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ADENOVIRUSES OF THE MOST ANCIENT PRIMATE LINEAGES SUPPORT THE THEORY ON VIRUS–HOST CO-EVOLUTION

Iva I. PODGORSKI[1a*], Laura PANTÓ[1b], Katalin FÖLDES[1c], Iris de WINTER[2], Máté JÁNOSKA[1†], Endre SÓS[3], Baptiste CHENET[4], Balázs HARRACH[1] and Mária BENKŐ[1]

[1]Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary; [2]Department of Environmental Sciences, Resource Ecology Group, Wageningen University, the Netherlands; [3]Budapest Zoo and Botanical Garden, Budapest, Hungary; [4]Zoo de Montpellier, Montpellier, France

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The scarcity or complete lack of information on the adenoviruses (AdVs) occurring in the most ancient non-human primates resulted in the initiation of a study for exploring their abundance and diversity in prosimians and New World monkeys (NWMs). In order to assess the variability of these AdVs and the possible signs of the hypothesised virus–host co-evolution, samples from almost every family of NWMs and prosimians were screened for the presence of AdVs. A PCR-screening of 171 faecal or organ samples from live or dead, captive or wild-living prosimians and NWMs was performed. The PCR products from the gene of the IVa2 protein were sequenced and used in phylogeny calculations. The presence of 10 and 15 new AdVs in seven and ten different species of prosimians and NWMs was revealed, respectively. Phylogenetic analysis indicated that the tentative novel AdVs cluster into two separate groups, which form the most basal branches among the primate AdVs, and therefore support the theory on the co-evolution of primate AdVs with their hosts. This is the first report that provides a comprehensive overview of the AdVs occurring in prosimians and NWMs, and the first insight into the evolutionary relationships among AdVs from all major primate groups.

Key words: Prosimian adenovirus, New World monkey adenovirus, virus–host co-evolution, phylogenetic analysis, wild prosimian

[a]Present address: Division of Molecular Medicine, Ruđer Bošković Institute, Zagreb, Croatia; [b]Present address: Laboratory of Genome Sciences, Graduate School of Information Science and Technology, Hokkaido University, Sapporo, Japan; [c]Present address: Ankara University Veterinary Faculty, Ankara, Turkey; [†]M. Jánoska, a gifted scientist and beloved colleague passed away during the research period of this paper

[*]Corresponding author; E-mail: ivapodgorski@gmail.com; Phone: 00385 (1) 456-1064, Fax: 00385 (1) 456-1010

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Adenoviruses (AdVs) are double-stranded, linear DNA viruses infecting a wide variety of vertebrate hosts (Harrach, 2014), and are grouped into five genera (*Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus*) of the family *Adenoviridae* (Harrach et al., 2011). An additional genus, named *Testadenovirus*, was proposed for novel AdVs found in testudinoid turtles (Doszpoly et al., 2013). The International Committee on Taxonomy of Viruses assigned the AdVs of primates into 17 accepted species within the genus *Mastadenovirus*, thus representing the best studied AdVs today (https://talk.ictvonline.org/taxonomy).

Based on molecular phylogeny, the order Primates can be divided into two suborders, Strepsirrhini (containing infraorders Lemuriformes and Lorisiformes) and Haplorhini (containing infraorders Tarsiiformes and Simiiformes). Infraorder Simiiformes includes parvorder Platyrrhini or New World monkeys (NWMs) and Catarrhini (including Old World monkeys (OWMs), lesser apes and great apes) (Roos and Zinner, 2017). Strepsirrhini and Tarsiiformes are called prosimians on the basis of various shared primitive characteristics. Although from the representatives of different species of the infraorder Simiiformes more than 150 AdV types are known (https://sites.google.com/site/adenoseq), only very limited, or no information is available regarding the AdVs belonging to the more ancient primates (NWMs and prosimians). Currently, seven human AdV (HAdV) species and nine OWM AdV species (*Simian mastadenovirus A* to *I*) are officially recognised (Kovács et al., 2004; Jones et al., 2007; Roy et al., 2012; Chiu et al., 2013; Malouli et al., 2014; Pantó et al., 2015; Podgorski et al., 2016; Tan et al., 2016). Very recently, the first AdV species was established for a NWM AdV, *Platyrrhini mastadenovirus A* (Adams et al., 2017). Contrarily, no AdVs have ever been published from any prosimian host.

