Title

AMDOPARVOVIRUS INFECTIONS ARE PREVALENT, PERSISTENT, AND GENETICALLY DIVERSE IN ZOO-HOUSED RED PANDAS (AILURUS FULGENS).

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Peer reviewed

AMDOPARVOVIRUS INFECTIONS ARE PREVALENT, PERSISTENT, AND GENETICALLY DIVERSE IN ZOO-HOUSED RED PANDAS (*AILURUS FULGENS*)

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Abstract

Red pandas (*Ailurus fulgens*) are a globally endangered small carnivoran species and subjects of a robust ex situ conservation effort that includes animals housed in zoos. In 2018, red panda amdoparvovirus (RPAV) was discovered by metagenomics analyses of tissues from two geriatric red pandas, and in one case it was associated with significant lesions. Because RPAV was discovered in a single zoo cohort, it was unclear whether these infections represented a widely distributed, enzootic virus of red pandas or a localized 'spillover' from a different host species into this collection. The first goal of this study was to estimate the prevalence of RPAV in US zoos. The authors amplified RPAV from feces of 104 individual red pandas from 37 US zoos, and the virus was detected in 52/104 samples (50.0%). Next, to establish persistence of infection in individual animals, the authors tested serial samples in a single cohort over a 4.5-yr period, and virus was consistently shed by infected animals throughout the sampling period. Finally, full viral coding sequences were amplified and sequenced from three cases, and partial sequences of both the nonstructural and capsid genes were obtained for an additional 19 cases. RPAV is a genetically

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diverse but monophyletic viral species, and multiple viral lineages are present in US zoo–housed red pandas. The authors do not know how red pandas were originally infected, but RPAV is very common in red pandas in the United States, and infections are persistent—presumably for the lifetime of the animal.

INTRODUCTION

Red pandas (*Ailurus* spp.) are small carnivorans native to temperate forests in the Himalayas and western China, and they are the only extant members of the family *Ailuridae*. With wild populations estimated at fewer than 10,000, they are classified as Endangered by the International Union for Conservation of Nature (IUCN) and are listed as Appendix 1 species according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora.[9] Two genetically distinct subpopulations exist: the Himalayan red panda (*Ailurus fulgens*), whose native range includes parts of Nepal, northern India, Tibet, Myanmar, Bhutan, and far-western China, and the Chinese red panda (*Ailurus styani*), native to parts of the Yunnan and Sichuan provinces in western China. While these were historically regarded as subspecies (*Ailurus fulgens fulgens* and *Ailurus fulgens styani*, respectively), this study recognizes recent genetic evidence that these are indeed two separate species.[7,13] The strategy for survival of these species includes an ex situ conservation effort that (as of 3 March 2021) relies on 850 individuals housed in zoos worldwide, 218 of which are in the United States and carefully managed by a Species Survival Plan.[25] Red pandas are charismatic and popular attractions in zoos and serve critical functions in terms of education and as safeguards for the continuity of the species. Understanding disease threats to the captive and free-ranging populations is imperative to ensuring their welfare and continued sustainability.

Amdoparvovirus is a genus in the family *Parvoviridae*.[6,22] They have a simple, small genome that is linear, single-stranded DNA and approximately 5,000 nucleotides long, with two open reading frames (ORFs) flanked by non-coding terminal hairpins.[6] A 5′ nonstructural (NS) ORF encodes proteins that function in replication, and a 3′ capsid (VP) ORF encodes two proteins that comprise the virion.[5,15,23] For over 50 yr, studies on genetics, pathogenesis of infection, and clinical impact of amdoparvoviruses were limited to Aleutian Mink Disease Virus (AMDV), which was first recognized in the 1950s as a cause of a chronic, progressive disease (Aleutian disease) in farmed mink (*Mustela vison*).[10,11] Recently, however, advances in sequencing technology and sensitive detection methods have facilitated a rapid expansion of the *Amdoparvovirus* genus, with novel members discovered in the past 10 yr in gray foxes (*Urocyon cinereoargenteus*),[20] raccoon dogs (*Nyctereutes procyonoides*) and arctic foxes (*Vulpes lagopus*),[24] and striped skunks (*Mephitis mephitis*).[2] Aside from AMDV, which is a significant cause of morbidity and mortality in mink, very little is known about the pathogenesis of amdoparvovirus infections in carnivores. Significant lesions have been attributed to skunk amdoparvovirus infections in striped skunks (*Mephitis mephitis*), and disease association has been suspected but not well established for other members of the genus.[18,20,24]

In 2018, the authors reported[1] the discovery of an amdoparvovirus infecting red pandas from a zoological collection. Species demarcation criteria in the *Parvoviridae* indicate a threshold of <85% amino acid sequence identity in the major nonstructural (NS1) protein for recognition of a novel viral species.[22] Red panda amdoparvovirus (RPAV) shares approximately 75% amino acid sequence identity in this protein with other members of the genus and was thus recognized as a novel species.[22] RPAV was detected by metagenomic analyses of tissues from two geriatric animals that died within a 1-yr period (2015–2016) and was detected during that same time span by PCR in fecal samples from all four healthy co-housed members of the cohort. Based on histopathology and in situ hybridization analyses, RPAV was abundant, strongly associated with inflammation, and considered the cause of death in at least one of the deceased pandas.

