

A new perspective on the evolution and diversity of the genus *Amdoparvovirus* (family *Parvoviridae*) through genetic characterization, structural homology modeling, and phylogenetics

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Abstract

Amdoparvoviruses (genus *Amdoparvovirus*, family *Parvoviridae*) are primarily viruses of carnivorans, but recent studies have indicated that their host range might also extend to rodents and chiropterans. While their classification is based on the full sequence of the major nonstructural protein (NS1), several studies investigating amdoparvoviral diversity have been focused on partial sequences, leading to difficulties in accurately determining species demarcations and leaving several viruses unclassified. In this study, while reporting the complete genomic sequence of a novel amdoparvovirus identified in an American mink (British Columbia amdoparvovirus, BCAV), we studied the phylogenetic relationships of all amdoparvovirus-related sequences and provide a comprehensive reevaluation of their diversity and evolution. After excluding recombinant sequences, phylogenetic and pairwise sequence identity analyses allowed us to define fourteen different viruses, including the five currently classified species, BCAV, and four additional viruses that fulfill the International Committee on Taxonomy of Viruses criteria to be classified as species. We show that the group of viruses historically known as Aleutian mink disease virus (species *Carnivore amdoparvovirus 1*) should be considered as a cluster of at least four separate viral species that have been co-circulating in mink farms, facilitating the occurrence of inter-species recombination. Genome organization, splicing donor and acceptor sites, and protein sequence motifs were surprisingly conserved within the genus. The sequence of the major capsid protein virus protein 2 (VP2) was significantly more conserved between and within species compared to NS1, a phenomenon possibly linked to antibody-dependent enhancement (ADE). Homology models suggest a remarkably high degree of conservation of the spikes located near the icosahedral threefold axis of the capsid, comprising the surface region associated with ADE. A surprisingly high number of divergent amino acid positions were found in the luminal threefold and twofold axes of the capsid, regions of hitherto unknown function. We emphasize the importance of complete genome analyses and, given the marked phylogenetic inconsistencies across the genome, advise to obtain the complete coding sequences of divergent strains. Further studies on amdoparvovirus biology and structure as well as epidemiological and virus discovery investigations are required to better characterize the ecology and evolution of this important group of viruses.

Key words: carnivore amdoparvovirus; Aleutian mink disease virus; skunk amdoparvovirus; viruses of carnivorans; parvovirus; virus taxonomy

1. Introduction

Amdoparvoviruses are viruses within the genus *Amdoparvovirus* (formerly *Amdovirus*) of the family *Parvoviridae* (subfamily *Parvovirinae*). Their virions are small, nonenveloped particles of T = 1 icosahedral symmetry. The capsid, formed by the major capsid virus protein 2 (VP2) and the minor capsid protein VP1, contains a single molecule of single-stranded DNA. The genome includes two gene cassettes, whose expression is driven by a single promoter, flanked by imperfect palindromes that fold into terminal

partially double-stranded hairpins. The gene cassette on the left side of the genome contains information for the three nonstructural proteins NS1–3, while the one on the right encodes the two capsid proteins. Alternative splicing plays a crucial role in the protein expression strategy of amdoparvoviruses, as several different transcripts are generated through this mechanism, extending the coding capacity of the small genome from 4.5 to 5 kb. The three NS proteins possess the same N-terminal sequence but have variable C-terminal sequences, while the two capsid proteins possess

the same C-terminal sequence, responsible for creating the capsid shell itself, but have different N-terminal truncations. In fact, VP1 contains a unique approximately forty additional amino acid (aa)-long extension designated as VP1 unique region (VP1u) (Canuti, Whitney, and Lang 2015; Cotmore et al. 2019).

As with all other parvoviruses, the major NS protein (NS1) of amdoparvoviruses does not have polymerase activities and hence these viruses rely on the cellular replication machinery for their DNA replication. However, NS1 is crucial for viral replication. This protein, in fact, contains an evolutionarily conserved HuH (His-hydrophobic-His) nuclease motif linked to rolling circle replication (RCR) and a highly conserved helicase superfamily 3 (SF3) domain with helicase and ATPase activity (Cotmore et al. 2019; Pénzes et al. 2020). Due to its high level of conservation, the NS1 protein is used to determine parvoviral taxonomy (Pénzes et al. 2020). On the other hand, unlike most members of the *Parvovirinae* subfamily, the VP1u of amdoparvoviruses lacks a phospholipase A2 (PLA2) domain that normally mediates virus egress across the endosomal bilayer during infection (Canuti, Whitney, and Lang 2015; Cotmore et al. 2019; Pénzes et al. 2020). The major capsid protein VP2, constituting about 90 per cent of the capsid, is highly immunogenic and contains domains that are relevant for antibody-dependent enhancement (ADE) during infections of the best-studied representative, Aleutian mink disease virus (AMDV) (McKenna et al. 1999; Bloom et al. 2001). ADE is a mechanism that facilitates viral entry into one of the virus' target replication cell types, circulating macrophages.

Amdoparvoviruses prevalently infect members of the mammalian order Carnivora (carnivorans). As mentioned above, the member of this genus that has been most well characterized is AMDV (species *Carnivore amdoparvovirus 1*), which has been a scourge of the mink-farming industry. AMDV is the causative agent of Aleutian disease, a progressive wasting syndrome characterized by hypergammaglobulinemia, weight loss, and anorexia, known to farmers since the late 1940s. After its genome was sequenced in 1988, AMDV remained the sole member of the genus *Amdovirus*, specifically named after this virus, until additional species were discovered in the 2010s (Canuti, Whitney, and Lang 2015). The genus now includes five species with official taxonomic designations (Pénzes et al. 2020), but a few additional recently discovered viruses possess the genetic characteristics typical of amdoparvoviruses. The closest known relatives to AMDV are skunk amdoparvovirus (SKAV, *Carnivore amdoparvovirus 4*) (Canuti et al. 2017) and Labrador amdoparvovirus 1 (LaAV-1) (Canuti et al. 2020a). Both viruses have been identified in North America and are found in animals that are close relatives to mink, specifically skunks and martens, respectively. However, LaAV-1 has also been found in foxes from the same area (Canuti et al. 2020a). SKAV, LaAV-1, and AMDV likely originated in and spread across North America, but AMDV now has a worldwide distribution due to international farming and movement of infected animals, followed by accidental escape or deliberate release of infected animals from farms (Canuti, Whitney, and Lang 2015; Franzo et al. 2021).