The very first NWM AdV was an isolate from owl monkeys, *Aotus* spp. (Shroyer et al., 1979). Three decades later, the titi monkey AdV (TMAdV) was isolated from red titi monkeys (*Callicebus cupreus*) with fatal pneumonia (Chen et al., 2011), and this AdV is still the only NWM AdV that has been sequenced completely. This was the first report about the potential cross-species transmission of an AdV from NWMs to humans. TMAdV was also capable of establishing infection in common marmosets (*Callithrix jacchus*), causing a mild, self-limiting disease (Yu et al., 2013), similar to the one seen in the infected humans. The presence of AdVs has also been reported in several wild-living NWMs, including a white-lipped tamarin (*Saguinus labiatus*) and three common marmosets (Wevers et al., 2011). All these NWM AdVs were found to be phylogenetically well separated from, and more ancient than, all the other primate AdVs. Two additional AdVs have been detected in a cotton-top tamarin (*Saguinus oedipus*) (Hall et al., 2012) and in a pygmy marmoset (*Cebuella pygmaea*) (Gál et al., 2013), but the phylogenetic calculations, based on the adenoviral DNA-dependent DNA polymerase gene (*pol*) fragment, showed both viruses to be

more closely related to non-primate than primate mastadenoviruses, implying the possibility of interspecies host switch(es) (Vidovszky et al., 2015).

The scarcity or complete lack of information on the AdVs occurring in the most ancient primates led us to initiate a study for exploring their abundance and diversity in prosimians and NWMs. Sampling of these primate groups is not an easy task since these species inhabit only Madagascar and parts of South America, respectively. For this reason, beside samples collected in Madagascar, we also examined captive animals kept in several European zoological gardens and research facilities. As expected, the presence of numerous hitherto unknown AdVs, even belonging to new lineages, was detected in representatives of many different species. The phylogenetic place of the newly detected AdVs was also estimated.

Materials and methods

Sample collection and processing, PCR and sequencing

Faecal samples from live or organ samples from dead, captive prosimians and NWMs were collected in one French, one Croatian and seven Hungarian zoos. Faecal and organ samples from a captive mouse lemur (*Microcebus murinus*) colony were kindly provided by Nadine Mestre-Francés. The examined collection consisted of 101 prosimian and 70 NWM samples (Table 1). The nucleic acid from the faecal samples and organs was extracted as described elsewhere (Doszpoly et al., 2014; Ballmann and Harrach, 2016). Two pairs of degenerate primers, targeting the IVa2 gene of mastadenoviruses (Pantó et al., 2015), were used to run nested PCRs. PCR products were sequenced and analysed by capillary electrophoresis performed by a commercial service on a 3500 Series Genetic Analyser (Life Technologies Inc., Warrington, UK).

Sequences and phylogenetic analyses

Newly gained sequences were analysed as described earlier (Pantó et al., 2015), assembled with the Staden Sequence Analysis Package, and translated to amino acid (aa) sequences. After removing the primer sequences, a 253-bp (84 aa) useful sequence was obtained. Phylogeny was inferred on partial IVa2 protein alignments by using the ProtDist and PhyML algorithms of the PHYLIP package (v3.5c) and Montpellier bioinformatics platform, respectively. The ProtDist analysis was run with the JTT substitution model, followed by Fitch-Margoliash analysis, applying the global rearrangements option (Harrach and Benkő, 2007). Bootstrap analysis with 100 sampling replicates was applied in the PhyML analysis which was based on a user tree obtained by the PHYLIP package. The resulting phylogenetic tree was visualised by the MEGA6 program. For construction of the primates' phylogenetic tree, nucleotide sequences of cytochrome c ox-

idase subunit I gene of each primate group representatives were used in a maximum likelihood analysis (GenBank accession numbers J01415, D38113, D38114, D38115, D38484, AF312703, AF312704, AF312708 and AF312709). Similarity plots and bootscanning analyses were performed with SimPlot 3.5.1 with window size 140 bp, step size 10 bp.