Taken together, the findings indicated that RPAV is a novel and potentially pathogenic virus in these endangered species. Amdoparvoviruses are increasingly recognized as multihost pathogens,[3,4] and it is unclear whether RPAV is native to red pandas or a spillover into this collection from a different host species. Establishing the epidemiological context, including prevalence, shedding (possible transmission), and viral genetic characteristics, is critical for interpreting the significance of infection in individual cases and for understanding the possible impact of RPAV on the health of zoo-housed red pandas at the individual and population level. To this end, the authors report a cross-sectional study among zoological collections in the United States and a single-institution longitudinal study with the goals of (1) estimating the prevalence of fecal shedding (potential transmission), (2) establishing the persistence of infection over a 4.5-yr time span in individual animals within a single cohort, and (3) characterizing the phylogenetic relationships and genetic diversity of RPAV. These studies are fundamental to the authors' estimation of the clinical impact of RPAV infection.

MATERIALS AND METHODS

Animals and samples

Fecal samples (~50–200 g) from 104 red pandas were collected by staff at participating institutions, labeled with the date of collection and identifying information (name, institutional identification number, and international studbook number), and shipped on ice to the laboratory. Samples were stored frozen at −20°C until use. Information on sex, subspecies, and date of birth for each red panda were obtained from the Zoological Information Management System[25] database based on identifying information provided by submitting zoos. Depending on the volume of submitted feces, one to six separate 1-g samples from each submission were collected and processed for DNA extraction. Extractions were either performed using a commercial kit (DNEasy Blood and Tissue Kit, Qiagen, Germantown, MD 20874, USA) according to protocols for tissue DNA extraction or were performed by a commercial laboratory (Idexx, West Sacramento, CA 95605, USA).

PCR detection

The target for PCR detection was a 154-nucleotide-long amplicon from the NS gene, as previously described.[1] The 25-μl PCR reactions contained 12.5 μl Apex Hot Start Taq BLUE Master Mix, 0.5 μM of each primer, and approximately 100 ng of purified DNA. Reactions

were performed in a C1000 Touch Thermal Cycler (BioRad, Hercules, CA 94547, USA) with an initial activation step at 95°C for 15 min followed by 35–40 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and elongation at 72°C for 30 s, with a final elongation at 72°C for 10 min. Primers are given in Table 1. Amplicons were evaluated by 1.4% agarose gel electrophoresis. Purified DNA from previously identified, PCR-confirmed RPAV cases were used as positive controls in each amplification, and negative (no template) controls were also included in each run. Samples were considered positive if at least one 1-g extraction yielded a detectable band of the correct size.

Sequencing

Various PCR reactions were used to amplify RPAV genomes in overlapping segments from positive samples. All reaction mixtures were composed as described above, with the exception that for degenerate primers, 1.0 μM was used. Primers used to generate amplicons for sequencing are included in Table 1. In most cases, PCR products were purified using either ExoSap-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA 02451, USA) or the QiaQuick PCR Purification Kit (Qiagen). In some cases, PCR products were cloned into TA TOPO cloning vectors (Thermo Fisher Scientific) and transformed into One Shot Top10 Chemically Competent *Escherichia coli* (Thermo Fisher Scientific) and cultured overnight in liquid media, then prepared for sequencing using the QiaPrep Spin MiniPrep kit (Qiagen). Samples were submitted for bidirectional Sanger sequencing at the UC Davis DNA Sequencing Facility.

Phylogenetic analysis

Sanger sequence traces were inspected and manually edited as necessary, and primer sequences were trimmed. Sequences were aligned in Geneious[16] using the MUSCLE algorithm[8] with default settings. For amino acid sequence comparisons in NS1, the authors reproduced splice patterns in silico based on experimental evidence from AMDV and predictions from SKAV.[2,23] The full NS1 amino acid sequences were compared to previously published RPAV sequences, representative sequences from other amdoparvoviruses, and a canine parvovirus-2 outgroup obtained from GenBank. The sequences used for these comparisons are listed in Table 2. The authors also analyzed partial nucleotide sequences in the NS and VP ORFs from 19 additional cases. Optimal substitution models for each nucleotide data set were determined using the Model Selection function in MEGAX,[17,26] and Maximum Likelihood phylogenies were generated in MEG-AX with 500 bootstrap replicates.

