Archives of Virology
https://doi.org/10.1007/s00705-022-05574-9

ANNOTATED SEQUENCE RECORD

Discovery of three cycloviruses in fecal samples from silver-haired bats (*Lasionycteris noctivagans*) in Arizona (USA)

Ciara Harding[1,2] · Brendan B. Larsen[3,4] · Sophie Gryseels[3,5,6] · Hans W. Otto[3] · Crystal Suazo[1,2] · Simona Kraberger[1] · Nathan S. Upham[2] · Michael Worobey[3] · Koenraad Van Doorslaer[7] · Arvind Varsani[1,2,8]

Received: 21 March 2022 / Accepted: 3 August 2022

Abstract

Bats harbour a diverse array of viruses, some of which are zoonotic, and are one of the most speciose groups of mammals on earth. As part of an ongoing bat-associated viral diversity research project, we identified three cycloviruses (family *Circoviridae*) in fecal samples of silver-haired bats (*Lasionycteris noctivagans*) caught in Cave Creek Canyon of Arizona (USA). Two of the three identified genomes represent two new species in the genus *Cyclovirus*. Cycloviruses have been found in a wide range of environments and hosts; however, little is known about their biology. These new genomes of cycloviruses are the first from silver-haired bats, adding to the broader knowledge of cyclovirus diversity. With continuing studies, it is likely that additional viruses of the family *Circoviridae* will be identified in Arizona bat populations.

Keywords *Circoviridae* · *Cyclovirus* · *Lasionycteris noctivagans*

GenBank accession nos. OM262453 · OM262454 · OM262459

Bats (order Chiroptera) are one of the most abundant and diverse groups of mammals, with 1,448 extant species currently recognized [1, 2]. Bats are associated with an abundant virome and have primarily been studied from the perspective of zoonotic transmission, with an emphasis on coronaviruses, filoviruses, paramyxoviruses, and rhabdoviruses [3–5]. Over the last decade, there has been a significant interest in analyzing bat-associated viruses using viral metagenomic approaches, which has resulted in the identification of numerous novel and known DNA and RNA viruses [4–6]. However, limited discovery work has been done on bat-associated viruses in Arizona [7–9], even through southern Arizona harbors the greatest diversity of bats of any comparably sized region in the United States of America [2].

Our field research was conducted in Madrean evergreen-woodland on Cave Creek in the Chiricahua Mountains (Cochise County, Arizona). To capture bats, we deployed mist nets (2.6, 4, and 6 m; Avinet Inc., Portland, ME) across areas of calm water on sections of Cave Creek where the

Handling Editor Roman Pogranichniy

✉ Arvind Varsani
arvind.varsani@asu.edu

[1] The Biodesign Center for Fundamental and Applied Microbiomics, Center for Evolution and Medicine, 85287-5001 Tempe, AZ, USA

[2] School of Life Sciences, Arizona State University, 85287-5001 Tempe, AZ, USA

[3] Department of Ecology and Evolutionary Biology, University of Arizona, 85721 Tucson, Arizona, USA

[4] Basic Sciences Division, Fred Hutchinson Cancer Research Center, 98102 Seattle, WA, USA

[5] Department of Microbiology, Immunology and Transplantation, Rega Institute, KU Leuven, 3000 Leuven, Belgium

[6] Department of Biology, University of Antwerp, 2000 Antwerp, Belgium

[7] School of Animal and Comparative Biomedical Sciences, The BIO5 Institute, Department of Immunobiology, Cancer Biology Graduate Interdisciplinary Program, UA Cancer Center, University of Arizona, 85724 Tucson, AZ, USA

[8] Structural Biology Research Unit, Department of Integrative Biomedical Sciences, University of Cape Town, 7701 Observatory, Cape Town, South Africa

Published online: 31 August 2022

Table 1 Summary of the primers used to recover the complete genomes of the three cycloviruses, with their GenBank accession numbers and genome lengths as well as their nonanucleotide, HUH endonuclease, and superfamily 3 helicase motifs