Results

Prevalence and diversity of adenoviruses in the samples

Specific amplicons, of approximately 300 bp in size, were obtained from 15 prosimian and 19 NWM samples (Table 2). Considering the total number (i.e. 171) of specimens, these values correspond to an overall positivity rate of almost 20%. Nucleotide (nt) and aa sequence comparisons showed that the prosimian samples contained 10, while the NWM samples 15 different, novel AdVs. Identical IVa2 sequences were sometimes derived from two or even three samples, which seldom had the same host species origin. The hitherto unseen putative AdVs were named tentatively after the common name of the host and the Arabic number if several AdVs were detected in the same species. If an AdV seemed to be capable of infecting several hosts, a less specified common name referring to a broader group of animal species (e.g. lemur or marmoset) was given (Table 2). DNA fragments with identical sequences were also recovered from different animals from the same place, marked as black lemur AdV-3 and lemur AdV, or, more often, from the same enclosure (red-fronted lemur AdV-2, marmoset AdV, tufted capuchin AdV-3, red-handed tamarin AdV-2). Partial IVa2 gene sequences of the newly detected AdVs were submitted to GenBank (accession numbers from MG574566 to MG574593).

The multiple alignment of the partial IVa2 aa sequences suggested a clear difference between the prosimian and NWM AdVs in certain amino acids (Fig. 1).

Phylogeny inference

A phylogenetic tree (Fig. 2), based on distance matrix and maximum likelihood analysis of partial adenoviral IVa2 aa sequences, confirmed the clear separation of the novel AdVs from all the earlier established AdVs of primates (except SAdV-WIV19 and the titi monkey AdV). Two new basal primate AdV (albeit not perfect) clusters appeared on the tree: prosimian AdVs as the most ancient ones of primate AdVs, and NWM AdVs, grouped together with previously described NWM TMAdV and, surprisingly, the recently described SAdV-WIV19 isolated from an OWM. The unexpected place of an OWM AdV on the phylogenetic tree prompted us to run the analysis of possible homologous recombination within the IVa2 gene. The analysis indicated that indeed the part of the IVa2 gene of SAdV-WIV19 which was used for this phylogenetic analysis, is more similar to TMAdV than to any other, closely related OWM AdV (Fig. 2).

Discussion

We successfully accomplished our primary aim of obtaining insights into the abundance, genetic diversity and evolutionary relationships of adenoviruses of the most ancient primate host lineages. Samples from every or almost every family of NWMs and the prosimian suborder Strepsirrhini were screened for the presence of AdVs, respectively. For the screening, we used primer pairs targeting the IVa2 gene of mastadenoviruses specifically, since these primers had proved to be the most robust for the detection of primate AdVs in our laboratory. For the sake of better detection performance, we had to accept the obvious drawback of the scarcer availability of the sequences of IVa2 gene in the GenBank, compared to the partial sequences from the *pol* and hexon genes of NWM and other AdVs (Hall et al., 2012; Gál et al., 2013; Lakatos et al., 2017). Previously we observed that TMAdV, the only NWM AdV with a fully sequenced genome, had appeared on a more basal branch of the phylogenetic tree, separated clearly from all the known human and simian AdVs (SAdVs) (Chen et al., 2011). This was in line with the remote taxonomic place of the titi monkey from all the OWMs and the more recent primates like apes (Perelman et al., 2011).

From the five prosimian families screened in this study (Table 1), AdVs were detected in the samples of only one family, i.e. the Lemuridae, probably due to the relatively small number of samples from the other four families. On the other hand, the presence of novel AdVs was detected in members of four of the five studied NWM families. Unfortunately, the separation of some individual clades was not supported by high bootstrap values (Fig. 2), most probably due to the shortness of the partial sequence of the IVa2 protein used for the analysis. Because of this later limitation, some ape and human AdVs supposed to belong to the same species, but which were not the main target of this study and therefore are collapsed in the phylogenetic tree for clarity, showed somewhat different clustering than those based on full gene sequences (Pantó et al., 2015). For example, the supposed ape members of species HAdV-C are separated in three groups depending on their host origin (human, chimpanzee/bonobo or gorilla, as marked with an asterisk in Fig. 2). However, they were still consistent with the broader evolutionary relationships observed earlier, thus we consider this analysis to be sufficient to obtain the first insights into the evolutionary relationships of all primate AdV groups.

Identical AdV sequences, only at aa level or sometimes even at nt level, were derived from samples collected from captive or wild animals from different parts of Europe and Madagascar, respectively (Table 2). In general, the diverse monkey species, in representatives of which the same virus was detectable, were usually very close evolutionarily (Perelman et al., 2011). On the other hand, sometimes we found rather diverse AdVs in representatives of a given host species.