Statistical analysis

Statistical analyses were performed in JMP version 15 (SAS Institute Inc., Cary, NC 27513, USA). Fisher's exact tests were used to evaluate the correlation between infection status and subspecies and sex. A Wilcoxon rank sum test was used to evaluate the correlation between infection status and age. *P*-values of   0.05 were considered statistically significant.

RESULTS

Prevalence

We had previously established that RPAV was detectable in the gastrointestinal tissues and feces of animals in a single zoo cohort.[1] Fecal samples were collected from 104 red pandas representing 37 zoological collections in 23 states. Each zoo submitted samples from one to eight red pandas (mean = 2.8). RPAV was detected in feces from 52/104 animals (50.0%), representing 25/37 zoos tested (67.6%). In 23 zoos, detection was consistent across all animals tested (either all positive or all negative). For 12 collections, RPAV was not detected in any samples. In 11 zoos, RPAV was detected in samples from all animals. In 14 zoos RPAV was detected in some, but not all, animals tested. Results and distributions by subspecies, sex, and age for the animals included in this study are shown in Table 3. Infection status was significantly associated with subspecies, with infections detected in 47/78 (60.3%) Himalayan red pandas (*A. fulgens*) and 5/26 (19.2%) Chinese red pandas (*A. styani*) ($P = 0.0005$). There was no significant difference in infection status by sex ($P = 0.70$). There was a trend toward increased infections in older animals, but this did not reach statistical significance ($P = 0.13$).

Persistence

To evaluate the persistence of fecal RPAV shedding, 422 samples were collected from six *A. fulgens* in a single zoo cohort between 15 January 2016 and 13 July 2020, for a total sampling period of 4.5 yr. Six animals were included in this study. Cases 1 and 2 were geriatric females that died during the sample collection period. Cases 3 and 4 were an 8-yr-old male and a 5-yr-old female, respectively, that remained clinically healthy throughout the sampling period. Cases 5 and 6 were a 1-yr-old female and a 2-yr-old male that were introduced to the cohort during year 2 of sample collection. The subset of samples tested and their PCR results are summarized in Table 4.

RPAV was consistently detectable in feces from cases 1 through 4 throughout the sampling period. Samples from cases 5 and 6 were PCR-negative prior to their introduction to the collection, and RPAV was not detected in their fecal samples collected during their first 2 mon of cohabitation with infected animals (0/23 and 0/32 for cases 5 and 6, respectively). When testing was resumed approximately 1 yr later, both introduced animals were infected, and RPAV was detectable in serial fecal samples (5/10 and 8/10 for cases 5 and 6, respectively).

Genetic diversity

Phylogenetic analysis based on complete NS1 amino acid sequences demonstrated clear monophyletic clustering of RPAV sequences (Fig. 1). Pairwise distances among RPAV sequences approached but did not exceed the species demarcation threshold, with up to 12.3% divergence between the two most divergent RPAV sequences.[22] RPAV amino acid sequences were between 62.0% and 74.4% similar to those of other members of the *Amdoparvovirus* genus. Thus, despite the remarkable sequence diversity among red panda samples, RPAV sequences are members of a single viral species based on established criteria for *Parvoviridae*. New full coding sequences have been added to GenBank under accession

numbers OK533470–OK533472. All sequences obtained for this study came from *Ailurus fulgens*.

We next amplified partial sequences from the NS and VP ORFs from 19 additional cases. The resulting Sanger traces were trimmed for read quality, resulting in an 852-nucleotide-long segment in NS and a 515-nucleotide-long segment of the VP ORF with the high-quality reads that were used for phylogenetic analyses. Phylogeny based on partial NS sequences demonstrated substantial genetic diversity (Fig. 2) and evidence of three genetically distinct RPAV clades (Fig. 2A). Notably, multiple clades were sometimes detectable from animals at the same institution (Fig. 2B). For most cases clade groupings were consistent in both genome segments, but there were several instances in which the NS and VP sequences obtained from individual samples belonged to different RPAV clades (Fig. 3).

DISCUSSION

We demonstrated that RPAV infections are highly prevalent (50%) among zoo-housed red pandas. The authors consider this to be a minimum estimate of the prevalence in this population for several reasons. First, feces from an individual bowel movement were voluminous (30–200 g), and the extracted sample represented only a fraction (1 g) of a sample at a single point in time. Either low quantities of viral shedding or uneven viral distribution in fecal samples may have led to missed cases. Second, quantities of shed virus are likely to be variable depending on diet, behavior, illness, stage of infection, the natural history of the virus, or other factors. Although shedding was consistently detectable among several persistently infected red pandas, negative samples were obtained from at least one animal known to be infected. Thus, sporadic negative samples in infected animals can occur, and clarification of the viral 'life cycle,' including identification of sites of persistence and frequency and quantitation of shed virus, are needed to determine optimal samples and sampling schedules for the most accurate detection of infected animals. Lastly, given the degree of sequence diversity observed in RPAV, the authors recognize that although they targeted a relatively conserved portion of the genome, cases may have been missed due to sequence variation in the primer binding sites of the screening assay. The authors therefore suggest that RPAV may be even more widespread in this population than the results indicate.