				HUH endonuclease				Superfamily 3 helicase		
Virus	Primer pair	Accession no.	Genome length (nt)	Nonanucleotide	Motif I	Motif II	Motif III	Walker A	Walker B	Motif C
Chifec virus UA15_35	F: AGATCGTGTTCATCGCCATCGTTATAGTT R: TAAAGCAAGACTGGGTGACACTGTCGTTT	OM262453	1758	AAGTATTAC	CFTKNN	RHLQGY	QNLTYCSK	GPPGTGKSRRFA	IIDDF	FISSN
Chifec virus UA15_517	F: TTGTACAATTCGTCGGTATTGATGTTAGAG R: ATTTGAAGGTTATGTAAACACAGCATTCGA	OM262454	1779	TAGTATTAC	VYTLNN	PHLQGF	DNQKYCSK	GEPGTGKSRTAL	IIDDF	WITSN
Chifec virus UA15_2320	F: GAGAGGATTTTGAGCAATAGTCCTTGTTTT R: AGTTTGAAGAATTTGGAATCCTACCAAGTC	OM262459	2320	TAGTATTAC	CWTKNN	RHLQCY	QNKDYCSK	GPTRTGKTRLAA	VLDDYIITSN	

tree canopy forced bats into corridors. We recorded species, time of capture, sex, length of right forearm (mm), and weight (g), and collected fecal samples from each bat. All bats were released where they we caught within 15 minutes of capture. On 10 June 2018, we captured 24 bats representing 11 species in mist nets deployed from 19:30 (MST) to midnight. Among those captures were three nonreproductive adult male silver-haired bats (*Lasionycteris noctivagans*), an insectivorous species characterized by blackish to dark brown pelage with a frosting of whitish silver on the back, two upper premolars, short rounded ears, and a dorsally furred interfemoral membrane [10].

Fecal samples collected from each individual silver-haired bat were pooled and processed as described by Male et al. [12]. In brief, the fecal samples were resuspended in 1 ml of SM buffer and homogenized. The homogenate was then filtered sequentially through 0.45- and 0.2-µm syringe filters. Two hundred µl of the filtrate was then used to extract viral DNA using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics, USA). We amplified circular DNA by rolling-circle amplification (RCA), using a TempliPhi 100 Amplification Kit (GE Healthcare, USA), and the amplified DNA was then used to generate an Illumina sequencing library using a TruSeq Nano DNA kit. The library was sequenced on a NovaSeq6000 sequencer at Psomagen Inc. (USA). The resulting raw reads were trimmed using Trimmomatic v0.39 [13] and then assembled *de novo* using metaSPAdes v3.14.0 [14]. The *de novo*-assembled contigs were analyzed using BLASTx (BLAST version 2.11.0) [15] against a viral Refseq protein database (downloaded September 2021). We identified three contigs with circovirus-like sequences (760, 1047, and 2320 nt). In addition, we also identified contigs with similarities to DNA bacteriophages of the family *Microviridae* (n = 1; 4404 nt) and those formerly assigned to the families *Myoviridae* (n = 72, 769–7771 nt), *Podoviridae* (n = 3; 1119–7008 nt), and *Siphoviridae* (n = 51; 767–3265 nt).

In this report, we focus on the three circovirus-like contigs for which, based on the *de novo*-assembled contigs, we designed abutting primer pairs (Table 1) in order to recover these full genomes. The RCA product was used as a template with the specific primer pairs (Table 1) to amplify the full genomes of the three circovirus-like sequences using Kapa HiFi DNA polymerase (Roche Diagnostics, USA) according to the manufacturer's recommendations. The amplicons were resolved by electrophoresis in a 0.7% agarose gel, purified, and cloned into the vector pJET 1.2 (Thermo Fisher Scientific, USA). Competent XL1-Blue *Escherichia coli* cells were transformed with the recombinant plasmids, which were then sequenced by the Sanger method at Macrogen Inc. (South Korea), using primer walking. The Sanger sequences were assembled using Geneious Prime 2021.0.3 (Biomatters Ltd., New Zealand). Open reading frames (ORFs) were identified using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/).