AMDV and SKAV are the only two viruses that have been the subject of studies evaluating the viral diversity and distribution. SKAV is likely endemic in skunks across North America, and the virus circulates with high local prevalence. Additionally, an ancient virus–host association has been postulated for SKAV, since it is characterized by a high genetic diversity and strains segregate based on their geographic origin (Britton et al. 2015; Nituch et al. 2015; Canuti et al. 2017; Glueckert et al. 2019; Alex et al. 2022). To the best of our knowledge, this virus has never been reported outside of Canada or the USA. On the contrary, AMDV

has been reported globally and its molecular epidemiology has been investigated extensively in farmed animals and somewhat among wild animal populations (reviewed in (Zaleska-Wawro et al. 2021)). The spread of AMDV as a direct consequence of fur animal import/export caused the same viral strains to circulate globally not only in farms, but also among free-ranging and wild mustelids since viruses are frequently exchanged between farms and wildlife (Zaleska-Wawro et al. 2021). However, once the virus has been introduced to wildlife, viral perpetuation also seems to occur among wild animals independently from farms (Leimann et al. 2015; Canuti et al. 2016, 2020a; Jakubczak et al. 2017; Virtanen et al. 2021). For these reasons, SKAV represents a better model than AMDV to study amdoparvoviral ecology and evolution in wildlife until divergent AMDV strains of non-North-American descent are identified.

The racoon dog and fox amdoparvovirus (RFAV, *Carnivore amdoparvovirus 3*) (Shao et al. 2014; Yang et al. 2021) and the red panda amdoparvovirus (RpAPV, *Carnivore amdoparvovirus 5*) (Alex et al. 2018), closely related to the AMDV-like viruses, were discovered in captive animals inhabiting fur farms and a zoo, respectively. Since there are no data about their distributions in wildlife, their host range and geographical distribution are still unconfirmed. Recently, a second red panda amdoparvovirus (referred to here as RpAPV-2) was discovered in captive Chinese red pandas (Zhao et al. 2022). More genetically distant viruses have been found in foxes. The gray fox amdovirus (GFAV, *Carnivore amdoparvovirus 2*) (Li et al. 2011), the red fox fecal amdovirus (RFFAV, only partially sequenced) (Bodewes et al. 2013), and the Labrador amdoparvovirus 2 (LaAV-2, only partially sequenced) (Canuti et al. 2020a) have been identified in foxes from North America (GFAV and LaAV-2) and Europe (RFFAV). Interestingly, amdoparvoviral fragments were also recently identified in rats (RtRn-ParV, only partially sequenced) (Wu et al. 2018) and horseshoe bats (Rp-BtAMDV1 and BtRI-PV/FJ2012, both only partially sequenced) (Wu et al. 2016; Lau et al., 2017), suggesting that the host range of amdoparvoviruses may not be restricted to carnivorans. This is further indicated by the presence of endogenous amdoparvoviral elements in rodents (Pénzes et al. 2018). Nevertheless, other than for AMDV and SKAV, there are no studies for amdoparvoviruses besides those describing their discovery, and knowledge about their diversity and distribution are still limited. Furthermore, AMDV is the only virus that has been grown in cell culture, and the biological properties of all other amdoparvoviruses can only be predicted based on sequence similarities within the genus at the moment.

Although sequences of the amdoparvoviral capsid proteins are relatively conserved across species, NS1 sequences are highly variable, even among viruses from the same species (Canuti, Whitney, and Lang 2015; Canuti et al. 2016, 2020a; Alex et al. 2022). For AMDV, different lineages have been observed that are characterized by an exceptionally high NS1 inter-lineage diversity and sometimes by different pathogenic potentials (Olofsson et al. 1999; Canuti, Whitney, and Lang 2015; Ryt-Hansen et al. 2017a; Prieto et al. 2020). Also, different SKAV lineages have been identified, but these seem to be more closely related to each other compared to AMDV and be geographically segregated (Canuti et al. 2017; Alex et al. 2022). For AMDV and SKAV, this antigenic stability has been hypothesized to be linked to ADE as the recognition of the virus by the host immune system would enhance viral replication (Canuti et al. 2016; Alex et al. 2022). However, as of yet no study has determined whether this could be theoretically applicable to other amdoparvoviral species. Besides this marked discrepancy in evolutionary dynamics of the two genes, the determination of the

phylogenetic relationships among amdoparvoviruses is further complicated by the occurrence of recombination, which has been frequently observed for AMDV, especially in farm settings (Canuti et al. 2016; Virtanen et al. 2019, 2021), and SKAV (Canuti et al. 2017; Alex et al. 2022). Furthermore, while the parvovirus classification is based on the phylogenetic analysis and sequence comparisons of NS1 protein sequences (Pénzes et al. 2020), several older studies (reviewed in (Zaleska-Wawro et al. 2021)) investigating the diversity of AMDV focused on VP2 or compared only partial sequences. This led to difficulties in accurately recognizing the species diversity spectrum of amdoparvoviruses and possibly to an overestimation of AMDV genetic diversity because the sequence pool of this virus could be ‘contaminated’ with sequences from different viruses. For example, SKAV was recognized as representing a separate species from AMDV only after phylogenetic analyses of the complete coding regions of the genome revealed that previously obtained partial genomic fragments were not sufficient to clearly differentiate the two species (Nituch et al. 2015; Canuti et al. 2017). Additionally, to the best of our knowledge, no study as of yet has compared all available amdoparvoviral sequences or presented a phylogenetic analysis that includes all recognized and presumed members, leaving aspects of amdoparvoviral diversity, ecology, and evolution largely unexplored.