The longitudinal cohort study demonstrates that RPAV is persistently (at least >4 yr) shed by healthy red pandas. Red pandas are frequently transferred among US zoos for breeding or management purposes, and it is suggested that these movements are a mechanism by which infections are propagated throughout the zoo-housed population. Indeed, the authors demonstrated two putative new infections after previously PCR-negative red pandas were introduced to an infected cohort. However, infection status could not be fully explained by exposure to other PCR-positive red pandas, since the authors detected multiple instances in which samples from co-housed animals yielded discordant results (i.e., individual collections housed both PCR-positive and PCR-negative red pandas). So while the trend toward increased infections in older animals may reflect a higher number of cumulative exposures or immunosenescence over the course of a lifetime, it is also suggested that infection and viral shedding likely depend on host or viral factors that influence susceptibility or viral production. Given the spectrum of possible clinical

outcomes, identifying the risk factors for infection and transmission will be important steps in clarifying the epidemiology and population-level impact of RPAV in red pandas.[1] It must be underscored, however, that because infection is present most often in healthy animals, the detection of RPAV must be interpreted cautiously with respect to clinical significance.

The phylogenetic analyses in this study indicate that the infections detected represent diverse variants of a single viral species. Because the origins of these infections are unknown, this is critical information for evaluating whether RPAV originated in wild-caught ancestors of the current zoo population or spilled over from a different host into red pandas in zoos. This question remains unresolved, but the widespread distribution and monophyly of RPAV suggest that RPAV is likely either a native virus of red pandas or spilled over many years ago and spread through the zoo population. Multiple genetic lineages were detected, presumably reflecting multiple distinct introductions into the zoo population, either via introductions from wild-caught ancestors of the current population or spillovers from other species acquired in zoos.

Clade groupings based on NS or VP sequences were generally consistent, but topologies did shift in several cases. This indicates the likely presence of recombinant genomes, potentially facilitated by high prevalence, persistence of infections, and management factors that promote novel exposures and close contacts between red pandas from distant collections. The discordant topologies could also plausibly be explained by undetected co-infections resulting in erroneous concatenations, but it is suggested that recombinations would be expected in this setting and could be a mechanism for the generation of novel genotypes with altered virulence or host tropism.

With regard to RPAV origin, either scenario could plausibly explain the significantly higher proportion of cases detected in Himalayan red pandas relative to Chinese red pandas. These populations are maintained separately in zoos, with limited opportunities for contact between them. Thus, if RPAV infections are native to or spilled over into Himalayan red pandas, the Chinese red panda subpopulation may have been relatively protected by their separate management. There are instances in which individual zoos have housed both species, however, and given the remarkable durability of parvoviruses in the environment, there may have been rare opportunities for crossover between subpopulations. A limitation of this study is that all sequences obtained were from Himalayan red pandas, so phylogenetic relationships between viral variants infecting the two subpopulations are unknown. An additional limitation is that for optimal sequence quality, portions of some genomes (~10% of all amplicons) were cloned into plasmid vectors prior to Sanger sequencing, possibly resulting in the inclusion of sequence segments from minor variants. For a subset of these amplicons, the authors sequenced multiple clones and found identical or nearly identical sequences from each (data not shown), but it is acknowledged that this approach does not account for viral intrahost diversity and the possibility of co-infections.

The growing database of RPAV sequence data, including the identification of multiple distinct clades, provides an important starting point for molecular epidemiological studies in this population. More detailed phylogenetic characterizations, including recombination analyses, coupled with medical records, pedigrees, and detailed historical records of

movements among zoo populations will facilitate a better understanding of the transmission dynamics and risk factors for infection in this population to better inform management decisions. Further research should also be focused on determining the prevalence and phylogenetic relationships of RPAV in other zoo cohorts and in free-ranging animals, including longitudinal comparative studies of the two subpopulations, to better understand the natural history of these infections.

Finally, while it must be emphasized that infections are prevalent in clinically normal animals, the consequences of infection are not well understood. In this longitudinal cohort, two red pandas that died within the study period had virus widely distributed in tissues, in one case clearly associated with inflammation. Other amdoparvoviruses are known to be pathogenic in small carnivores, and the possibility that RPAV may be contributing to morbidity or mortality in red pandas must be carefully evaluated.

