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Comprehensive phylogenetic analysis of newly detected rodent adenoviruses sheds light on ancient host-switches

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ABSTRACT

Here we provide a comprehensive update on the diversity and genetic relatedness of adenoviruses occurring in rodents. Extensive PCR screenings revealed the presence of adenoviral DNA in samples originating from representatives of 17 rodent species from four different suborders of Rodentia. Distinct sequences of 28 different adenoviruses were obtained from the positive samples. Out of these, 20 were from hitherto unknown, putative novel adenoviruses, whereas 6 were variants of previously published murine adenoviruses. Additionally, two known viruses, guinea pig adenovirus 1 and squirrel adenovirus 1 were also detected. By PCR and primer walking, we determined the sequence of a considerable part of the genomic DNA of squirrel adenovirus 1, detected in red squirrel (*Sciurus vulgaris*) samples from Germany previously. We annotated the almost complete genome sequence of a novel mastadenovirus found by data mining in the bulk data of the Ord's kangaroo rat (*Dipodomys ordii*) genome project. We revisited the sequence of the gene of E1B 19 K protein of mouse adenovirus 3. In contrast to the prototype strain, where a truncated version of this gene has been found, in our sample of mouse adenovirus 3, it seemed to be intact. Based on phylogeny reconstructions, all rodent adenoviruses clustered in the genus *Mastadenovirus*. Interestingly, however, there wasn't a common monophyletic clade encompassing every adenovirus of rodent origin. Instead, three major lineages were observed. Because two lineages contained viral sequences deduced from samples of three suborders, and one consisted almost exclusively of adenoviruses from the family Muridae, we hypothesize there has been a long-term coevolution with the rodent hosts, as a result of possible ancient host-switch events. Several putative viruses appeared in distinct branches further away from the three clades. Thus, the evolutionary past of the adenoviruses of rodents remains to be studied further.

1. Introduction

Adenoviruses (AdVs) are non-enveloped, medium-sized, icosahedral viruses with double stranded, linear DNA genomes ranging in size from 25 to 48 kbp and containing genes on both strands. AdVs are widespread throughout the world and occur commonly in humans and most vertebrates. With some exceptions, they rarely cause severe disease or mortality. Primary infection usually manifests in mild, self-limiting upper respiratory or enteric signs. Nonetheless, there are multiple examples for serious, sometimes even lethal diseases caused by AdV infection (Cole, 1971; Dastjerdi et al., 2022; Vidovszky et al., 2019 Zahradnik, 1987). Serologically distinct, but genetically closely related AdVs are classified into common species. To date, more than 100 AdV species have been approved or proposed, but this number probably reflects targeted detection efforts rather than actual occurrence (Benkő et al., 2022; Harrach et al., 2019). According to the International Committee on

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Taxonomy of Viruses, the family *Adenoviridae* is divided into 6 genera (*Aviadenovirus*, *Barthadenovirus*, *Ichtadenovirus*, *Mastadenovirus, Siadenovirus*, *Testadenovirus*), out of which the genus *Mastadenovirus* encompasses the AdVs of mammals including different rodents (Hartley and Rowe, 1960; Hashimoto et al., 1966; Hemmi et al., 2011; Klempa et al., 2009; Zheng et al., 2016).

Currently, five rodent AdV species are officially established. Representatives of *Mastadenovirus encephalomyelitidis* and *M. muris* (former *Murine mastadenovirus A* and *B*), namely MAdV-1, strain FL (Davison et al., 2003) and MAdV-2 strain K87 (Hemmi et al., 2011), respectively, have been isolated from house mice (*Mus musculus*), while MAdV-3 (Klempa et al., 2009) originating from the striped field mouse (*Apodemus agrarius*) is grouped in species *M. cordis* (former *Murine mastadenovirus C*). MAdV-1 induces fatal encephalomyelitis in newborn mice or in adult specimens of certain specific mouse strains (Guida et al., 1995). Infection with MAdV-2 typically leads to mild diarrhea. MAdV-3 is cardiotropic in mice, based on the dominant presence of viral DNA in the myocardium, while in the original (striped field mouse) host, the virus is found in larger amounts in the lung tissue (Klempa et al., 2009). Although MAdV-1 and −2 have been isolated from house mice and MAdV-3 from the striped field mouse, molecular analysis revealed that MAdV-1 and MAdV-3 are more closely related to each other, whereas MAdV-2 seems to be more distant (Hemmi et al., 2011). A receptor determination study has shown that the amino acid (aa) motif RGD in the fiber gene of MAdV-1 and −3 is responsible for the binding to αvβ6 and αvβ8 integrins (Bieri et al., 2021). A study on the high-resolution crystal structure and binding affinity of the carboxy-terminal head domain of the MAdV-2 fiber protein has revealed its binding to N-acetylglucosamine, which may play a role in the viral penetration through the mucin layer in the mouse gut (Singh et al., 2018).

Two additional rodent AdV species are *M. caviae* (former *Guinea pig mastadenovirus A*) and *M. sciuri* (former *Squirrel mastadenovirus A*). *M. caviae* has been established to contain guinea pig adenovirus 1 (GPAdV-1), an isolate from guinea pig (*Cavia porcellus*) with complete genome sequence analysis (Hofmann-Sieber et al., 2020). AdV-like particles have been detected by electron microscopy in guinea pigs presenting respiratory signs. The relationship to AdVs has been confirmed by PCR targeting the hexon gene (Pring-Åkerblom et al., 1997), but no further characterization of the virus has been performed for more than two decades. During attempts to isolate and propagate the virus in various guinea pig cell lines from archival frozen tissue samples, two GPAdV-1 isolates have been obtained, identifying the pathogen that has caused severe respiratory disease among laboratory guinea pig colonies worldwide (Kajon et al., 2019). The species *M. sciuri* includes red squirrel adenovirus 1 (SqAdV-1). The presence of AdVs in red squirrels (*Sciurus vulgaris*) that had died following translocation in the wild from west to east was first reported in 2001 (Sainsbury et al., 2001). In the following years, this virus has been found at several geographical locations of Great Britain (Everest et al., 2010a, 2010b; Martínez-Jiménez et al., 2011) in association with severe diarrhea of the native red squirrels. Interestingly, SqAdV-1 has also been detected in healthy grey squirrels (*Sciurus carolinensis*), representatives of an invasive species introduced to the UK from North America (Everest et al., 2009). Subsequently, the emergence of SqAdV-1 has been documented among red squirrels in Germany and Italy (Abendroth et al., 2017; Romeo et al., 2014); Recently, sequences from a different AdV, which does not seem to be a close relative of SqAdV-1 have been reported from Korea and Portugal. The novel virus is classified tentatively as SqAdV-2, and obviously represents a novel species (Côrte-Real et al., 2020; Kim et al., 2017).