A BLASTn web search against the NCBI nt database of the three complete Sanger-sequenced genomes (1758–2320 nt; OM262453, OM262454, OM262459) revealed that they shared the highest similarity with various cycloviruses of the family *Circoviridae*. *Circoviridae* is a family of single-stranded circular DNA viruses with an ambisense genome organization and two ORFs coding for the capsid protein (Cp) and replication-associated protein (Rep) [16]. There are two genera within this family, *Circovirus* and *Cyclovirus* [16]. Unlike members of the genus *Circovirus* that have been implicated in various diseases in mammals and birds (e.g., postweaning multisystemic wasting syndrome in pigs and psittacine beak and feather disease in parrots), relatively little is known about members of the genus *Cyclovirus* [17]. A feature that distinguishes the genomes of circoviruses and

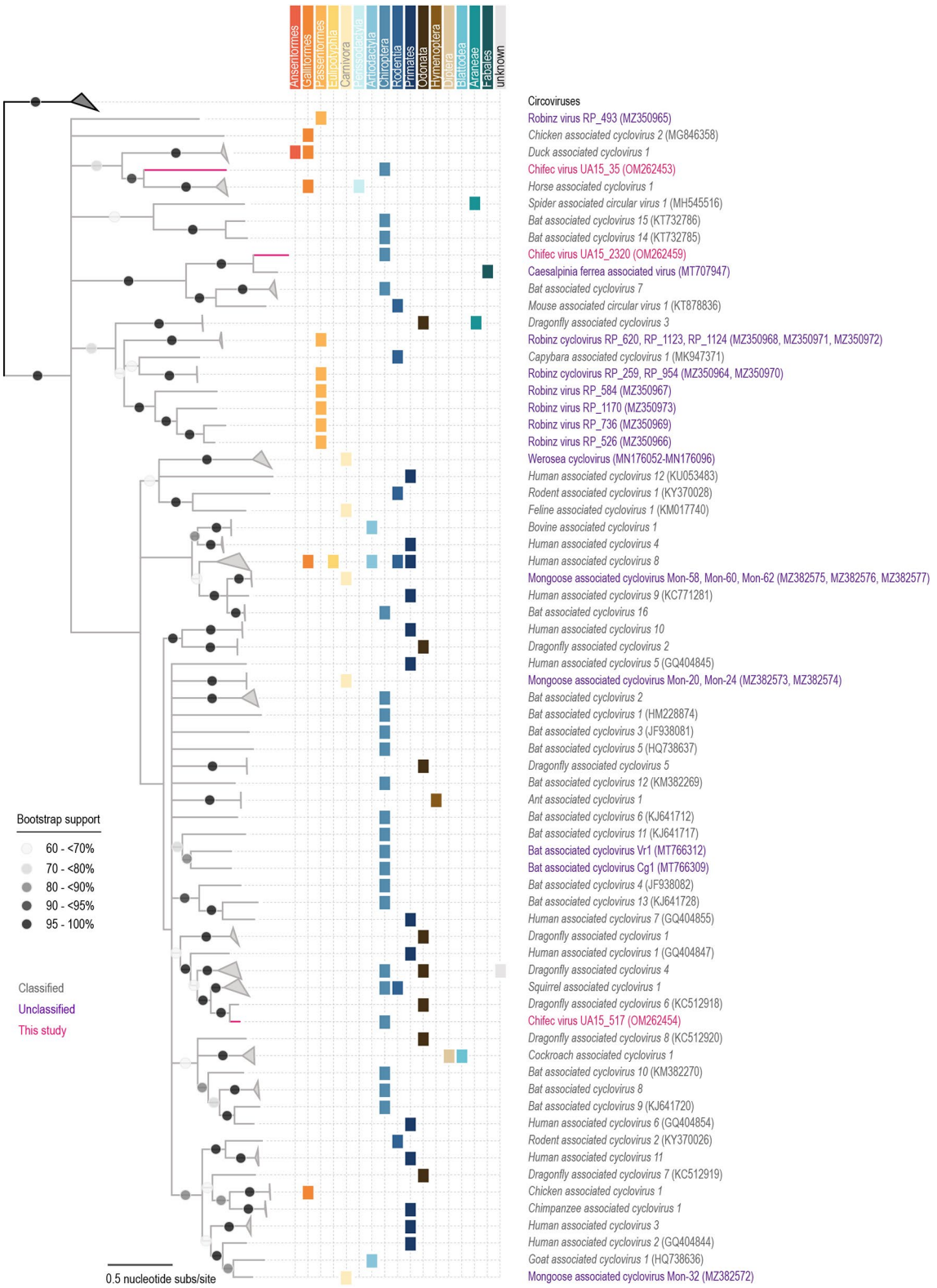

Fig. 1 Maximum-likelihood phylogenetic tree inferred from the alignment of the genome sequences of cycloviruses and rooted with the reverse completement sequences of members of the genus *Circovirus*. The source at the level of order (Anseriformes, Araneae, Artiodactyla, Blattodea, Carnivora, Chiroptera, Diptera, Eulipotyphla, Galliformes, Hymenoptera, Odonata, Passeriformes, Perissodactyla, Primates, Rodentia and Fabales) of the genomes is shown in color-coded boxes. Branches with more members within a species have been collapsed. Accession numbers are provided for unclassified cycloviruses and for species that only has a single member. The phylogenetic tree was rooted with reverse complement genome sequences of representative members of the genus *Circovirus*

cycloviruses is the orientation of the *rep* and *cp* genes relative to the conserved nonanucleotide motif. The *rep* gene is on the virion sense strand, whereas the *cp* gene is on the complementary strand for members of the genus *Circovirus* and vice versa for those of the genus *Cyclovirus* [17]. Although cyclovirus genomes have been identified in various environmental and animal samples [17], including those obtained from members of the orders Anseriformes (n = 1), Araneae (n = 2), Artiodactyla (n = 7), Blattodea (n = 1), Carnivora (n = 52), Chiroptera (n = 29), Diptera (n = 1), Eulipotyphla (n = 7), Galliformes (n = 11), Hymenoptera (n = 3), Odonata (n = 35), Passeriformes (n = 10), Perissodactyla (n = 1), Primates (n = 27), Rodentia (n = 7), and from a plant of the order Fabales (n = 1), no definite hosts have been identified so far, and thus, their biology is unknown.