Table 1: Species origin of the 171 specimens screened for the presence of adenoviruses: 101 from prosimians (suborder Strepsirrhini), 70 from New World monkeys (suborder Haplorhini, parvorder Platyrrhini)

Suborder	Infra-/parvorder	Family	Subfamily	Latin name	English name	Sample type[a]	No. of samples
Strepsirrhini	Lorisiformes	Galagidae	–	*Otolemur crassicaudatus*	brown greater galago	F	1
		Lorisidae	Lorisinae	*Nycticebus coucang*	greater slow loris	F	1
				Nycticebus pygmaeus	pygmy slow loris	F	1
	Lemuriformes	Cheirogaleidae	–	*Microcebus murinus*	mouse lemur	F/O	13
		Indridae	–	*Indri indri*	indri	F	4
		Lemuridae	–	*Eulemur albifrons*	white-headed lemur	F	1
				Eulemur coronatus	crowned lemur	F	5
				Eulemur fulvus	common brown lemur	F	11
				Eulemur macaco	black lemur	F	9
				Eulemur mongoz	mongoose lemur	F	6
				Eulemur rubriventer	red-bellied lemur	F	13
				Eulemur rufifrons	red-fronted lemur	F	22
				Eulemur sanfordi	Sanford's brown lemur	F	1
				Hapalemur griseus	eastern lesser bamboo lemur	F	1
				Lemur catta	ring-tailed lemur	F	9
				Varecia rubra	red ruffed lemur	F	1
				Varecia variegata	black-and-white ruffed lemur	F	2
Haplorhini	Platyrrhini	Aotidae	Aotinae	*Aotus azarae*	Azara's night monkey	F	1
				Aotus lemurinus griseimembra	gray-bellied night monkey	F	2
				Aotus trivirgatus	three-striped night monkey	F	1
		Atelidae	Alouattinae	*Alouatta caraya*	black howler	F	1
			Atelinae	*Ateles paniscus*	red-faced spider monkey	F	1
		Cebidae	Cebinae	*Cebus apella*	tufted capuchin	F	5
				Cebus capucinus	white-headed capuchin	F	1
			Saimiriinae	*Saimiri boliviensis*	black-capped squirrel monkey	F	1
				Saimiri sciureus	common squirrel monkey	F/O	17
		Callitrichidae	Callitrichinae	*Callithrix jacchus*	common marmoset	F/O	14
				Callithrix geoffroyi	white-headed marmoset	F	1
				Callithrix penicillata	black-tufted marmoset	F	1
				Cebuella pygmaea	pygmy marmoset	F	2
				Leontopithecus chrysomelas	golden-headed lion tamarin	F/O	4
				Saguinus imperator	emperor tamarin	F	2
				Saguinus labiatus	red-bellied tamarin	F	3
				Saguinus midas	red-handed tamarin	F/O	3
				Saguinus oedipus	cotton-top tamarin	F	9
		Pitheciidae	Pitheciinae	*Pithecia pithecia*	white-faced saki	F	1