In addition to their potential to induce severe disease in certain rodent populations, rodent AdVs have attracted attention as model candidates in vector research for gene therapy and next-generation vaccines (Hemmi and Spindler, 2019; Robinson et al., 2009). Rodents themselves serve as excellent model systems for studying innovative vectors (De Vleeschauwer et al., 2018).

Adenoviral sequences, detected by virome analysis or by PCR in samples from animals belonging to rodents, including various mouse species, rats, voles, ground squirrels and hamsters without the successful isolation of the viruses have also been published (Kumakamba et al., 2019; Phan et al., 2011; Wu et al., 2018; Zheng et al., 2016). However, the number of known AdV types in rodents is still very low especially compared to the number (>2000) of the potential host species. Therefore, our goal was to detect AdVs from as many host species as possible belonging to the most diverse mammalian order, Rodentia. The more adenoviral sequences we know, the more accurate inferences we can make about the ancestral origin of rodent AdVs and their coevolution with their hosts.

2. Materials and Methods

2.1. Samples

We screened approximately 400 various rodent samples (feces or organs of dead animals), collected from different pet shops (Hungary and the Netherlands), zoos and natural habitats (Hungary and Germany) over the past two decades from 2004 onwards. Genomic DNA was extracted applying the E.Z.N.A. DNA Stool Kit (OMEGA Bio-Tek) for fecal samples or the NucleoSpin® Tissue Kit (Macherey-Nagel) for organ samples, according to the manufacturer's instructions.

2.2. PCR analyses

The presence of AdVs in the samples was tested by nested PCRs using consensus degenerate primers targeting the most conserved region of the DNA-dependent DNA polymerase (pol) (Wellehan et al., 2004) and IVa2 genes (Vidovszky et al., 2015). The sizes of the PCR amplicons were 318–324 bp and 253 bp, respectively. To further characterize the positive samples for both pol and IVa2, additional PCR was performed to link the two short gene fragments whose positions in the AdV genome are relatively close to each other (~2500 bp). Additional consensus PCRs were also performed targeting the genes of hexon (Kiss et al., 1996) and pVIII (Vidovszky et al., 2022). Specific PCRs were performed to link the sequenced parts of SqAdV-1 by designing appropriate primer pairs. The investigation of the completeness of the E1B 19 K gene, in the case of MAdV-3 was also performed using PCR primers based on the nucleotide (nt) sequence of MAdV-3 available in GenBank (EU835513).

The reaction mixtures contained 12.5 µl of 2 × Dream Taq Hot Start PCR Master Mix (Thermo Scientific), 0.5 µl (25 pmol/µl) of each primer and 2 µl (~100 ng) of target DNA. Milli-Q water was added to a final volume of 25 µl. In the second round of nested PCRs, 2.5 µl of the product from the first round was used as template. For amplification of longer DNA fragments, custom designed, specific primers and the PrimeSTAR Max DNA Polymerase (Takara) were used.

For nested PCRs, the thermocycling program consisted of an initial denaturation step at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 46 °C for 1 min and elongation at 72 °C for 1 min with a final elongation step at 72 °C for 3 min. For the PrimeSTAR Max enzyme, the PCR program and the contents of the reaction mixture were adjusted and optimized according to the manufacturer's instructions. The amplicons were visualized by agarose gel electrophoresis.

2.3. DNA sequencing

PCR amplicons were excised from the agarose gels and DNA was purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Sequencing was performed for both strands by primer walking using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) then the electrophoresis was carried out on an ABI Prism 3500 Genetic Analyzer by a commercial service.

2.4. Bioinformatics and phylogenetic analysis

The CLC Genomics Workbench 8.5 (CLC bio) program was used for annotation and other genome analyses.

Two maximum likelihood phylogenetic trees were constructed, based on the derived partial aa sequences of the pol and IVa2 proteins. A third tree was based on the nt sequences of the genome parts joining the partial fragments of the two adjacent genes. To aid proper phylogenetic placement in the third analysis, the genomic regions of the complete IVa2 and DNA polymerase genes were used from the reference strains for the tree inference, and these were aligned with the partial sequences. The aa sequences were aligned using the G-INS-i algorithm in MAFFT v7.490 (Katoh and Standley, 2013), while the nt sequences were aligned using MUSCLE v3.8.425 (Edgar, 2004). The nt alignment was edited using the Automated1 algorithm of trimAl v1.3 (Capella-Gutiérrez et al., 2009), while no editing of the aa alignments was required. The final length of the alignments was 84 and 92 aa for the IVa2 and DNA polymerase, respectively. The length of the joint fragment alignment was roughly 3600 nt, containing the GenBank available complete pol and IVa2 genes and the newly determined, IVa2-pol linked (~2500 nt) sequences. Using ModelTest-NG v0.1.5, the LG + G evolutionary model was selected for the two aa alignments and the GTR + I + G model for the nt alignments (Darriba et al., 2020). The trees were derived using RAxML-NG v1.1.0, and the robustness of the trees was determined with non-parametric bootstrap calculations using 1000 replicates Kozlov et al., 2019). Transfer bootstrap expectation values were applied to the trees (Lemoine et al., 2018). Phylogenetic trees were visualized using MEGA 7, rooted at the midpoint, and only bootstrap values $\geq$ 75 % are shown (Kumar et al., 2016).