Acknowledgments:

LITERATURE CITED

1. Alex CE, Kubiski SV, Li L, Sadeghi M, Wack RF, McCarthy MA, Pesavento JB, Delwart E, Pesavento PA. Amdoparvovirus infection in red pandas (Ailurus fulgens). Vet Pathol. 2018;55(4):552–561. [PubMed: 29433401]

2. Canuti M, Doyle HE, P Britton A, Lang AS. Full genetic characterization and epidemiology of a novel amdoparvovirus in striped skunk (Mephitis mephitis). Emerg Microbes Infect. 2017;6(5):e30. [PubMed: 28487558]

3. Canuti M, McDonald E, Graham SM, Rodrigues B, Bouchard É, Neville R, Pitcher M, Whitney HG, Marshall HD, Lang AS. Multi-host dispersal of known and novel carnivore amdoparvoviruses. Virus Evol. 2020;6(2). doi:10.1093/ve/veaa072

4. Canuti M, Todd M, Monteiro P, Van Osch K, Weir R, Schwantje H, Britton AP, Lang AS. Ecology and infection dynamics of multi-host amdoparvoviral and protoparvoviral carnivore pathogens. Pathogens. 2020;9(2). doi:10.3390/pathogens9020124

5. Clemens DL, Wolfinbarger JB, Mori S, Berry BD, Hayes SF, Bloom ME. Expression of Aleutian mink disease parvovirus capsid proteins by a recombinant vaccinia virus: self-assembly of capsid proteins into particles. J Virol. 1992;66(5):3077–3085. [PubMed: 1313919]

6. Cotmore SF, Agbandje-McKenna M, Canuti M, Chiorini JA, Eis-Hubinger A-M, Hughes J, Mietzsch M, Modha S, Ogliastro M, Pénzes JJ, Pintel DJ, Qiu J, Soderlung-Venermo M, Tattersall

P, Tijssen P, and ICTV Report Consortium. ICTV virus taxonomy profile: Parvoviridae. J Gen Virol. 2019;100(3):367–368. [PubMed: 30672729]

7. Dalui S, Khatri H, Singh SK, Basu S, Ghosh A, Mukherjee T, Sharma LK, Singh R, Chandra K, Thakur M. Fine-scale landscape genetics unveiling contemporary asymmetric movement of red panda (Ailurus fulgens) in Kangchenjunga landscape, India. Sci Rep. 2020;10(1):15446. [PubMed: 32963325]

8. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 2004;5:113. [PubMed: 15318951]

9. Glatston A, Wei F, Than Zaw (IUCN SSC Cat SG/Wildlife Conservation Society (WCS), Myanmar Program, Yangon, Myanmar), Sherpa AP. Ailurus fulgens. IUCN Red List of Threatened Species. 2015. https://www.iucnredlist.org/species/714/110023718

10. Gorham JR, Leader RW, Henson JB. The experimental transmission of a virus causing hypergammaglobulinemia in mink: sources and modes of infection. J Infect Dis. 1964;114(4):341–345. [PubMed: 14217934]

11. Hartsough GR, Gorham J. Aleutian disease in mink. Nat Fur News. 1956;28:10–11.

12. Hasegawa M, Kishino H, Yano T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol. 1985;22(2):160–174.13. [PubMed: 3934395]

13. Hu Y, Thapa A, Fan H, Ma T, Wu Q, Ma S, Zhang D, Wang B, Li M, Yan L, Wei F. Genomic evidence for two phylogenetic species and long-term population bottlenecks in red pandas. Sci Adv. 2020;6(9):eaax5751. [PubMed: 32133395]

14. Huang Q, Deng X, Best SM, Bloom ME, Li Y, Qiu J. Internal polyadenylation of parvoviral precursor mRNA limits progeny virus production. Virology. 2012;426(2):167–177. [PubMed: 22361476]

15. Huang Q, Luo Y, Cheng F, Best SM, Bloom ME, Qiu J. Molecular characterization of the small nonstructural proteins of parvovirus Aleutian mink disease virus (AMDV) during infection. Virology. 2014;452–453:23–31.

16. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28(12):1647–1649. [PubMed: 22543367]

17. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 2018; 35(6):1547–1549. [PubMed: 29722887]

18. LaDouceur EEB, Anderson M, Ritchie BW, Ciembor P, Rimoldi G, Piazza M, Pesti D, Clifford DL, Giannitti F. Aleutian disease: an emerging disease in free-ranging striped skunks (Mephitis mephitis) from California. Vet Pathol. 2015;52(6):1250–1253. [PubMed: 25445322]

19. Le SQ, Gascuel O. An improved general amino acid replacement matrix. Mol Biol Evol. 2008;25(7):1307–1320. [PubMed: 18367465]

20. Li L, Pesavento PA, Woods L, Clifford DL, Luff J, Wang C, Delwart E. Novel amdovirus in gray foxes. Emerg Infect Dis. 2011;17(10):1876–1878. [PubMed: 22000359]

21. Parrish CR. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. Virology. 1991;183(1):195–205. [PubMed: 1647068]

22. Pénzes JJ, Söderlund-Venermo M, Canuti M, Eis-Hübinger AM, Hughes J, Cotmore SF, Harrach B. Reorganizing the family Parvoviridae: a revised taxonomy independent of the canonical approach based on host association. Arch Virol. 2020;165(9):2133–2146. [PubMed: 32533329]