[a]F = faeces, O = organ

Table 2

Novel adenoviruses detected in this study by nested PCR

	Host	Proposed adenovirus name	Sample type[a]	Place of sample origin
Prosimians	black-and-white ruffed lemur	black-and-white ruffed lemur AdV	F	Hungarian zoo 1
	black lemur 1	black lemur AdV-1	F	Nosy Komba Island, Madagascar
	black lemur 2	black lemur AdV-2	F	
	black lemur 3	black lemur AdV-3	F	
	black lemur 4		F	
	crowned lemur	crowned lemur AdV	F	French zoo
	eastern lesser bamboo lemur	eastern lesser bamboo lemur AdV	F	
	red-fronted lemur 1	red-fronted lemur AdV-1	F	Ranomafana National Park, Madagascar
	red-fronted lemur 2	lemur AdV	F	
	red-bellied lemur 1		F	
	red-bellied lemur 2		F	
	red-fronted lemur 3	red-fronted lemur AdV-2	F	Kirindy Mitea National Park, Madagascar
	red-fronted lemur 4		F	
	red-fronted lemur 5		F	Hungarian zoo 7
	ring-tailed lemur	ring-tailed lemur AdV	F	Hungarian zoo 1
New World monkeys	common marmoset 1	common marmoset AdV	O	Hungarian zoo 1
	common marmoset 2	marmoset AdV	O	Hungarian zoo 2
	common marmoset 3		O	
	white-headed marmoset		F	Hungarian zoo 3
	common squirrel monkey 1	common squirrel monkey AdV-1	F	Hungarian zoo 5
	common squirrel monkey 2	common squirrel monkey AdV-2	O	Hungarian zoo 2
	common squirrel monkey 3	common squirrel monkey AdV-3	F	Hungarian zoo 4
	cotton-top tamarin	cotton-top tamarin AdV	F	Hungarian zoo 5
	golden-headed lion tamarin	golden-headed lion tamarin AdV	F	Hungarian zoo 5
	gray-bellied night monkey	gray-bellied night monkey AdV	F	French zoo
	red-bellied tamarin	red-bellied tamarin AdV	F	Hungarian zoo 3
	red-faced spider monkey	red-faced spider monkey AdV	F	Hungarian zoo 1
	red-handed tamarin 1	red-handed tamarin AdV-1	O	Hungarian zoo 6
	red-handed tamarin 2	red-handed tamarin AdV-2	O	Hungarian zoo 1
	red-handed tamarin 3		F	French zoo
	tufted capuchin 1	tufted capuchin AdV-1	F	Croatian zoo
	tufted capuchin 2	tufted capuchin AdV-2	F	Hungarian zoo 4
	tufted capuchin 3	tufted capuchin AdV-3	F	Hungarian zoo 1
	tufted capuchin 4		F	Hungarian zoo 5

[a]F = faeces, O = organ

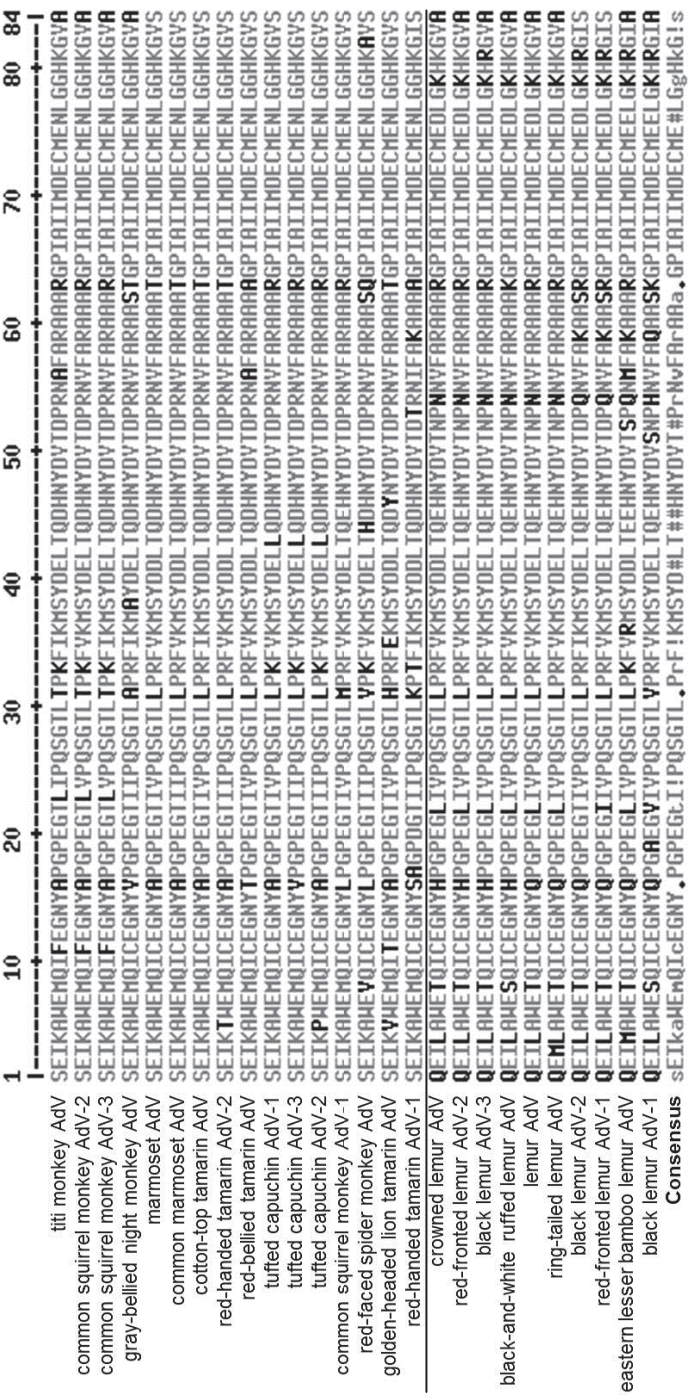

Fig. 1. Alignment of the amino acid sequences of the partial IVa2 protein identified in the newly detected adenoviruses (AdVs) compared to titi monkey AdV. New World monkey AdVs are presented above, and the prosimian AdVs under the black line