In this study, we report the full coding sequence of a novel amdoparvovirus identified in an American mink from British Columbia, Canada, and studied its potential geographic range through sequence database investigations. Furthermore, we investigated all amdoparvoviral sequences available to reconcile novel discoveries and long-known viruses. We assessed common amdoparvoviral molecular features and phylogenetic relationships to evaluate the extent of amdoparvoviral diversity, elucidate relationships among the currently known viruses, and incorporate this new information in the species-level taxonomy within this genus. Furthermore, to gain more insights on the mechanisms driving amdoparvoviral capsid evolution, we constructed models of the VPs and characterized the possible location of various variable and conserved structural regions.

2. Materials and methods

2.1 Sample

Organ samples were collected from one American mink (*Neovison vison*) that was trapped in the winter of 2015 in the Elk Valley, in the southeastern part of British Columbia, Canada. The carcass was part of a collection by wildlife researchers for various sampling protocols. On submission to a diagnostic laboratory, DNA was isolated from tissues as described (Britton et al. 2015) and amdoparvovirus screening polymerase chain reactions (PCRs) and complete genomic sequencing were subsequently done as previously reported (Canuti et al. 2016, 2020a, 2020b). The sample processing was carried out in accordance with guidelines of the Canadian Council on Animal Care, with approved protocol 15-04-AL from the Memorial University Institutional Animal Care Committee. In accordance with the Canadian Council on Animal Care guidelines, this research was exempt from Animal Research Ethic Board review in Canada because samples were collected from an animal previously harvested for non-research purposes.

2.2 Sequence analyses

The study involved 1,919 sequences, including all amdoparvoviral sequences available in GenBank on 24 November 2021 ($N = 1,894$), twenty-four recently obtained SKAV sequences (Alex et al. 2022),

and the novel genome obtained in this study. Sequence alignments obtained with Clustal W (Larkin et al. 2007) were screened for recombination with recombination detection program (RDP) (Martin et al. 2015), and maximum-likelihood phylogenetic trees were built with IQ-TREE 2 (Minh et al. 2020) using the best-fit substitution model identified as the one with the lowest Bayesian information criterion with the ModelFinder function (Kalyaanamoorthy et al. 2017). For each tree, branch support was assessed with both ultrafast bootstrap approximation (ufBoot) (Hoang et al. 2018) and Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT) (Guindon et al. 2010). Recombination events were further explored with a bootscanning analysis performed with Simplot (Lole et al. 1999). Identities between sequence pairs were calculated as $1 - p$ distance and used to construct histograms, representing the frequency distributions of pairwise sequence identities between and within groups, with PAST 4 (Hammer, Harper, and Ryan 2001). The sequence motif identification and open reading frame (ORF) annotations were performed in Geneious R11 (Biomatters), and figures were finalized with INKSCAPE (<https://inkscape.org>).

2.3 Homology modeling

As there has not been any amdoparvoviral high-resolution structure deposited to the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) thus far, we relied on homology modeling to assess the location of certain structural elements of the amdoparvovirus capsid. This approach was greatly facilitated by the high structural conservation among parvoviral capsid proteins, characteristic of the entire *Parvovirinae* subfamily (Mietzsch, Pénez, and Agbandje-McKenna 2019). Template searching was carried out by the pGenThreader (Lobley, Sadowski, and Jones 2009) profile-based fold recognition algorithm of the PSIPred suite (Moffat and Jones 2021). The prediction was executed independently in case of the VP2 of each representative of prospective or established amdoparvoviral species, and the template of the overall highest score was used ubiquitously in subsequent protein modeling applications to avoid possible false structural differences introduced by template bias. Protein modeling was carried out with Modeller v10.2 (Webb and Sali 2016). The models obtained were visualized using University of California at San Francisco (UCSF) Chimera (Pettersen et al. 2004) and complete 60mers were generated using the suite’s built-in icosahedral matrix generator based on the I₂22r orientation. The pseudo-atomic surface rendition of the AMDV capsid model was created using ChimeraX.

3. Results and discussion

3.1 Detection of a novel amdoparvovirus

Screening organ samples collected from a dead mink with a pan-amdoparvovirus PCR (Canuti et al. 2020a) allowed us to identify a novel amdoparvovirus. Since this virus was found in only one mink and considering that amdoparvoviruses sometimes lack host specificity (Shao et al. 2014; Canuti, Whitney, and Lang 2015; Nituch et al. 2015; Canuti et al. 2017, 2020a, 2020b), we could not conclusively assign a maintenance host to this virus. We decided to name it British Columbia amdoparvovirus (BCAV), according to the origin of the sample where the virus was identified. Because of sequence conservation between amdoparvoviruses, we were able to obtain the near-complete genomic sequence of BCAV, covering the full coding sequence but lacking the termini, whose sequences are difficult to obtain from uncultured parvoviruses. The obtained sequence was 4281 nt long and a Blast analysis showed it was 90.5 per cent identical to its closest relative, an

AMDV strain from the Netherlands (accession number KY997041). The molecular features of this virus will be analyzed in more detail in the following sections.

3.2 Recombination detection and final sequence datasets

From the original dataset of 1,919 amdoparvoviral sequences, all sequences that encompassed the full NS1 or VP2 ORFs were extracted for the following analyses. From this set, duplicate sequences (100 per cent nt identity), sequences with long stretches of undetermined bases, and sequences with indels causing frameshifts were removed. This process resulted in the selection of 329 full NS1 and 241 full VP2 sequences. For VP2, the area of the polyglycine stretch, which is highly variable in length between and within species (see below) and hence can interfere with phylogenetic reconstructions, was removed when phylogenetic trees were built and for pairwise identity calculations.