To estimate the divergence times of rodent-associated adenoviral lineages, we employed BEAST v2.7.7 (Bouckaert et al., 2019), using the partial DNA polymerase amino acid sequences as input. The analysis applied the LG + G evolutionary model, an optimized relaxed clock model (Douglas et al., 2021), and a birth–death tree prior. An exponential distribution with a mean of 10.0 was specified as the prior for the mean branch rates (ORCucldMean.c). Molecular clock calibration was achieved by assigning ages to two viral clades, assuming coevolution and codiversification with their respective hosts: Old World monkey adenoviruses were assigned an age of 35 million years (Schrago and Russo, 2003), and Arctoidea (bears, seals, and weasels) adenoviruses were assigned an age of 46 million years (Nyakatura and Bininda-Emonds, 2012). Markov Chain Monte Carlo simulations were run for 10 million generations, sampling trees and parameters every 10,000 steps. The final maximum clade credibility tree was constructed using TreeAnnotator v2.7.7, with the first 20 % of sampled trees discarded as burn-in. Tree visualization was performed using FigTree v1.4.4.

3. Results

3.1. Adenoviruses in rodent samples and their phylogeny

As presented in detail in Table 1, 28 distinct AdV sequences were obtained from 46 positive samples belonging to 17 host species from four suborders of the order Rondentia. The distribution of the species of the positive samples among the suborders were as follows: Castorimorpha (1 species), Hystricomorpha (4), Myomorpha (10) and Sciuromorpha (2). A sample was considered positive if adenoviral sequence was obtained at least from one PCR. From the 46 positive samples, 39 (84.8 %) were positive for IVa2 and 30 (65.2 %) for pol. Both PCRs were successful in 23 (50 %) samples. For 16 samples (34.8 %) only IVa2 was amplified, while for seven samples (15.2 %) only the pol PCR worked. The PCR to join the two gene fragments was successful in ten of the double-positive 23 cases. Attempts for the PCR detection of two additional genes were less fruitful, in only 17.4 % of the positive samples: hexon and pVIII gene sequences were amplified from 8 samples. Based on the determined sequence parts, 20 of the identified rodent AdVs are novel, while six are variants of previously identified AdVs: MAdV-2 (5), and MAdV-3 (1) variants. In two cases, we detected previously identified rodent AdVs: SqAdV-1 and GPAdV-1 (Table 1). In addition to the aforementioned MAdV-3 variant and the previously known GPAdV-1, we identified also novel AdVs in samples of guinea pigs and striped field mice. The accession numbers of the sequences submitted to GenBank are listed in **Supplementary document 1**.

The phylogenetic trees are presented in Fig. 1. Every newly detected AdV belonged to the genus *Mastadenovirus*. At least three monophyletic rodent AdV clades appeared in all four phylogeny reconstructions. Two of these clades consisted of virus strains from two or three different rodent suborders (Hystricomorpha, Myomorpha, Sciuromorpha), while the third consisted almost exclusively of viruses from the family Muridae (suborder Myomorpha). Bayesian inference (Fig. 2) corroborated the evolutionary history reconstructed through maximum likelihood analyses. It estimated the divergence of the Muridae clade at approximately 82 million years ago, while the two further rodent clades were dated to 64 and 49 million years ago, respectively.

3.2. MAdV-2 variants

When screening samples from house mice, we detected only MAdV-2 variants and no MAdV-1 or other AdVs at all. Of the seven positive house mouse samples, five different MAdV-2 sequence variants were identified. They all differed from the reference strain MAdV-2 (K87) available in GenBank (HM049560) and showed quite high diversity. When examining the amplified partial pol sequence (270 nt), we found nt differences among the five MAdV-2 variants at 23 different positions, but only three (nt positions 148, 170, 202) of these resulted in aa changes, two in ‘variant 4′ and one in ‘variant 5′. Apart from these positions, all the other 20 nt differences proved to be silent mutations as shown in Table 2.

3.3. Amplification of E1B 19 K gene of MAdV-3

In the complete genome sequence record of the reference strain of MAdV-3 (GenBank No. EU835513), the gene of the E1B 19 K protein contains a stop codon after the twenty-third aa. This results in a predicted truncated, short product instead of the average-sized (150–200-aa) protein. We examine this gene in the MAdV-3 variant (OR405365) detected in Hungarian striped field mouse samples. The sequence of the corresponding, PCR-amplified fragment did not contain the stop codon, indicating that in the Hungarian MAdV-3 sample a full-length 19 K gene is present.

3.4. Coding-complete genome of a novel AdV found through data mining

During a comprehensive search for AdV sequences in the GenBank, we came across the Ord’s kangaroo rat (*Dipodomys ordii*) genome sequencing project, conducted by scientists in the Baylor College of Medicine, Houston. This project identified an almost complete, unspecified AdV genome sequence (ABRO02087964) in the dataset. With the generous permission of the authors, we annotated and analyzed this genome sequence, supposedly originating from an AdV of the animal because the sequence was derived from the kidney. We present the genomic map in Fig. 3.

The complete scaffold consists of 27,746 bp with an average G + C content of 40.8 % that correlates with the overall base composition (42 % G + C) of the chromosomal DNA of the kangaroo rat. Inversely repeated sequence stretches of 161 bp were found at both ends of the contig, however at the putative left genome end, an additional, non-repeated 23-bp addition exists. The genome was predicted to contain 28 genes in an arrangement typical for mastadenoviruses. The annotated genome of the kangaroo rat adenovirus 1 (KRAdV-1), provided in **Supplementary document 2**, was submitted to GenBank (BK067721) as a TPA (third party annotation).