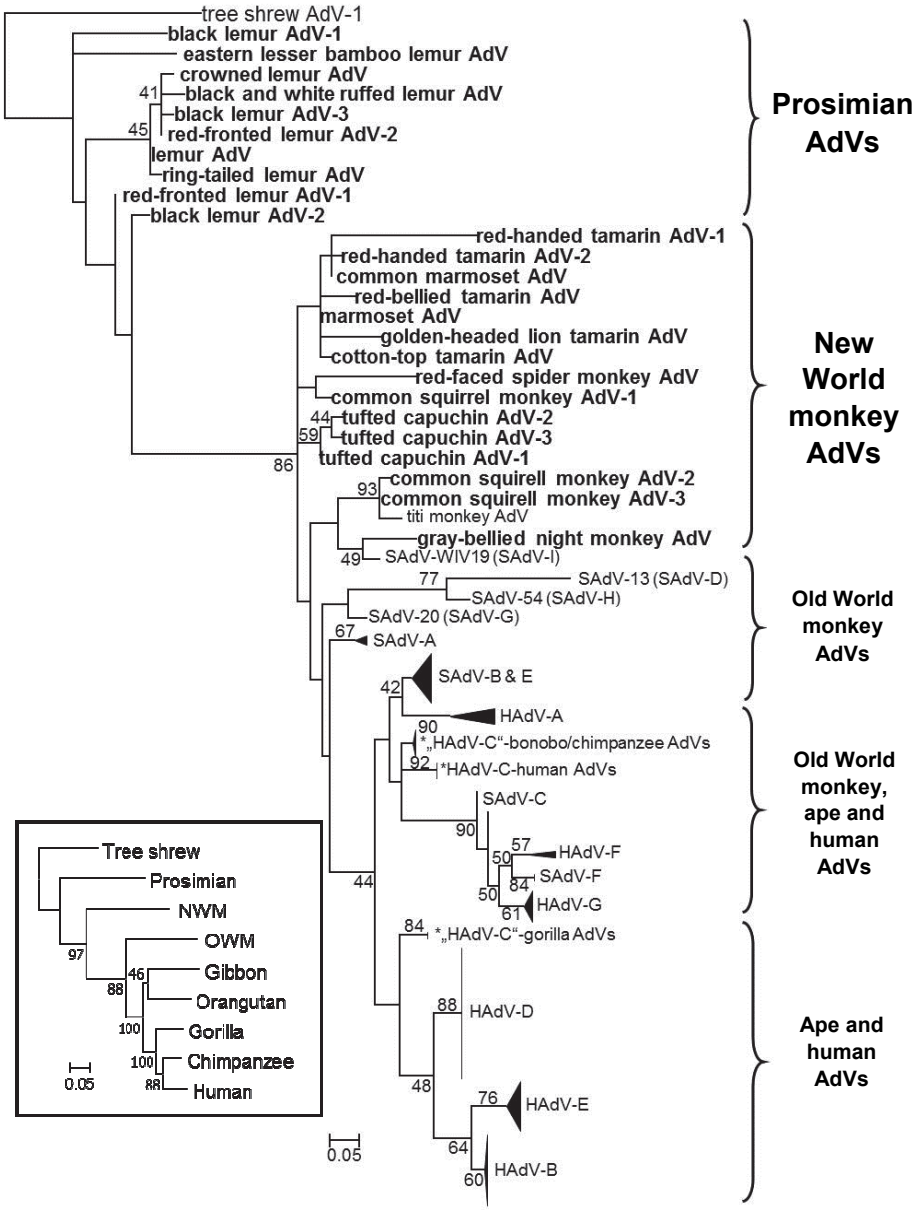

Fig. 2. Phylogeny reconstruction (ML analysis) based on partial (84-aa) sequences of the IVa2 protein of primate adenoviruses (AdVs). A phylogenetic tree of primates is shown in the bottom left corner to demonstrate the parallel evolution of AdVs and hosts. For visualisation tree shrew AdV-1 was used as outgroup. Bootstrap analysis with 100 sampling replicates was applied. The novel AdVs, discovered in this study, are highlighted in bold. Abbreviated species names are shown for human or simian AdV species which were collapsed for clarity. *Simian mastadenovirus A*: SAdV-A; *Human mastadenovirus B*: HAdV-B, etc. Hosts of AdVs presumably belonging to species HAdV-C are marked with an asterisk due to their separation