A recombination analysis was performed to identify and remove potentially chimeric sequences before building trees and achieve a better resolution of the phylogenetic clades, which is made problematic by the presence of recombinant sequences (Posada 2000), rather than to examine the recombination potential of amdoparvoviruses, which has previously been investigated (Canuti et al. 2016, 2017; Virtanen et al. 2019, 2021; Alex et al. 2022). These sequences could represent both recombinant viruses evolved during the co-infection of the same cell by two different viruses as well as artificial chimeras generated during sequence assembly from samples containing more than one virus. Nucleotide sequences were converted into protein sequences and used to build maximum-likelihood phylogenetic trees, while individual gene alignments were used for recombination detection with RDP. Each event detected by RDP was carefully inspected by assessing the alignments and the generated graphs and trees to make sure that the potentially recombinant as well as parental sequences were correctly identified. Sequences identified as potentially recombinant that showed parents located in different main branches (i.e. inter-species chimeras) or for which one parent was unknown were removed from the dataset and new phylogenetic trees were built. This process was repeated until no further recombinant sequences were found. The analysis evidenced the presence of several recombination signals involving many different sequences with many different predicted breakpoints. As an example, the bootscanning analysis of a few potentially recombinant sequences is provided in supplementary material (Supplementary Fig. S1). Potentially chimeric NS1 sequences were mainly found among SKAV and AMDV (between the three main clades), confirming previous studies (Canuti et al. 2016, 2017; Virtanen et al. 2019, 2021; Alex et al. 2022). A potential recombination involving AMDV and RFAV was also detected (Supplementary Fig. S1). This consisted of a 400-nt AMDV-like sequence in a RFAV NS1 backbone. Given the low number of RFAV available sequences, this potentially recombinant sequence (strain SD17, accession number KY421419) was not removed from the final dataset as its presence did not affect the overall tree topology. For VP2, recombination was detected among AMDV, SKAV, BCAV, and LaAV-1. The lack of recombination detection for other viruses was likely related to the low number of available sequences, rather than the absence of this phenomenon in the evolutionary history of those groups. The final dataset included 280 full NS1 and 207 full VP2 sequences after the exclusion of 49 and 34 potentially recombinant AMDV NS1 and VP2 sequences, respectively.

The partially sequenced NS1 of Bt-RI-PV (only approximately 30 aa shorter on the N-terminal side) was also included in the NS1 phylogenetic analyses. Furthermore, to achieve a more complete overview of amdoparvoviral diversity, partially sequenced viruses (RFFAV, LaAV-2, RtRn-ParV, and BtRI-PV) were used for additional phylogenetic analyses, as well as for within- and between-group identity analyses and sequence motif comparisons. However, only full sequences from viruses that were either already classified within species or that could be eligible for classification were used to make the histograms. Because we identified clades that were populated by many highly identical sequences for AMDV, all but one sequence from those clades were removed for calculating distance frequencies to avoid an overrepresentation of the signal caused by these strains. Finally, the whole non-redundant dataset was used for genome structure analyses and motif conservation assessment. Accession numbers of sequences used are available in Supplementary Tables S1 and S2.

3.3 Phylogenetic analyses

Phylogenetic relationships among groups of viruses were studied considering the two main ORFs separately because the structural and non-structural proteins are subject to different evolutionary forces and the mutation rates of genes encoding NS1 and VP2 are considerably different (Canuti et al. 2016, 2017). Therefore, we built two trees with the NS1 protein and nucleotide sequence alignments, the first normally also used for parvoviral taxonomic purposes (Pénzes et al. 2020), and a separate one with the nucleotide alignment of VP2 because the high protein sequence identity, resulting in the overrepresentation of invariable sites, made VP2 proteins unsuitable for group delineation (Fig. 1, Supplementary Fig. S2). Additional trees were built with partial alignments to estimate the phylogenetic placement of partially sequenced viruses (Supplementary Fig. S3). The results of these analyses are summarized in Fig. 1, where the positions of partially sequenced viruses in supplementary trees are indicated by dotted lines.

Although the topology of the NS1 and VP2 trees was slightly different and differences between groups in the VP2 tree were less noticeable, the same clades corresponding to different groups of viruses were clearly distinguishable in all trees. Specifically, viruses belonging to each of the five classified viral species are clearly separated into highly supported clades and additional clades corresponding to potential novel species (indicated by a star in Fig. 1) can also be seen. Novel groups corresponded to the recently discovered LaAV-1 (Canuti et al. 2020a) and RpAPV-2 (Zhao et al. 2022) and the virus described for the first time in this study (BCAV). Additionally, these trees show that viruses historically defined as AMDV could be divided into three well-separated groups, indicated here as AMDV-1, AMDV-2, and AMDV-3.

These trees also show that all viruses of mustelids and skunks (AMDVs, SKAV, LaAV-1, and BCAV) were closely related and always formed one highly supported clade, where SKAV is the outlier. Similarly, all viruses from foxes (GFAV, RFFAV, and LaAV-2) clustered closely together while viruses from red pandas (RdPAV and RdPAV-2) occupied an intermediate position. This distribution reflects the phylogenetic relationships among these groups of carnivorans and may reflect long-term virus–host co-evolution throughout the evolution of these hosts. Since RFAV has so far only been identified in foxes, raccoon dogs, and mink housed in farm settings, its reservoir host remains uncertain, and we prefer not to speculate about the position of this virus in the tree in relationship to its unknown reservoir host.