Table 1
Samples positive for AdV pol and IVa2 by PCR. Colours indicate different G + C %. Dark blue: very low (<40 %), light blue: low (40–45 %), green: balanced (45–55 %), orange: high (55–60 %), burgundy: very high (60–70 %), red: extremely high (70 %<).

	host species	common name	AdV strain	IVa2 fused	pol	AdV name	G+C%
Myomorpha	*Thallomys paedulcus*	acacia rat	I118	+	-	acacia rat adenovirus 1	34.52
	Microtus arvalis	common vole	Ma3	-	+	common vole adenovirus 1	43.33
	Phodopus sungorus	Djungarian hamster	I39	+	+	Djungarian hamster adenovirus 1	48.08
			I103	+	+		
	Cricetus cricetus	European hamster	H5	-	+	European hamster adenovirus 1	52.96
	Mesocricetus auratus	golden hamster	MT04		+	golden hamster adenovirus 1	48.09
			MGR04	+	-		
			I42	+	-		
			I43	+	-		
			I44	+	-		
			MB1454	+	+	golden hamster adenovirus 2	52.30
	Mus musculus	house mouse	MT01	-	+	murine adenovirus 2 variant 1	65.90
			I104_v1	+	+		
			I104_v2	+	+	murine adenovirus 2 variant 2	64.94
			I109	+	+	murine adenovirus 2 variant 3	65.13
			MT18	+	+	murine adenovirus 2 variant 4	65.33
			MT22	-	+		
			I40	+	+	murine adenovirus 2 variant 5	65.13
	Meriones unguiculatus	Mongolian gerbil	MGR03	+	-	Mongolian gerbil adenovirus 1	52.78
	Apodemus agrarius	striped field mouse	Ma16	+	+	murine adenovirus 3 variant 1	43.49
			Ma95	-	+		
			Ma91	+	+	striped field mouse adenovirus 1	51.15
			Ma92	+	+		
	Apodemus sylvaticus	wood mouse	MTE1	+	-	wood mouse adenovirus 1	64.87
			MTE2	+	-		
			MTE4		+		
			MTE7	+	-		
			I114	+	-		
			I180	+	-		
			MTE3		+	wood mouse adenovirus 2	54.30
	Apodemus flavicollis	yellow-necked mouse	L2	+	-	yellow-necked mouse adenovirus 1	49.21
Hystrico-morpha	*Hydrochoeris capybara*	capybara	Ma40	+	+	capybara adenovirus 1	51.92
	Octodon degus	common degu	MB1453	-	+	common degu adenovirus 1	71.11
	Cavia porcellus	guinea pig	TM1	+	+	guinea pig adenovirus 1	65.71
			MGR06		+	guinea pig adenovirus 2	50.54
	Dasyprocta leporina	red-rumped agouti	JMag08	-	+	red-rumped agouti adenovirus 1	51.85
Sciuromorpha	*Spermophilus citellus*	European ground squirrel	BK133		+	European ground squirrel adenovirus 1	53.51
			BK135		+	European ground squirrel adenovirus 2	56.06
			BK138		+	European ground squirrel adenovirus 3	44.87
	Sciurus vulgaris	red squirrel	15357		+	squirrel adenovirus 1	45.71
			15358		+		
			15359		+		
Castori-morpha	*Castor fiber*	Eurasian beaver	BK139	+	-	Eurasian beaver adenovirus 1	30.56
			BK140	+	-		
			BK141	+	-		
			MG2	+	-	Eurasian beaver adenovirus 2	30.56