The lineage of NWM AdVs contains several clusters (Fig. 2), which we compared, if supported by higher bootstrap values, to that of the corresponding hosts (Perelman et al., 2011). One cluster contains all the tamarin and marmoset AdVs (similar hosts), a further one the tufted capuchin AdVs, and yet another the common squirrel monkey and titi monkey AdVs. The position of TMAdV on the tree in a common clade with common squirrel monkey AdVs might be considered unexpected since these hosts are not closely related evolutionarily (Perelman et al., 2011). Moreover, since AdVs usually cause only mild disease or subclinical infections in their hosts, the case of high fatality rate (83%) associated with the TMAdV outbreak in the titi monkey colony had already indicated that the titi monkey is perhaps not the native host for TMAdV (Chen et al., 2011). Furthermore, it has been described repeatedly that cross-species transmission can result in elevated pathogenicity in the new host compared to that in the original host (Jánoska et al., 2011; Kohl et al., 2012; Vidovszky et al., 2015). Another AdV, reported to be derived from a NWM but most probably having origin in some other host, is the pygmy marmoset AdV (Gál et al., 2013; absent from the phylogenetic tree due to the lack of the IVa2 protein gene sequence in the GenBank). Its host was found dead with signs of respiratory infection. The partial *pol* sequence of this virus is identical to that of an AdV found in dead skunk (*Mephitis mephitis*) and in four-toed hedgehogs (*Atelerix albiventris*) (Kozak et al., 2015; Madarame et al., 2016), thus placing the virus far from the primate AdV lineages in phylogeny reconstructions. Finally, this virus is hypothesised as coevolved originally with bats based on inferred phylogeny and genome organisation characteristics (i.e., the genes in the E3 and E4 regions) (Kozak et al., 2015; Vidovszky et al., 2015). These data indicate that certain AdVs can cross the host species barrier, and can be transmitted between primate hosts, and sometimes even between primate and non-primate animals.

Interestingly, an OWM SAdV (WIV19) from golden snub-nosed monkey (*Rhinopithecus roxellana*) clustered with the newly detected NWM AdVs. The original report on SAdV-WIV19 pointed out that the virus represents a distinct branch separated from all the other OWM AdVs (Tan et al., 2016), which can indicate that either this virus has another host origin, or it could be an intermediate between OWM and NWM AdVs. However, all phylogenetic analyses from the previous studies based on other genes placed this AdV closer to OWM AdVs than to the only fully sequenced NWM AdV, TMAdV (Tan et al., 2016). Consequently, the results of our phylogenetic analysis, based on partial IVa2 protein sequence, prompted us to screen this gene for possible homologous recombination(s) between SAdV-WIV19 and two other closely related OWM AdVs, as well as NWM TMAdV. Homologous recombinations have been already demonstrated in numerous studies in primate AdVs (Crawford-Miksza and Schnurr, 1996; Walsh et al., 2011; Chiu et al., 2013; Dehghan et al., 2013; Podgorski et al., 2016), and are known as an important driver of AdV evolution. Indeed, we could