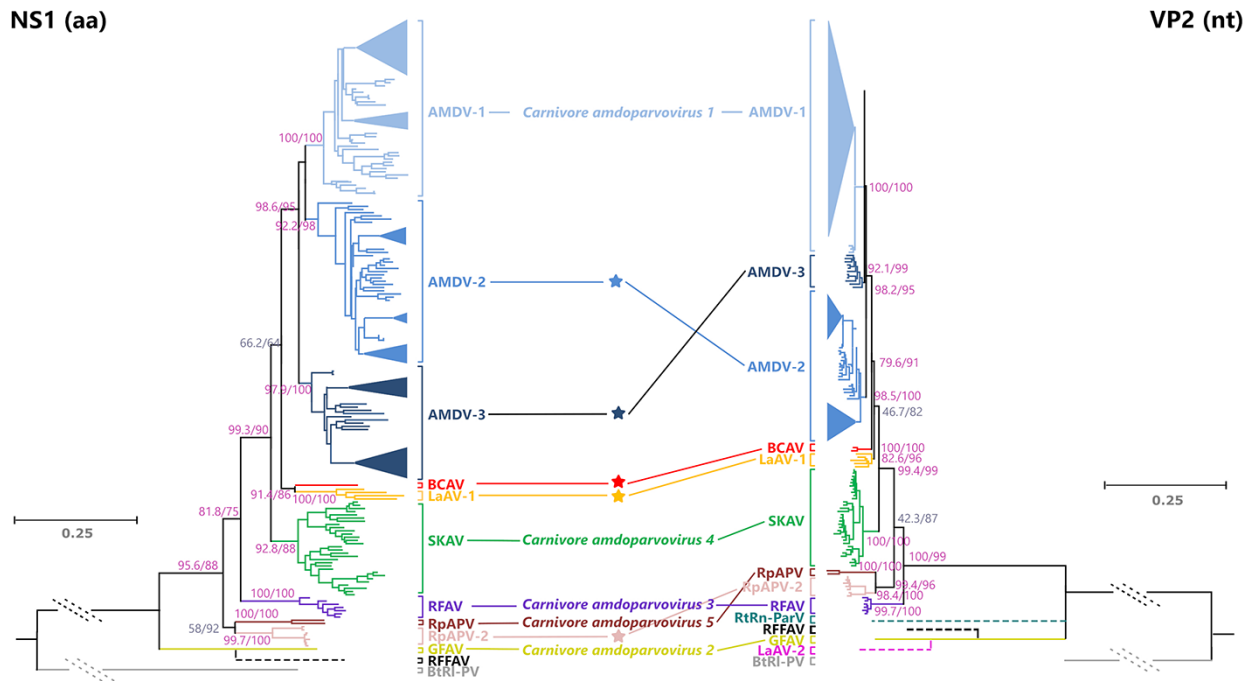


Figure 1. Phylogenetic analyses of amdoparvoviruses. The phylogenetic trees based on the NS1 protein sequences (left) and VP2 nucleotide sequences (right) were built with the maximum-likelihood method with the JTTDCMut + F + R5 and TVM + F + R7 models, respectively, using IQ-TREE. The outcomes of the SH-aLRT and bootstrap test (1,000 replicates) are shown for the main nodes. Triangles represent collapsed redundant clades, and branch lengths are proportional to genetic distances as indicated by the scale bar. The predicted phylogenetic placements of partially sequenced viruses assessed with different analyses (Supplementary Fig. S3) are shown with dotted lines. The groups of viruses are color-coded and labeled according to virus abbreviations listed in Table 1. Official taxonomic names are indicated for species when available. The phylogenetic positions in the two trees of viruses whose genomes were fully sequenced are connected by straight lines and viruses that are eligible for official classification are indicated by a star.

Table 1. List of amdoparvoviruses and their current classification status.

Virus name	Virus abbreviation	Species ^a	Reference strain	Maintenance host ^b	Reference sequence AN ^c
Aleutian mink disease virus 1	AMDV-1	<i>Carnivore amdoparvovirus 1</i>	AMDV-G	Mink	NC_001662
Gray fox amdovirus	GFAV	<i>Carnivore amdoparvovirus 2</i>	–	Fox	NC_038533
Raccoon dog and fox amdoparvovirus	RFAV	<i>Carnivore amdoparvovirus 3</i>	HS-R	Raccoon dog?	NC_025825
Skunk amdoparvovirus	SKAV	<i>Carnivore amdoparvovirus 4</i>	SK-23	Skunk	NC_034445
Red panda amdoparvovirus	RpAPV	<i>Carnivore amdoparvovirus 5</i>	–	Red panda	NC_031751
Aleutian mink disease virus 2	AMDV-2	Unassigned	HY 327	Mink	MG821261
Aleutian mink disease virus 3	AMDV-3	Unassigned	LM	Mink	KY680280
British Columbia amdoparvovirus	BCFAV	Unassigned	BCWM-1	Mink?	ON375541
Labrador amdoparvovirus 1	LaAV-1	Unassigned	MART4	Marten?	MT770849
Red panda amdoparvovirus 2	RpAPV –2	Unassigned	patient12amdo01-4	Red panda	MZ357124
Red fox fecal amdovirus	RFFAV	Not eligible	S40	Fox	KF823809
Labrador amdoparvovirus 2	LaAV-2	Not eligible	F6	Fox	MT770902
Rattus nitidus parvovirus	RtRn-ParV	Not eligible	GZ2016	Rat	KY432922
Rhinolophus lepidus parvovirus	BtRI-PV	Not eligible	FJ2012	Bat	KJ641663

^aWhen a species lacks taxonomic designation but is eligible for classification, it is indicated as ‘unassigned’ while ‘not eligible’ indicates that the virus cannot be classified as a species because it has only been partially sequenced.

^bA question mark indicates that not enough data are available to determine the reservoir host with certainty.

^cAccession number.

Interestingly, the virus identified in rats (RtRn-ParV) was closely related to fox viruses. A previous study hypothesized that foxes may acquire amdoparvoviral infection through carnivory (Canuti et al. 2020a), and the presence of a rodent-specific amdoparvovirus clade that is the source of infection for foxes could explain this proximity. However, other types of cross-species transmission as well as the fact that viral particles could reach the digestive tract of rodents through the ingestion of contaminated food can also be hypothesized. Finally, as expected, the virus found in bats proved to be the most divergent sequence. Undoubtedly, studies on amdoparvoviruses in animals other than carnivores are still

lacking and future studies investigating the potential presence of amdoparvoviruses in other animal orders should be performed to increase our knowledge of the ecology and host spectrum of these viruses.