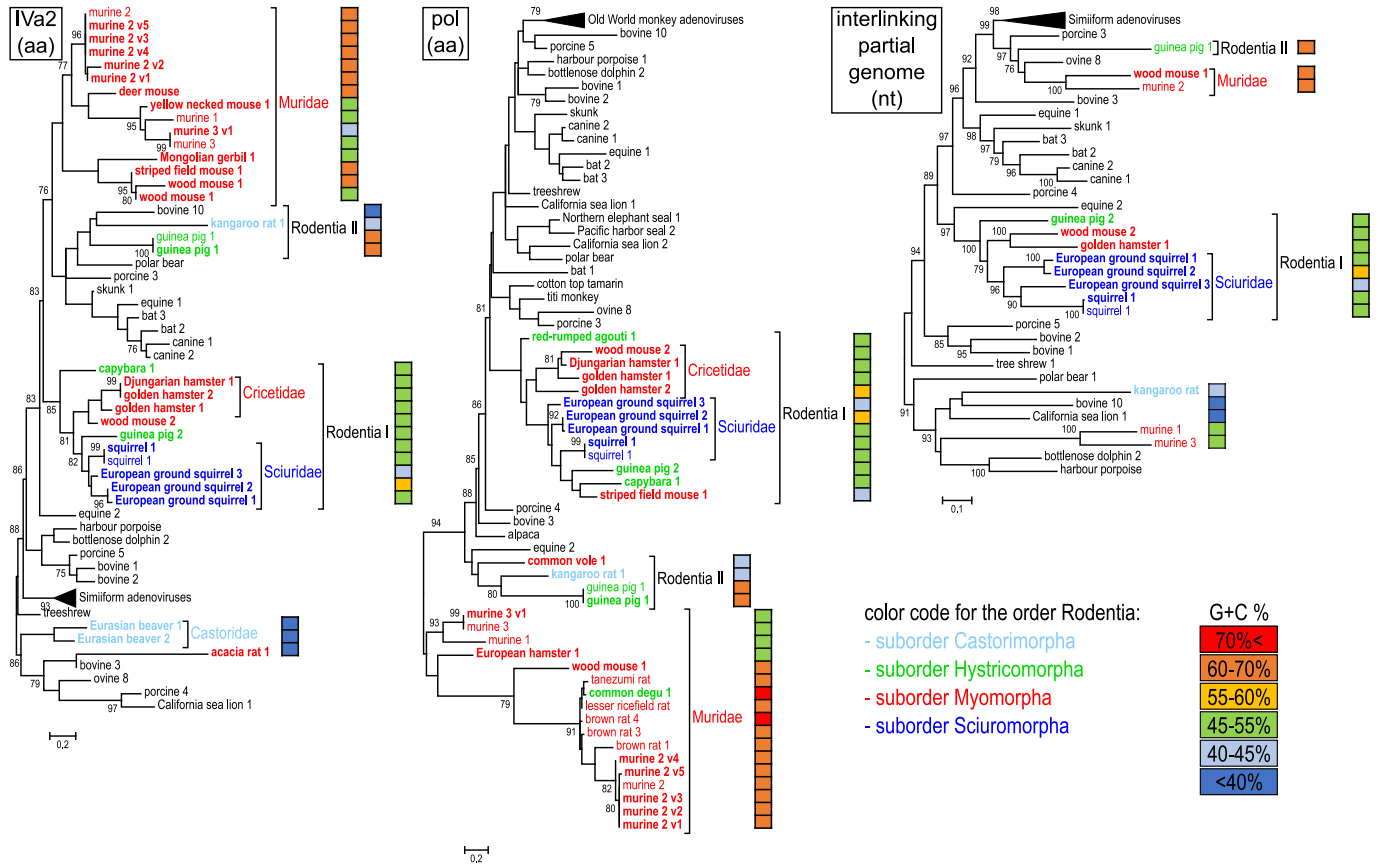


Fig. 1. Phylogenetic analysis of rodent adenoviruses. Phylogenetic tree reconstructions based on partial IVa2 and DNA-dependent DNA polymerase (pol) protein amino acid sequences and on the nucleotide sequences of the genome fragment interlinking the gene fragments. The newly discovered AdVs are highlighted in bold. The color of AdV names of rodent origin corresponds to the color representing the given rodent suborder. Virus names include the host species and type serial number. The color of the bars next to the phylogenetic trees indicates the different G + C % of the AdV next to it. Dark blue: very low (<40 %), light blue: low (40–45 %), green: balanced (45–55 %), orange: high (55–60 %), burgundy: very high (60–70 %), red: extremely high (70 %<).

3.5. Squirrel AdV

A PCR study was performed on the genome of an AdV detected in red squirrels in Germany in 2011 (Peters et al., 2011). The samples originated from three squirrels died of necrotizing enteritis. After amplification of parts of the pol and IVa2 genes, fragments from the hexon and pVIII genes were also obtained by PCR. Because the samples seemed to contain sufficient viral DNA, complete genome sequencing, using PCRs with custom designed specific primers, was initiated. We determined the nt sequence of more than half of the genome, from the IVa2 to the pVIII genes (PP157592). Meanwhile, the complete genome sequence (35,030 bp, KY427939) of another SqAdV-1 strain, detected in Germany in 2013 was published (Abendroth et al., 2017). The 20,600 bp-long sequence we obtained was identical to the fully sequenced SqAdV-1 (only 2 nt differences in non-coding regions were noted), so its investigation was discontinued.

4. Discussion

The use of two consensus PCR analyses, targeting the pol and IVa2 genes, significantly improved the success rate of AdV detection in rodents. Although the pol PCR is generally considered an ideal diagnostic tool in screening avian and reptilian samples (Wellehan et al., 2004), its performance in detecting AdVs in mammals, i.e., members of the genus *Mastadenovirus* seems to be slightly less reliable. In mammals, it is therefore questionable whether IVa2 or pol PCR is more effective, but it is recommended to use both. Based on our study's experiences the hexon and pVIII PCRs seemed less suitable for detecting AdVs in mammals. This is presumably due to the lower sensitivity of the hexon PCR (non-nested) and the fact that the pVIII gene is less conserved. This is important to point out, because hexon PCRs are still used in many AdV screening of mammals, which is clearly much less effective than the pol or IVa2 PCRs.

An unexpected finding was the presence of a 27-nt-long insertion in the pol sequence of every newly identified, putative wood mouse AdV-1, which was detected in 6 different wood mouse (*Apodemus sylvaticus*) samples. Interestingly, in the sequence of the PCR product joining the two partial gene fragments (from sample MTE4, OR405363), an additional 9-aa-long insertion was detected in the longer pol segment at a different location. To investigate the cause of this unusual size difference, further sequencing, preferably of the whole genome, would be required. These aa insertions were cut out from the multiple alignments created for the phylogenetic calculations to avoid bias.

Identical IVa2 sequences, likely representing the putative golden hamster AdV-1, were obtained from five samples of golden hamsters, originating from three enclosures from the Netherlands and Hungary. This finding strongly supports that the golden hamster is the natural host for the novel virus.

In the five newly detected variants of MAdV-2, the almost 10 % differences in the nt sequence leads to only three differences at the aa level only in two variants, with no more than two differences in a single variant. This indicates that we are dealing with a virus that has existed in this host for a very long time, persisting for millions of years. The variations in the nt sequence can persist, but at the aa level it is important to maintain the important properties of the coded protein. At 3 sites (nt 97, 109, 253), all the detected MAdV-2 variants were identical, but differed