In the three cyclovirus genomes from silver-haired bats, we identified a conserved nonanucleotide motif, "NAGTATTAC". Additionally, in the Rep sequence, we identified RCR endonuclease and superfamily 3 (SF3) helicase motifs (Table 1). To determine the phylogenetic relationship of the viruses to other cycloviruses, 195 genome sequences of cycloviruses were downloaded from the GenBank database on 10 Jan 2022 and aligned with those from silvered-haired bats identified in this study. Reverse complement genome sequences of two circoviruses (porcine circovirus 1 and 2) were used as an outgroup. These were aligned using MAFFT v7.113 in AUTO mode [18], and the resulting alignment was used to infer a maximum-likelihood phylogenetic tree using PhyML 3.0 [19] with the GTR + I + G substitution model. Branches with less than 60% bootstrap support were collapsed using TreeGraph2 [20]. The phylogenetic tree was visualized and edited in iTOL v6 [21]. The three cyclovirus genomes from silvered-haired bats share ~ 57–60% genome-wide pairwise identity (calculated using SDT v1.2 [22]) and are phylogenetically distinct from each other (Fig. 1; Supplementary Data 1). Chifec virus UA15_517 (OM262454) shares 87% genome-wide pairwise identity with dragonfly cyclovirus 6 (KC512918), which is the sole known member of the species *Dragonfly associated cyclovirus 6* (Supplementary Data 1). Members of the family *Circoviridae* are classified into species based on their genome-wide pairwise identity with a species demarcation threshold of 80% [17]; thus, chifec virus UA15_517 would be a member of the species *Dragonfly associated cyclovirus 6*. Chifec virus UA15_35 (OM262453) shares < 61% identity with all other cycloviruses and clusters phylogenetically with members of the species *Horse associated cyclovirus 1* (Fig. 1; Supplementary Data 1). Chifec virus UA15_2320 (OM262459) shares < 70% identity with all other cycloviruses and clusters phylogenetically with Caesalpinia ferrea associated virus (MT707947) and members of the species *Bat associated cyclovirus 7* and *Mouse associated cyclovirus 1* (Fig. 1; Supplementary Data 1). Chifec virus UA15_35 and UA15_2320, based on the species demarcation threshold, each represent a new species of cycloviruses.