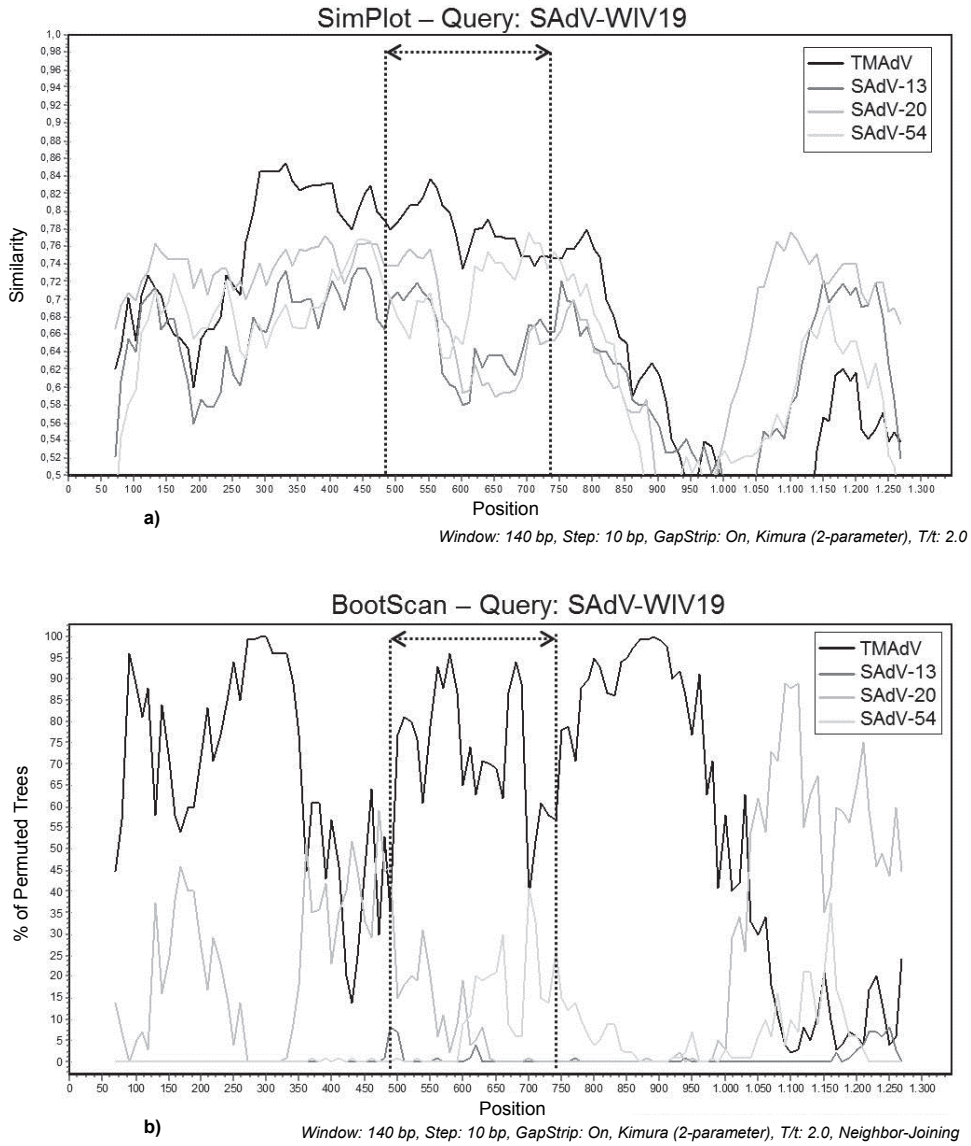

Fig. 3. (a) SimPlot and (b) BootScan analyses of the IVa2 gene of simian adenovirus 13 (SAdV-13), SAdV-20, SAdV-54 and titi monkey AdV (TMAdV) relative to SAdV-WIV19. The part of the IVa2 gene used in the phylogenetic analysis in Fig. 2 is marked with dotted lines

observe that the part of the IVa2 gene of SAdV-WIV19 used for our phylogenetic analysis is more similar to its counterpart in TMAdV than in the OWM AdVs (Fig. 3). However, it is also obvious that there are very few OWM AdVs, and even less fully sequenced NWM AdVs (actually only a single one), to which the

SAdV-WIV19 could be compared to. Consequently, at this point we can only state that the reason for its position on the phylogenetic tree, based on partial IVa2 protein, might be a homologous recombination.

In this study we detected numerous hitherto unknown AdVs in NWMs and, for the very first time, also in prosimian hosts. In phylogeny inference, every novel AdV clustered into novel clades of the primate AdVs. Thus the known lineages of primate AdVs were broadened significantly. None of the two groups (of the prosimian or NWM AdVs) showed clear monophyly. The presence of several paraphyletic branches in both clades might have resulted from the relative shortness of the partial, well-conserved sequence of the IVa2 protein, used for the initial characterisation and classification. In the majority of the cases, the theory on the co-evolution of adenoviruses with their hosts seemed to be confirmed; however, the traces of host switches were also recognised occasionally. Such events usually involved phylogenetically closely related host species whereas larger-scale host switches were rarely recognised. We plan more detailed genome sequence sampling and analyses of selected AdVs from each representative group.

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