23. Qiu J, Cheng F, Burger LR, Pintel D. The transcription profile of Aleutian mink disease virus in CRFK cells is generated by alternative processing of pre-mRNAs produced from a single promoter. J Virol. 2006;80(2):654–662. [PubMed: 16378968]

24. Shao X-Q, Wen Y-J, Ba H-X, Zhang X-T, Yue Z-G, Wang K-J, Li C-Y, Qiu J, Yang F-H. Novel amdoparvovirus infecting farmed raccoon dogs and arctic foxes. Emerg Infect Dis. 2014;20(12):2085–2088. [PubMed: 25417672]

25. Species360 [Internet]. Zoological Information Management System; 2021. https://www.species360.org/

26. Stecher G, Tamura K, Kumar S. Molecular Evolutionary Genetics Analysis (MEGA) for macOS. Mol Biol Evol. 2020;37(4):1237–1239. [PubMed: 31904846]
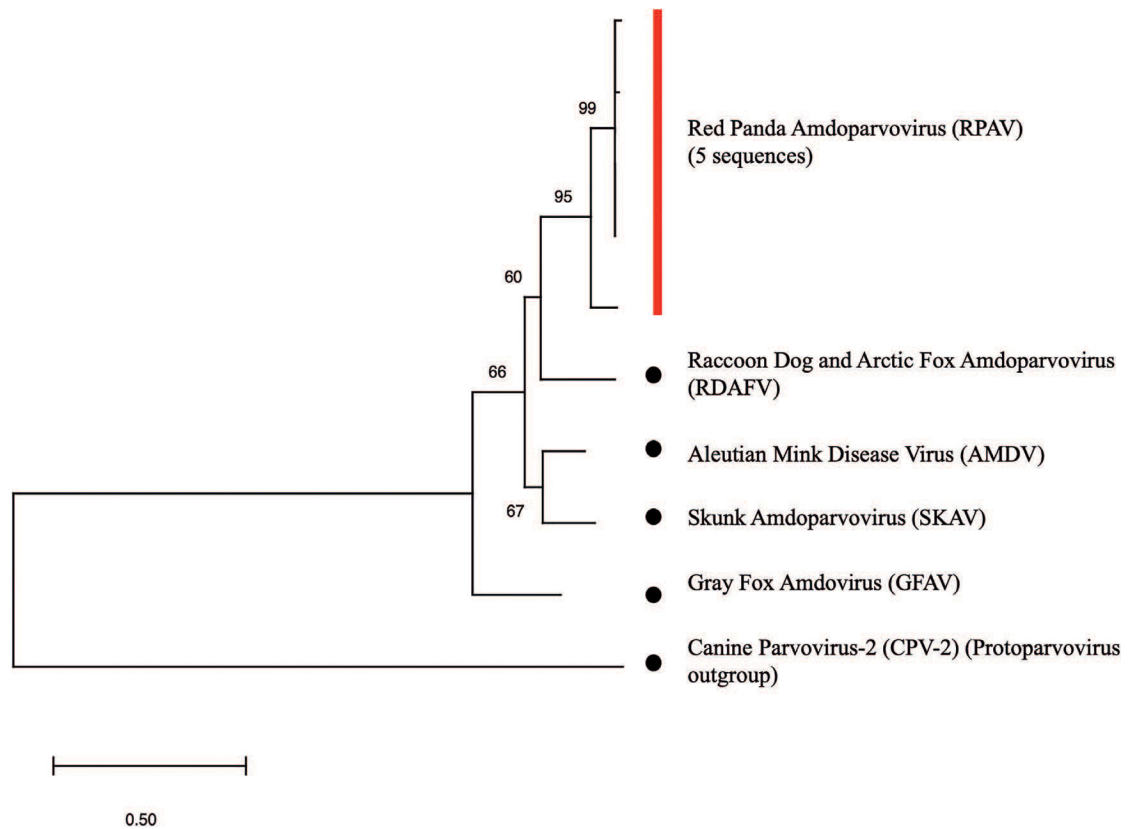
Figure 1.
Phylogenetic analysis based on full amino acid sequences of the NS1 gene. Sequences isolated from red panda (*Ailurus fulgens*) samples cluster as a monophyletic amdoparvovirus lineage. The Maximum Likelihood method and Le Gasquel model were used, and the tree with the highest log likelihood (−6678.69) is shown.[19] A discrete Gamma distribution was used to model evolutionary rate differences among sites (+G, four categories, parameter = 1.2784). Five hundred bootstrap replicates were performed, and bootstrap support values are shown next to nodes.
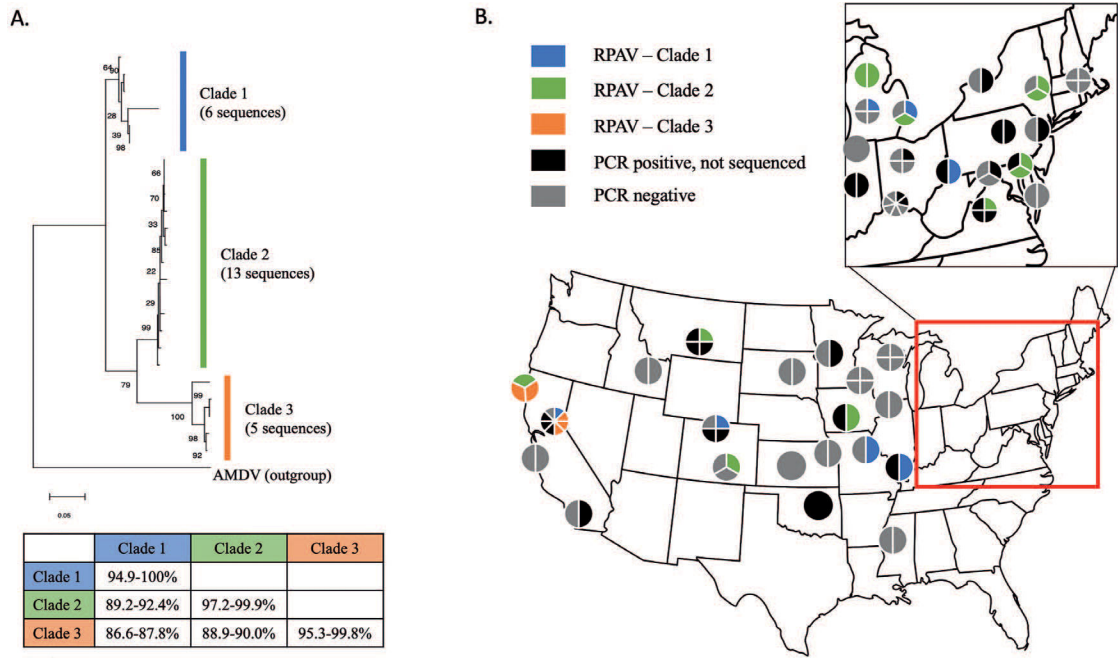
Figure 2.