The three cycloviruses identified in the fecal samples of silver-haired bats are the first from this bat species, and only two other cycloviruses from bats have been described in the USA, one from the pallid bat (*Antrozous pallidus*) [23] and one from the Mexican free-tailed bat (*Tadarida brasiliensis*) [24]. Besides these, other cycloviruses have been identified in bat guano and tissue from bats of various species (*Chalinolobus gouldii, Molossus molossus, Myotis* spp., *Nyctalus noctula, Pipistrellus nathusii, Plecotus auritus, Pteropus tonganus, Rhinolophus ferrumequinum, Rhinolophus pusillus, Tadarida brasiliensis, Tylonycteris pachypus, Vespertilio superans*) from Australia, Brazil, China, Hungary, Tonga, and Ukraine [12, 25–28]. Since we detected the cycloviruses in bat feces, we are unable determine whether these are diet-related viruses or ones that truly infect silver-haired bats. Examination of feces across their range has shown that silver-haired bats feed on a variety of insects, including representatives of the orders Lepidoptera, Hemiptera, Coleoptera, Diptera, and Trichoptera [10, 11]. Thus, it is likely that the cycloviruses identified in silver-haired bat feces infect these insects. Nonetheless, given the increased focus on bats as reservoirs of various viruses, it is likely that the identification of novel cycloviruses in bat samples will continue.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-022-05574-9.

Funding The work described here was supported with startup funds awarded to AV from the Arizona State University and funds from Barrett's Honors College (Arizona State University) awarded to CH.

Data availability The sequences described in this study have been deposited in the GenBank database under accession numbers OM262453, OM262454, and OM262459.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Permits All capture and handling of mammals was approved by the University of Arizona Institutional Animal Care and Use Committee (Approval number 15–583) and followed guidelines of the American Society of Mammalogists. A state permit was issued by the Arizona Department of Game and Fish (permit number SP506475).

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

Supplementary Data 1 Pairwise identity matrix of the cycloviruses identified in this study together with to all available cyclovirus sequences in GenBank.