3.4 Identities within and between groups

Pairwise aa sequence identities were calculated for NS1 and VP2 proteins using the whole dataset (Fig. 2A). For NS1, the pairwise identity ranges show that sequences within each group are at least 83 per cent identical, a percentage that is slightly lower than the cut-off set as species demarcation criteria for parvoviruses (85 per

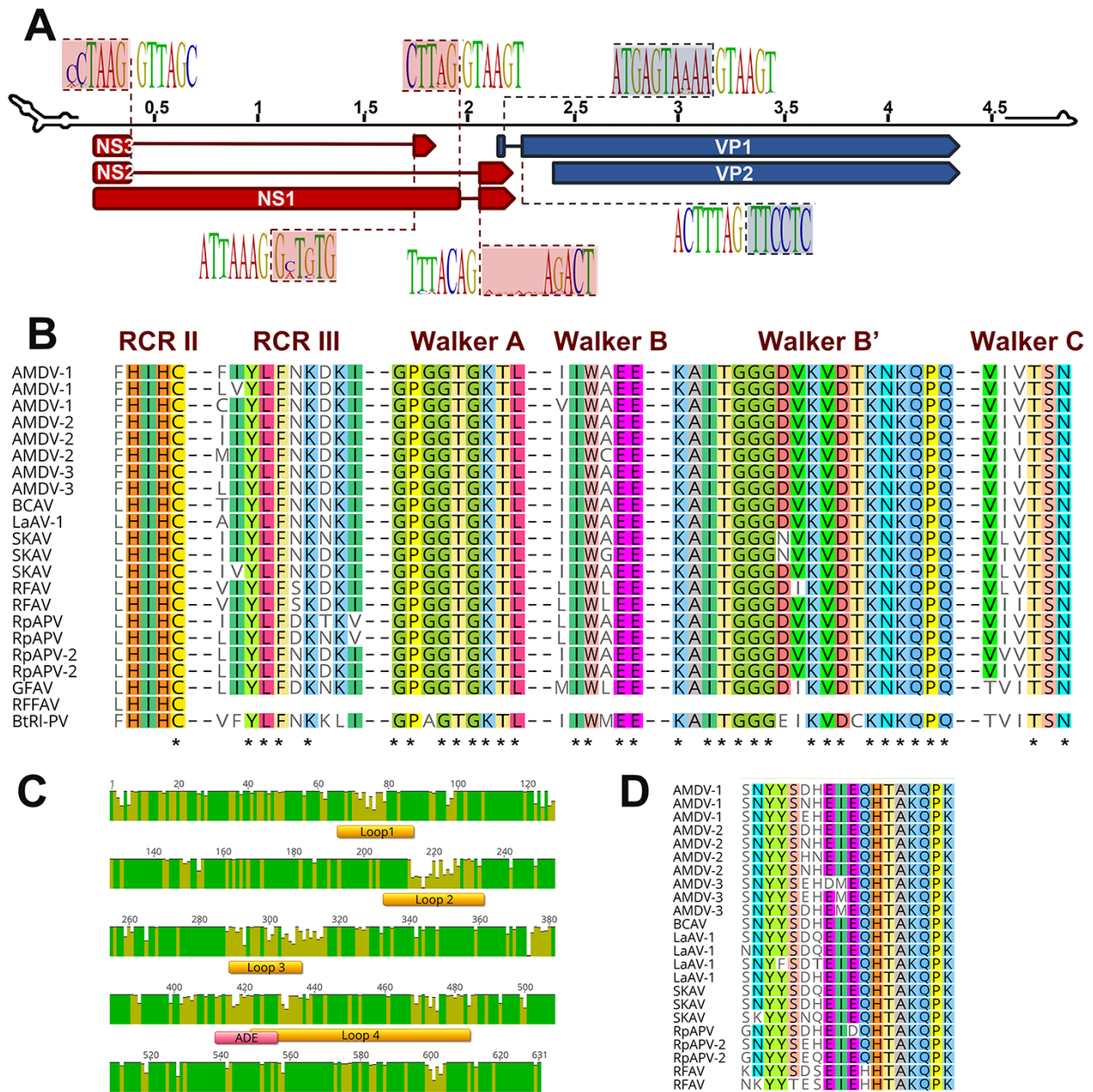


Figure 4. Genomic features of amdoparvoviruses. (A) Schematic representations of amdoparvoviral genomes with ORFs for NS (left) and structural (right) proteins. A sequence logo depicts sequence conservation across viruses at the level of each splicing donor (top) and acceptor (bottom) sites. Genomic positions are indicated in kb. (B) RCR and Walker motifs typical of parvoviral NS1 proteins are shown for representative strains of each virus group as indicated on the left. The sequence of RFFAV was partial. An asterisk at the bottom indicates that the residue was conserved across the whole alignment (280 sequences). (C) VP2 protein sequence conservation among viruses of Musteloidea. A dark tall bar corresponds to a residue conserved in each sequence, while lighter and shorter bars correspond to less conserved residues and the height of each bar is proportional to the conservation level. The locations of the four antigenic loops and of the immunogenic domain involved in ADE, experimentally determined for AMDV, are indicated with rectangles (D) Sequences corresponding to the immunogenic domain involved in ADE are shown for representative strains of each Musteloidea virus as indicated on the left.

