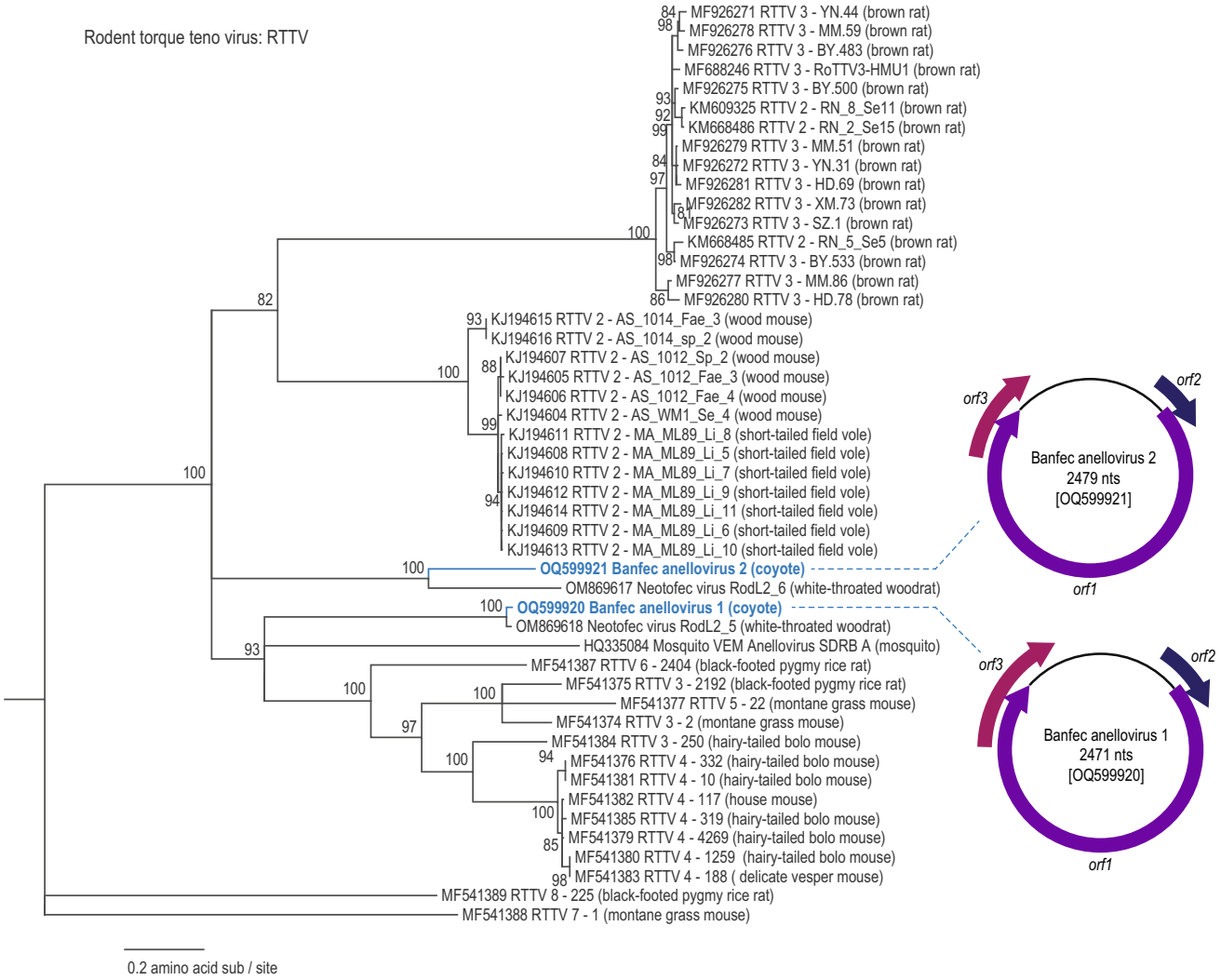


Fig. 6 Maximum-likelihood tree (inferred using the LG+G substitution model) based on ORF1 protein sequences of anelloviruses in the genus *Wawtorquevirus*, rooted with members of the genus *Thetatorquevirus*. The sequences from this study are indicated in bold blue font, and the genome organizations of the circoviruses identified in this study are shown to the right of the phylogenetic tree. Common names of the sources of the viruses are shown in parentheses next to the virus names.

similarity to predicted proteins of two protists (*Histomonas meleagridis* and *Monocercomonoides exilis*), are likely to infect enteric protists of coyotes or their prey. Endogenized Rep-like sequences have been identified in the genomes of various organisms [90–92], suggesting virus-host interactions in their evolutionary history.

This study focused on viruses identified in coyote scats associated with a major metropolitan area; however, scat samples were collected in regions of relatively low levels of urbanization on the outskirts of a highly urbanized area (i.e., Phoenix and adjacent cities). Coyotes can travel many kilometers on a given day, and thus, the pathogens detected in this study could have originated within either lower or higher levels of urbanization. Further work could include sampling of pathogens in coyote scats across the entire gradient of urbanization and relate viruses to specific animals with telemetry collars to track their movements and better understand how habitat use and diet relate to pathogens in wildlife.

Although we are unable to determine whether any of the cressdnaviruses actively infect coyotes, the genome sequences of the two circoviruses can be used to develop molecular probes for screening coyote tissue or blood samples for pathogens, either in the wild by trapping or in natural history collections. Overall, using non-invasive sampling of a native predator found in urban environments, this study provides sequence information on some of the small circular DNA viruses circulating in coyotes within the Greater Phoenix region of Arizona, USA.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-023-05937-w.

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Data availability The sequences described in this study have been deposited in the GenBank database under accession numbers OQ599920-OQ599930.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Research involving human participants and/or animals The research did not involve human participants or animals. This study is a non-invasive study sampling feces of coyotes.

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