(**A**) Phylogenetic analysis based on 852 nucleotides in the major nonstructural gene (NS1) gene. RPAV sequences form three distinct clades. The range of percent identity within and between groups based on pairwise comparisons is shown. The Maximum Likelihood method and Hasegawa-Kishino-Yano model were used, and the tree with highest log likelihood (−3183.46) is shown.[12] A discrete Gamma distribution was used (+G, parameter=0.4382), and 500 bootstrap replicates were performed. Bootstrap support values are shown next to nodes. All RPAV sequences are from samples from *Ailurus fulgens*. (**B**) Map of testing and results. Each pie chart represents a zoo cohort, and segments represent individual animals. One to eight animals were present in the cohorts tested. RPAV lineages (blue, green, and orange) were determined based on partial NS1 sequence.
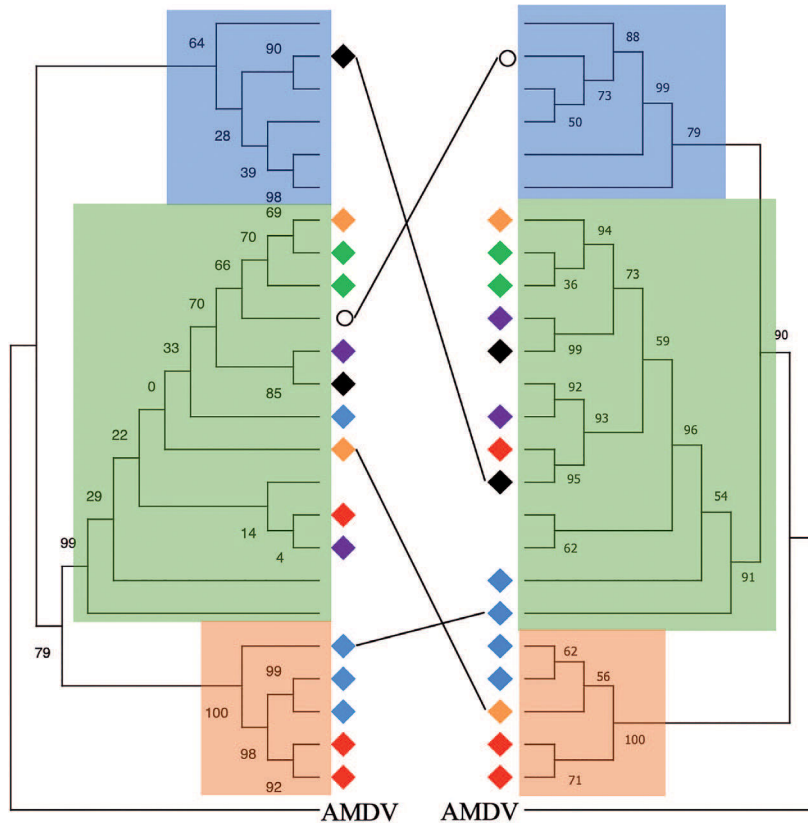
Figure 3.
Maximum likelihood trees of partial NS (left) and VP (right) sequences. Diamond tip
markers indicate zoos from which the sequences originated (grouped by color). Tips without
markers were the only sequences obtained from their respective institutions. Several zoos
(black, red, blue) had multiple RPAV lineages represented in their collections. Clade
grouping is generally maintained between trees, but in several cases (black lines) sequences
from individual animals shift clades depending on the segment examined. The empty circle
is the only sequence from its institution but is marked to show the shift in clade grouping.
All RPAV sequences are from samples from *Ailurus fulgens*. The NS tree is the same as that
shown in Figure 3, with transformed branches. The Maximum Likelihood VP tree (left) was
generated from 563 nucleotides in the capsid coding sequence using the Hasegawa-Kishino-
Yano model with a discrete Gamma distribution (parameter = 0.6857) and allows for
invariant sites ($+I$, 63.25%).[12] Five hundred bootstrap replicates were performed. Branches
were transformed, and bootstrap support values are shown next to nodes.