(Fig. 4C and 4D, Fig. 5A), this domain is suspected to maintain structural conservation, as shown in Fig. 5B. The terminal part of the ADE-associated domain was the most conserved among musteloid-infecting viruses (Fig. 4D), while this was not true for viruses whose maintenance hosts are suspected to be a canid or a non-carnivoran (Supplementary Table S6). However, the Loop 4 region, flanked by VRs 7 and 8, is characterized by an almost identical 20-aa-long sequence even in divergent amdoparvoviruses, such as GFAV and BtRI-PV. Although this should be corroborated by *in vitro* experiments, these findings further support

the possibility that ADE is an evolutionary conserved pathogenic mechanism among all members of the genus *Amdoparvovirus* and imposes an important constraint on the evolution of the capsid proteins (Canuti et al. 2016; Alex et al. 2022). Furthermore, it is plausible that the entire Loop 4 plays a role in this process, involving the top and the inner wall of the threefold spikes. Because only a few sequences from viruses of foxes and non-carnivorans are currently available, further studies providing more sequence information from these underrepresented groups will allow a better assessment of the conservation of this domain

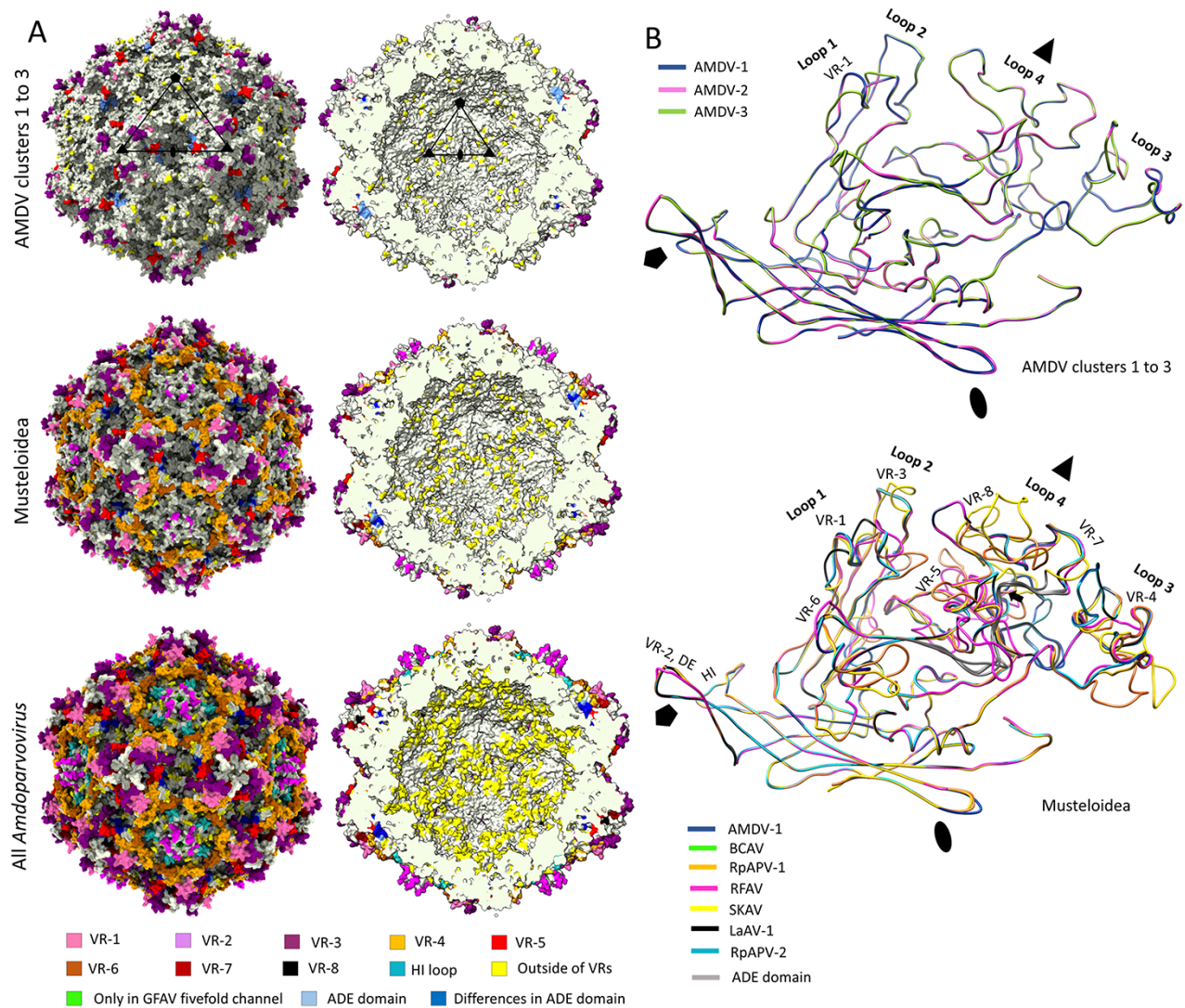


Figure 5. Homology modeling of the VP2-derived aa sequence throughout the genus *Amdoparvovirus*. (A) Rendered capsid surface (left) and lumen (right) of the AMDV-1 VP2 homology model is shown at a simulated atomic resolution. The large triangle highlights an asymmetric unit, with the fivefold symmetry axis marked by a pentagon, the twofold axis by an ellipsoid, and two threefold axes by triangles. On the top panel, differences among the three prospective AMDV species are presented, using the coloring code in the bottom of the figure. The middle panel displays the sequence comparison of VP2 proteins throughout amdoparvoviruses of musteloid hosts, while the bottom panel presents the same analysis encompassing the entire genus *Amdoparvovirus*. The region previously associated with ADE and the VRs and differences outside of these are colored according to the legend. Sequence differences concerning the conserved surface loop linking the β H and β I sheets together are also shown. The unaltered capsid regions are not colored. (B) Ribbon diagrams comparing the homology models of a single capsid monomer between representative members of prospective species AMDV-1 to -3 (top) and between amdoparvoviruses of musteloids. The region previously linked to ADE is indicated by the arrow (bottom). Symmetry axes, to orient the monomer, are indicated as in Panel A.

among closely related viruses. Furthermore, additional sampling efforts will be fundamental for obtaining amdoparvoviral strains (other than AMDV) that can be cultured on lymphatic cells in the presence of sera or monoclonal antibodies to repeat the experiments performed for AMDV (Bloom et al. 2001) and confirm or disprove that other species in this genus are capable of ADE and specifically define the domains involved. Furthermore, sera from infected animals are suitable for the purification of amdoparvoviral capsid-specific antibodies, which could be utilized to structurally characterize their interactions with various amdoparvoviral capsids, given that parvoviral capsid proteins can be effectively expressed and assemble spontaneously to virus-like particles *in vitro* (Mietzsch, Pénczes, and Agbandje-McKenna 2019).