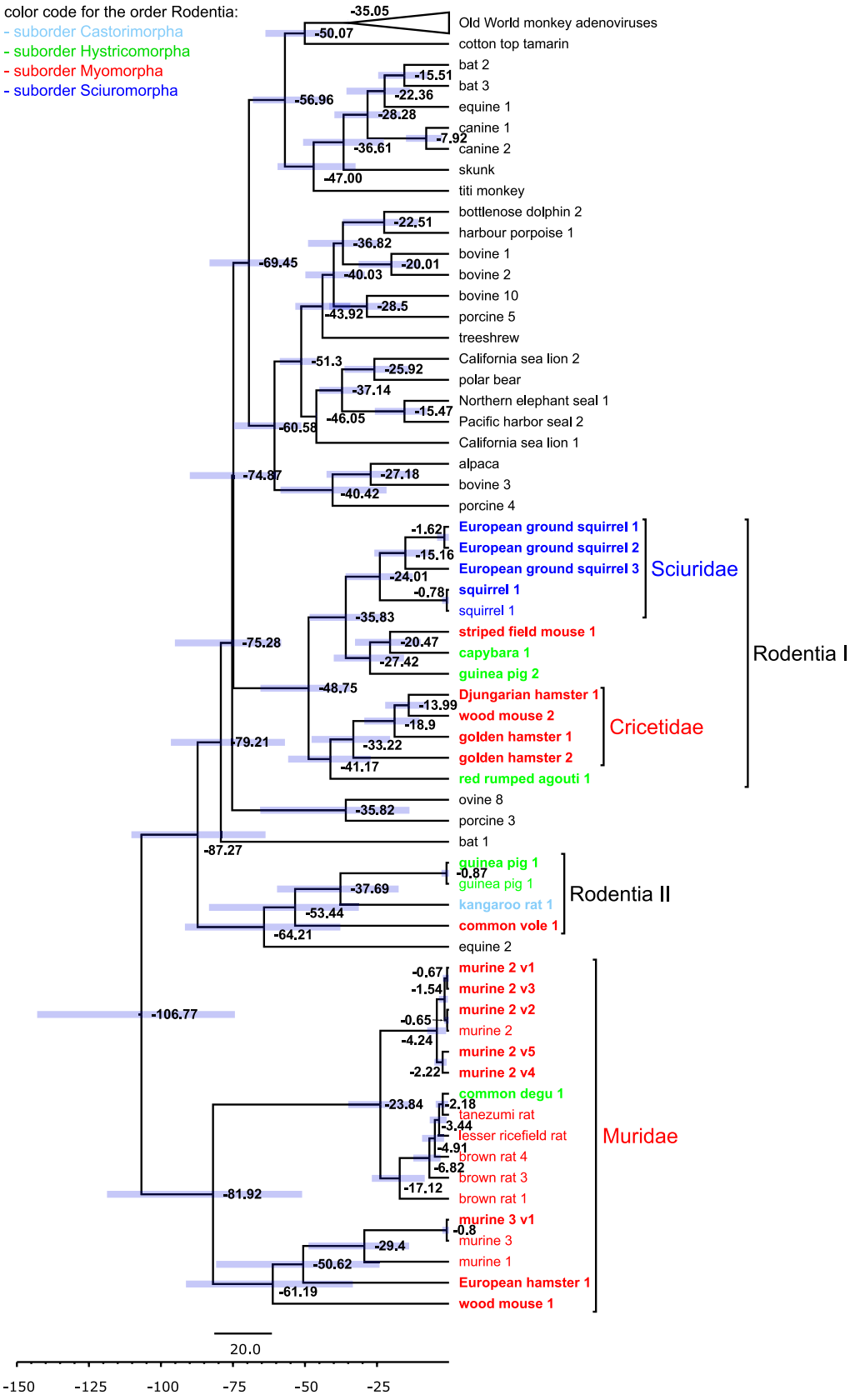


Fig. 2. Maximum clade credibility tree of rodent adenoviruses based on partial DNA-dependent DNA polymerase (pol) amino acid sequences. The newly discovered AdVs are highlighted in bold. The color of AdVs of rodent origin corresponds to the color representing the given rodent suborder. Virus names include the host species and type serial number. Node labels represent the estimated divergence times in million years and the 95% credible intervals for these node ages are shown with transparent blue bars.

Table 2
Point mutations in the pol sequence of the detected MAdV-2 variants.

MAdV-2 strain	nucleotide position#																					nt diff.	aa diff.
	25.	31.	55.	61.	67.	73.	79.	94.	97.	100.	109.	136.	139.	142.	148.	151.	169.	170.	187.	202.	220.	247.	253.		
K87	c	g	c	t	c	g	g	c	t	c	a	a	g	c	t	g	a	g	t	c	c	g	a	ref	ref
*MT01						a		g	c	t	g	c							g				g	8	0
*I104_v1						a		g	c	t	g	c							g				g	8	0
+MT18	g	c	g	c	t		a	a	c		g					t	g	a		g	t		g	15	2
+MT22	g	c	g	c	t		a	a	c		g					t	g	a		g	t		g	15	2
I40	a		t					g	c	t	g	c			g								g	9	1
I104_v2				g					c		g	c	t									t	g	7	0
I109									c		g		t	g							t	c	g	7	0

The framed positions (148, 170, 202) show point mutations causing aa substitutions. The * and + symbols show identical MAdV-2 variants, detected in different samples.

from the reference MAdV-2 (strain K87). This finding suggests that the reference strain may not represent the most ancient variant of MAdV-2.

The MAdV-3 variant found in Hungary differs by 1 nt in the partial pol and by 3 nt in IVa2 gene, although the aa sequences share 100 % identity with their counterparts in the reference MAdV-3 strain in GenBank. Amplification of the corresponding sequence part of its E1B 19 K gene confirmed that the wild strain MAdV-3, unlike the reference strain, contains a full-length 19 K gene. To rule out a previous sequencing error, we re-sequenced the 19 K gene of the reference strain available at the University of Zurich, which confirmed the presence of the truncated gene. The discrepancy between the 19 K genes of the wild and isolated viruses could be due to a mutation during the isolation of the reference strain. In an *in vitro* environment during the propagation of the virus, it may not have needed to retain the E1B 19 K gene, which plays a crucial protective role during virus infection and enhances gene expression (White et al., 1988). Another possibility is that there is a variant of MAdV-3 existing in nature in which the E1B 19 K gene is truncated.