Table 1.

PCR primers used for red panda amdoparvovirus detection and sequencing.

Purpose and primer	Binding site [a]	Amplicon size (nt)	Target ORF	Primer sequence
Screening assay				
RP Amdo NSF2	936–955	154	NS	CGCCAAAACCAACCGACCAA
RP Amdo NSR2	1069–1089			AACACGCCCTTAGCTGTGCT
Full coding sequence				
Amdo30F	32–52	467	NS	TGGAATGMGGAAGTKCTRGTG
Amdo392R	479–498			CCHAMSMAACARTGRATATG
AmdoNS1.130F	236–255	893	NS	AAACCAACCAMAGMAACACC
AmdoNS1.1058	1108–1128			TCTTCTGGAGTAAAGCAACCA
Amdo948F	950–969	1052	NS	GACCAAACTGACTCAGCAAC
Amdo1999R	1981–2001			GTGAGCTGCCAGAACTCTTGT
Amdo4F	1572–1590	677	NS-VP	AAGCYATTACTGGAGGWGG
Amdo5R	2229–2248			TTGCTGRATAAAYACGTGTC
Amdo5F	2094–2111	754	NS-VP	AAGAGACCTCGGCATGAG
Amdo2844R	2826–2847			CCTGTAACGACGCAGTTAAGTC
SKAV1F	2636–2647	660	VP	CCAACAAGTAATGACACCWTGG
SKAVR1	3308–3324			TCACCCCAAAAGTGACC
Amdo8Fb	3181–3200	827	VP	CCAAAGTACACGTTGCTTGG
Amdo4692R	3985–4009			CAGTGAGGTTCTCTGTTAGTTTAA
Amdo4556F	3906–3927	422	VP	CTGATGTGGACCACAAACCTAG
Amdo8R	4308–4329			GGTTRTTCACTDTTYATGTAAC

[a] All nucleotide positions refer to the previously published genome NC_031751.

Table 2.

Published sequences used for phylogenetic comparisons.

Viral species	GenBank accession No.	Reference
Canine parvovirus	M38245	21
Aleutian mink disease virus	JN040434	14
Gray fox amdovirus	NC_038533	20
Raccoon dog and arctic fox amdoparvovirus	KJ396347	24
Skunk amdoparvovirus	KX981920	2
Red panda amdoparvovirus (CA1)	NC_031751	1
Red panda amdoparvovirus (CA2)	KY564173	1

Table 3.

Results of PCR screening of feces from US zoo-housed red pandas.

Category	Total tested (No.)	Positive (No.)	Negative (No.)
All	104	52	52
Species			
Ailurus fulgens	78	47	31
Ailurus styani	26	5	21
Sex			
Male	51	24	27
Female	53	28	25
Age (yr)			
0–1	8	4	4
1–2	20	7	13
3–5	33	16	17
6–9	20	11	9
10+	23	14	9

Table 4.

Red panda amdoparvovirus (RPAV) detection in serial samples (samples positive/samples tested).

Case	2016	2017	2019	2020	Total
1	8/10[a]				8/10
2	10/10[a]				10/10
3	7/7	23/23	10/10	2/2	42/42
4	8/8	23/23	11/11	2/2	44/44
5		0/23[b]	5/5	1/2	6/30
6		0/32[b]	8/8[c]		8/40

[a] Cases 1 and 2 were geriatric animals that died in 2016.

[b] Cases 5 and 6 were introduced to the collection in 2017. Samples collected before their introduction were PCR-negative for RPAV.

[c] Case 6 was transferred to a different collection in 2019.