Single aa mutations and polymorphic regions outside of the VRs were not limited exclusively to the capsid surface. According to our models, on the luminal surface of the AMDV-1, 2, and 3 capsids such divergent residues are positioned in areas exclusively underneath the icosahedral threefold symmetry axis, while polymorphisms are accumulated in large numbers along the twofold axis as well in the entire genus. Apart from being the location of the VP1u and VP N-terminal externalization, the fivefold symmetry axis and the associated channel also serve as the sight of genome packaging and of uncoating, as shown for other parvoviral genera (Bleker, Sonntag, and Kleinschmidt 2005; Plevka et al. 2011). The high conservation of the luminal fivefold area and the inside wall of the DE loop (Fig. 5A) suggests the mechanisms of DNA movement into and out of the capsid are highly conserved.

Moreover, it also supports the idea of a ubiquitous VP1u externalization, even in the absence of the PLA2. The luminal threefold and twofold interfaces, on the other hand, have been associated with transcription initiation, DNA binding, and high pH sensitivity in some parvoviruses (Nam et al. 2011; Saiganik et al. 2012, 2014). Considering the likelihood of differences in intracellular trafficking, virus tissue tropism, and host spectrum, these areas are highly likely to be under stronger diversifying selective pressure, which is in concordance with the variable nature of these luminal regions in an otherwise conserved environment.

3.8 Taxonomic implications

According to the ICTV rules for parvoviral classification, the NS1 proteins of viruses within the same genus are monophyletic and share at least 35–40 per cent sequence identity, while members of the same species are monophyletic and their NS1 proteins are over 85 per cent identical to each other. Additionally, it is a prerequisite for a virus to be classified as species to have its coding sequence fully sequenced from its likely host (Cotmore et al. 2019; Pénczes et al. 2020). According to these criteria, AMDV cannot be considered a single species as NS1 pairwise identities between AMDV strains are as low as 77 per cent. Although defining the taxonomy of these viruses is complicated by the presence of recombinant strains in the databases, the availability of many AMDV sequences made it possible to define more precisely the phylogenetic relationships among strains, even after removing a large number of potential recombinants. We could, therefore, identify three distinct phylogenetic clades of AMDV, which we called AMDV-1 to -3, and we propose that they have the characteristics to be classified as separate species, despite some overlap in pairwise identities between and within groups. Additionally, BCAV can also be considered an independent species, although its VP2 is highly identical (97.5 per cent) to a viral strain previously classified as AMDV.

Although a clear 85 per cent aa identity cut-off is not always met, we demonstrate here that LaAV-1 and RpAV-2 can also be classified as their own species. However, partially sequenced viruses are not eligible for classification. Thus, in addition to the five amdoparvoviral species currently accepted by the ICTV, we propose the definition of five additional species, which are indicated with a star in Fig. 1 and listed in Table 1. Proposed reference strains were selected to fulfill ICTV criteria for species classification (Supplementary Table S7), for not having detectable recombination signal in either of the two ORFs, and for showing consistent clustering in the two trees in Fig. 1 and Supplementary Figs S1 and S2.

3.9 Conclusions

For over 20 years after its genome was fully sequenced, AMDV was the only known amdoparvovirus, but the genus *Amdoparvovirus* has experienced a rapid and steady expansion during the last 10 years. Additionally, until a few years ago, only amdoparvoviruses of carnivorans were known, but today we are aware that rodents and chiropterans could also be hosts for these viruses. Sequence and structural similarities at key sites indicate that biological properties of amdoparvoviruses are preserved across the genus and imply that amdoparvoviral replication and pathogenicity strategies may be evolutionarily conserved, regardless of the host type. Specifically, a high sequence and structural

conservation of the capsid proteins may be connected to the use of ADE as a viral replication strategy for most, if not all, amdoparvoviral species. Additionally, our analyses showed evidence for virus–host co-evolution.

In this study we report the sequencing and characterization of a novel amdoparvoviral species that we identified in an American mink and showed that the VP2 sequence of this virus is highly identical to a partially sequenced AMDV strain that has been extensively used in pathogenicity experiments, highlighting again the importance of complete genome sequencing when studying amdoparvoviruses and the cruciality of performing NS1-based analyses, since the high VP2 conservation may result in failure to distinguish separate viral species. Our analyses identified a total of five viral candidates that can be classified as novel amdoparvoviral species and four additional viruses that, although currently not eligible for classification, will hopefully be further characterized in future studies and become officially recognized species. Finally, our study highlights how the traditional viewpoint that describes AMDV as a virus with an exceptionally diverse NS1 should be shifted toward a new perspective, more genetically coherent, of a cluster of at least four different viruses with an exceptionally conserved capsid. These viruses, which may have different pathogenic potentials and may or may not have been co-circulating in the wild, started forcedly interacting with each other in mink farms, likely at a higher frequency than in naturally less-crowded environments. Intense farming facilitated the occurrence of recombination, making the distinction between the different viruses harder to perceive, and likely altered the selection pressure forces acting on the viral capsid, *de facto* interfering with the direction of viral evolution.

While much progress has been made during the last years in clarifying the diversity and host range of amdoparvoviruses, additional studies evaluating emerging and novel potential hosts should be performed to further our understanding of the ecology and evolutionary histories of these viruses and to set the ground for *in vitro* experiments to assess how pathogenicity and the pressure of the host immune response influence viral evolution.

Data availability

The sequence obtained in this study is available in GenBank under accession number ON375541.

Supplementary data

Supplementary data are available at *Virus Evolution* online.

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