References

1. Burgin CJ, Colella JP, Kahn PL, Upham NS (2018) How many species of mammals are there? J Mammal 99:1–14
2. MMM (2022) Mammal Diversity Database v1.8. Zenodo https://doi.org/10.5281/zenodo.4139818
3. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T (2006) Bats: Important Reservoir Hosts of Emerging Viruses. Clin Microbiol Rev 19:531–545
4. Letko M, Seifert SN, Olival KJ, Plowright RK, Munster VJ (2020) Bat-borne virus diversity, spillover and emergence. Nat Rev Microbiol 18:461–471
5. Van Brussel K, Holmes EC (2022) Zoonotic disease and virome diversity in bats. Curr Opin Virol 52:192–202
6. Tan CW, Yang X, Anderson DE, Wang LF (2021) Bat virome research: the past, the present and the future. Curr Opin Virol 49:68–80
7. Kuzmin IV, Shi M, Orciari LA, Yager PA, Velasco-Villa A, Kuzmina NA, Streicker DG, Bergman DL, Rupprecht CE (2012) Molecular inferences suggest multiple host shifts of rabies viruses from bats to mesocarnivores in Arizona during 2001–2009. PLoS Pathog 8:e1002786
8. Larsen BB, Gryseels S, Otto HW, Worobey M (2022) Evolution and Diversity of Bat and Rodent Paramyxoviruses from North America. J Virol 96:e0109821
9. King KM, Larsen BB, Gryseels S, Richet C, Kraberger S, Jackson R, Worobey M, Harrison JS, Varsani A, Doorslaer KV(2022) Co-evolutionary analysis implicates TLR9 in the restriction of papillomavirus infection. mBio 13:e0005422
10. Kunz TH(1982) Lasionycteris noctivagans. Mammalian Species:1–5
11. Jones JK (1973) Notes on the distribution and natural history of bats in southeastern Montana. Museum, Texas Tech University
12. Male MF, Kraberger S, Stainton D, Kami V, Varsani A (2016) Cycloviruses, gemycircularviruses and other novel replication-associated protein encoding circular viruses in Pacific flying fox (Pteropus tonganus) faeces. Infect Genet Evol 39:279–292
13. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120
14. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA (2012) SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J Comput Biol 19:455–477
15. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
16. Breitbart M, Delwart E, Rosario K, Segales J, Varsani A, Ictv Report C (2017) ICTV Virus Taxonomy Profile: Circoviridae. J Gen Virol 98:1997–1998
17. Rosario K, Breitbart M, Harrach B, Segales J, Delwart E, Biagini P, Varsani A (2017) Revisiting the taxonomy of the family Circoviridae: establishment of the genus Cyclovirus and removal of the genus Gyrovirus. Arch Virol 162:1447–1463
18. Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780
19. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321
20. Stover BC, Muller KF (2010) TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinformatics 11:7
21. Letunic I, Bork P (2019) Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res 47:W256–W259
22. Muhire BM, Varsani A, Martin DP (2014) SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. PLoS ONE 9:e108277
23. Li L, Victoria JG, Wang C, Jones M, Fellers GM, Kunz TH, Delwart E (2010) Bat guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. J Virol 84:6955–6965
24. Li L, Shan T, Soji OB, Alam MM, Kunz TH, Zaidi SZ, Delwart E (2011) Possible cross-species transmission of circoviruses and cycloviruses among farm animals. J Gen Virol 92:768–772
25. Ge X, Li J, Peng C, Wu L, Yang X, Wu Y, Zhang Y, Shi Z (2011) Genetic diversity of novel circular ssDNA viruses in bats in China. J Gen Virol 92:2646–2653
26. Kemenesi G, Kurucz K, Zana B, Foldes F, Urban P, Vlaschenko A, Kravchenko K, Budinski I, Szodoray-Paradi F, Bucs S, Jere C, Csosz I, Szodoray-Paradi A, Estok P, Gorfol T, Boldogh S, Jakab F (2018) Diverse replication-associated protein encoding circular DNA viruses in guano samples of Central-Eastern European bats. Arch Virol 163:671–678
27. Lima FE, Cibulski SP, Dos Santos HF, Teixeira TF, Varela AP, Roehe PM, Delwart E, Franco AC (2015) Genomic characterization of novel circular ssDNA viruses from insectivorous bats in Southern Brazil. PLoS ONE 10:e0118070
28. Wu Z, Yang L, Ren X, He G, Zhang J, Yang J, Qian Z, Dong J, Sun L, Zhu Y, Du J, Yang F, Zhang S, Jin Q (2016) Deciphering the bat virome catalog to better understand the ecological diversity of bat viruses and the bat origin of emerging infectious diseases. ISME J 10:609–620