In the genome sequence of the putative KRAdV-1, inverted terminal repeats (ITRs) of 161 bp were found. However, a 23-bp-long, proximal extension, at the left genome end, had no repetition in the right ITR. Because the canonical consensus starting sequence of ITRs (Dán et al., 2001) is missing even at the left end, the full length of ITRs likely exceeds 184 bp. Accordingly, the complete genome size of KRAdV-1 is estimated to be around 27,800 bp. This may be the shortest known mastadenovirus genome, while the second shortest is the 29,952-bp-long DNA of the polar bear AdV-1 (Böszörményi et al., 2020). Although the genome organization of KRAdV-1 is generally typical for mastadenoviruses, there are differences such as the lack of the gene for protein IX. Previous examples for such an absence include SqAdV-1 (Abendroth et al., 2017) or polar bear AdV-1 (Böszörményi et al., 2020). These three AdVs, have an ORF at the position of protein IX gene but without homology to each other or to any adenoviral genes. As expected, the left-hand end and middle region of the KRAdV-1 genome are conserved, and the normally unique right-hand end (the E4 region) contains mostly unknown ORFs. Interestingly, KRAdV-1 has no identifiable E3 region between the pVIII and fiber genes. Based on studies of human AdVs, genes in the E3 region play a role in evading the immunosurveillance of the host (Wold and Gooding, 1991). In rodents, a MAdV-1 mutant, in which expression of E3 proteins was inhibited, is less virulent than the wild strain in both mouse cell lines and mice (Cauthen et al., 1999). Neither the gene of protein 12.5 K nor the U-exon, which are almost generally present in the E3 region of mastadenoviruses, could be detected in the genome of KRAdV-1. The absence of these genes is remarkable, but neither is unprecedented. There are some known AdVs without U-exon (e.g, MAdV-1 and −3; Hemmi et al., 2011) or 12.5 K (reindeer AdV-1; Dastjerdi et al., 2022; polar bear AdV-1; Böszörményi et al., 2020). Yet this is the first AdV known to date that lacks both genes, with the complete absence of the E3 region.

The splicing sites in the genes IVa2, DNA-dependent DNA polymerase, pTP, which are composed of multiple exons in mastadenoviruses, were identified in KRAdV-1. The E1A seemed to be the only exception. The homologous sequence regions for the alternatively spliced 33 K and 22 K genes in the L4 region were also determined, but a start methionine could not be found. A possible reason for this could be that 22 K and 33 K proteins are encoded here with an alternative start codon. According to the results of BLASTp, the most closely resembling counterpart of the 33 K protein of KRAdV-1 is in bovine adenovirus 10 (BAdV-10). At the position of the starting methionine of the 33 K of BAdV-10, a threonine, coded by an ACG triplet, was found in KRAdV-1. Although the ACG triplet is widely accepted as a start codon only in bacteria and archaea, its function has been tested and confirmed, albeit with limited efficiency, in a eukaryotic translation system, too (Anderson and Buzash-Pollert, 1985). Our results indicate that the ACG triplet is used to initiate protein synthesis, supporting this nearly 40-year-old finding, but mRNA and translation studies would be needed to confirm it.

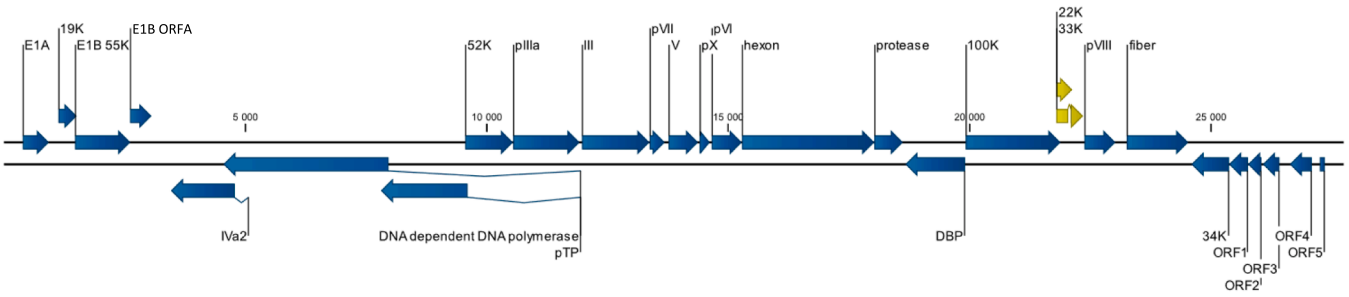


Fig. 3. Schematic genome map of the putative kangaroo rat adenovirus. The genome is represented by two horizontal lines with vertical markings of 5-kbp units. Putative genes and ORFs are indicated by arrows of proportional size. The exons in spliced genes are linked. Two genes (of the 22 K and 33 K proteins) lacking the canonical start codon (ATG) are shown in yellow. The presence of a hypothetical gene (E1B ORFA) was found in the traditional position of the protein IX gene.

Interestingly, the RGD motif, which is responsible for binding to αvβ6 and αvβ8 integrins in MAdV-1 and MAdV-3 (Bieri et al., 2021), is not present in the KRAdV-1 fiber gene.

We proposed to establish a new species for the annotated kangaroo rat adenovirus 1 due to the adequate phylogenetic distance, more than 15 % (Benkő et al., 2022), and the unique host. The proposed name of *Mastadenovirus dipodomysis*, based on the general binomial renaming of virus species and applying the genus name of the host has been accepted by ICTV (Benkő et al., 2023; Postler et al., 2022).

The increasing incidence of SqAdV-1 associated with enteritis and mortality in red squirrels Peters et al., 2011; Sainsbury et al., 2001) suggests that it is a common pathogen in Europe. The fact that the same AdV has also been detected in grey squirrels (Everest et al., 2009), raises the possibility that SqAdV-1 may have been transferred from introduced grey squirrels to the more susceptible red squirrel through host-switching (Everest et al., 2023). However, to prove this hypothesis, the AdV detected in grey squirrels should be studied in more detail.

Based on phylogenetic tree reconstructions, all rodent AdV strains have been placed in the genus *Mastadenovirus*, which is consistent with the fact that mastadenoviruses have coevolved with mammals through 100-millions of years (Kaján et al., 2020). However, the independent and distant monophyletic placement of these viruses suggests that multiple AdV lineages have coevolved with rodents; and since three different host suborders are detected in both major lineages (Fig. 1: Rodentia I and II), a relatively long-term virus-host coevolution can be hypothesized. The over-representation of the family Muridae among host species can be explained by the fact that this is the largest family of mammals. The distinct monophyletic clade of their AdVs may be the original, most ancient mastadenoviral lineage that has coevolved with members of the family Muridae.

We examined the G + C ratio of the available sequences of the detected viruses (Fig. 1, **Supplementary table 1** and Table 1), as it is suggested that a low G + C% may be a sign of host switching (Farkas et al., 2002; Papp et al., 2009). Fig. 1 clearly shows that viruses belonging to each monophyletic clade typically have similar G + C ratios. For example, the G + C% of viruses belonging to the Rodentia I clade is normally balanced. While the G + C% of rodent AdVs, that are closer to AdVs detected from non-rodent host species and being far from the rodent clades have not balanced G + C% (e.g. Eurasian beaver AdVs or acacia rat AdV-1 have a distinctly low G + C%, which could be a sign of host switching). Interestingly, the G + C% of AdVs in the Rodentia II clade is either low or high, different from balanced. The Muridae group, especially in the pol tree, has a distinct clade with balanced G + C content (MAdV-1, MAdV-3, and the European hamster), while the other subclade members of Muridae AdVs possess a high or extremely high (above 70 %) G + C%. Although the variation in the G + C ratio and its underlying causes remain unexplored, the similar G + C content of AdVs within the same phylogenetic clade is notably significant.

The long-term coevolution of adenoviruses and their mammalian hosts suggests that the common ancestor of all mammals was likely infected by the progenitor of mastadenoviruses (Kaján et al., 2020). In the absence of host-switching events, phylogenetic analyses would be expected to reveal a single Rodentia adenovirus clade. Contrary to this expectation, our analyses identified at least three ancient monophyletic clades of rodent adenoviruses, with node ages corresponding to the evolutionary timeline of rodents. The evolutionary age of rodents remains uncertain, with one study estimating their origin at approximately 61.7–62.4 million years ago (Wu et al., 2012), while another study dates the major bifurcation within the rodent clade to as early as 75 million years ago (Adkins et al., 2001).

It is important to note that the evolutionary conclusions and divergence time estimates presented here are inherently constrained by the limited sequence length and varying evolutionary rates across the phylogenetic tree. Despite these limitations, the observation of ancient, monophyletic adenovirus clades across all four phylogenetic reconstructions supports the hypothesis of long-term coevolution between at least three adenovirus lineages and rodents.

The origins of these adenoviral lineages remain unclear. While based on BLAST searches, close relatives of the newly identified rodent adenoviruses were incorporated into the phylogenetic analyses, all three lineages occupy deep, monophyletic branches, with no identifiable proximal relatives to suggest potential spillover events. Based solely on clade age, the Muridae adenovirus clade could represent the original coevolving adenovirus lineage. However, given host diversity, the other two lineages may also warrant similar consideration. Future research focusing on the biodiversity of adenoviruses in wild mammals and the complete genome sequences of these viruses will likely provide deeper insights into the origins and evolutionary trajectories of rodent adenoviruses. To take into account the possible geographic, ecological, and environmental impacts of host-switching events, the origin of the samples should be investigated. In the samples collected from animals living in the forests of Europe, the overlap of habitats of different species naturally plays a role, especially during the winter period when rodents move from forests and fields to human settlements in search of easier foraging. In the case of zoo samples, it is important to note the presence of free-living populations of house mice (*Mus musculus*), house rats (*Rattus rattus*), and Norway rats (*R. norvegicus*) in zoos, which are known to be excellent virus reservoirs of viral host-switching events for exotic rodents in zoos. In the case of samples from the pet trade, breeders often breed animals in inappropriate and unhygienic conditions, often in crowded conditions with several species close to each other. In pet shops, the immune systems of individual animals can be weakened due to the smoothing of visitors.

In summary, we detected 20 novel and several previously described AdVs in samples from 17 rodent species belonging to four suborders of the order Rodentia. In 13 out of the 17 species, no AdV sequences have been reported previously. Moreover, from some animals, including the acacia rat, no virus of any type has ever been reported to date.

Considering the wealth of extant rodent species, continued efforts foreshadow a strong increase in the number of novel adenoviruses. We plan to sequence the whole genome of selected novel AdVs to further characterize their specific features in the rodent host.

The GenBank accession numbers for the newly determined sequences are OR405311 to OR405365, BK067721 and PP157592.

CRediT authorship contribution statement

Márton Z. Vidovszky: Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis, Data curation, Conceptualization. **Győző L. Kaján:** Visualization, Methodology, Formal analysis, Conceptualization. **Kinga P. Böszörményi:** Writing – original draft, Formal analysis, Data curation. **Iva I. Podgorski:** Formal analysis, Data curation, Conceptualization. **Andor Doszpoly:** Formal analysis, Data curation, Conceptualization. **Mónika Z. Ballmann:** Investigation, Data curation. **Gergő Mitró:** Investigation, Data curation. **Gabriella Skoda:** Data curation. **Marije Turk:** Data curation. **Matthijs K. Groothuizen:** Data curation. **Marina Bidin:** Data curation. **Rodinde Hendrickx:** Data curation. **Silvio Hemmi:** Investigation, Formal analysis, Conceptualization. **László Egyed:** Data curation. **Mária Benkő:** Writing – original draft, Supervision, Project administration, Investigation, Formal analysis, Conceptualization. **Balázs Harrach:** Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2025.108287.

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