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RANDOM SAMPLING OF THE CENTRAL EUROPEAN BAT FAUNA REVEALS THE EXISTENCE OF NUMEROUS HITHERTO UNKNOWN ADENOVIRUSES[+]

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From over 1250 extant species of the order Chiroptera, 25 and 28 are known to occur in Germany and Hungary, respectively. Close to 350 samples originating from 28 bat species (17 from Germany, 27 from Hungary) were screened for the presence of adenoviruses (AdVs) using a nested PCR that targets the DNA polymerase gene of AdVs. An additional PCR was designed and applied to amplify a fragment from the gene encoding the IVa2 protein of mastadenoviruses. All German samples originated from organs of bats found moribund or dead. The Hungarian samples were excrements collected from colonies of known bat species, throat or rectal swab samples, taken from live individuals that had been captured for faunistic surveys and migration studies, as well as internal organs of dead specimens. Overall, 51 samples (14.73%) were found positive. We detected 28 seemingly novel and six previously described bat AdVs by sequencing the PCR products. The positivity rate was the highest among the guano samples of bat colonies. In phylogeny reconstructions, the AdVs detected in bats clustered roughly, but not perfectly, according to the hosts' families (Vespertilionidae, Rhinolophidae, Hipposideridae, Phyllostomidae and Pteropodidae). In a few cases, identical sequences were derived from animals of closely related species. On the other hand, some bat species proved to harbour more than one type of AdV. The high prevalence of infection and the large number of chiropteran species worldwide make us hypothesise that hundreds of different yet unknown AdV types might circulate in bats.

Key words: Molecular virology, phylogeny, bat viruses, adenovirus

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[+]The GenBank accession numbers for the sequences are KM043057 to KM043112

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Adenoviruses (AdVs) are non-enveloped, dsDNA viruses occurring commonly in different vertebrate hosts from fish to man all over the world. The family *Adenoviridae* is divided into five approved genera mostly according to phylogenetic distance, genome organisation, and host origin (Davison et al., 2003; Harrach et al., 2011). The genera *Mastadenovirus*, *Aviadenovirus* and *Ichtadenovirus* contain AdVs derived from mammals, birds and a fish, respectively. The first identified members of the genus *Atadenovirus* were found in birds, ruminants and a marsupial, but targeted screenings revealed that these viruses are most prevalent in snakes and lizards, and the virus lineage seems to have originated in scaled reptiles (Farkas et al., 2008; Papp et al., 2009). The genus *Siadenovirus* comprises a continuously increasing number of avian AdV species together with a frog isolate, the only amphibian AdV known so far (Davison et al., 2000; Kovács et al., 2009; Park et al., 2012). Moreover, a siadenovirus has been described to cause mass mortality among confiscated Sulawesi tortoises *(Indotestudo forsteni)* (Rivera et al., 2009) and to infect other tortoise species, too (Schumacher et al., 2012). The evolutionary provenance of this genus is considered unclear at the moment (Kovács and Benkő, 2011). Recently, in different species of testudinoid turtles, candidate members of a sixth AdV lineage have been discovered, for which the establishment of a sixth genus (with the putative name *Testadenovirus*), has been proposed (Farkas and Gál, 2009; Doszpoly et al., 2013).

To further explore the biodiversity represented by AdVs, we conduct PCR screening of different vertebrate animals. In the past few years, bats have emerged as an especially rich source of novel viruses, including AdVs (Jánoska et al., 2011; Vidovszky and Boldogh, 2011).

In respect of the number of species, bats (Chiroptera) comprise the second richest mammalian order, with over 1250 extant species, right after that of rodents (Rodentia). There are 25 and 28 bat species that have been confirmed to occur in Germany and in Hungary, respectively. The majority of Central European bats belong to the family Vespertilionidae, whereas only three species are members of the family Rhinolophidae and merely one belongs to Miniopteridae.

In the USA, the little brown bat *(Myotis lucifugus)* as an experimental animal had been recognised to be susceptible to infection by a wide range of human and animal viruses more than half a century ago (Reagan et al., 1950), yet the important role of bats, as reservoirs for many significant viruses, has been uncovered relatively recently. In addition to being recognised as carriers of the pathogenic agents of several fearsome diseases such as rabies, SARS, influenza as well as Nipah, Hendra and Ebola viruses, bats may harbour a large variety of less dangerous viruses (Baker and Murcia, 2014; Kohl and Kurth, 2014; Reuter et al., 2014). The first bat AdV isolate was obtained from a Ryukyu flying fox *(Pteropus dasymallus yayeyamae)* in Japan during the establishment of bat cell cultures for the isolation of other viruses present in bats (Maeda et al., 2008).

Only short fragments from the DNA-dependent DNA polymerase (pol) and the hexon genes are known from this virus, which was proposed to be named bat adenovirus 1 (BtAdV-1). In 2008, another novel AdV was isolated on Vero cells in Germany from the organ samples of common pipistrelles *(Pipistrellus pipistrellus)* (Sonntag et al., 2009). The full genome analysis of this AdV, designated BtAdV-2, has been published recently (Kohl et al., 2012). Almost the entire genome sequence of a third bat AdV (BtAdV-3; strain TJM), isolated from *Myotis ricketti* in China, has also been published (Li et al., 2010*b*). Finally, a fourth bat AdV isolate has been obtained from a fruit bat *(Rousettus leschenaultii)* in India recently. From this strain (isolate 1050597), short partial sequences of the pol and hexon genes were published (Raut et al., 2012). Besides these four AdV isolates, partial sequences obtained by PCR and viral metagenomics from about twenty further bat AdVs have been published in the USA, China, Germany, Hungary, and Brazil (Li et al., 2010*a*; Li et al., 2010*b*; Drexler et al., 2011; Jánoska et al., 2011; Vidovszky and Boldogh, 2011; Lima et al., 2013; Yang et al., 2013). Moreover, a collection of partial pol sequences, obtained from AdVs of different bats in Spain, has been deposited to the GenBank (Casas et al., 2010).

The aim of the present study was to assess, by PCR and sequencing, the prevalence and diversity of AdVs in bats occurring naturally in Germany and Hungary, and to compare them with bat AdVs reported previously.

Materials and methods

Ethics

Since all German and Hungarian bats are covered by species protection through the European Commission (http://ec.europa.eu/environment/nature/legislation/habitatsdirective) and through the Agreement on the Conservation of Populations of European Bats (www.eurobats.org), investigative research required special permission by local government bodies. Animals were found dead, injured or moribund near roosting sites or human habitations in urban and suburban areas of different regions in Germany (Bavaria, Lower Saxony and Berlin greater metropolitan area). All bat carcasses were kindly provided by bat researchers and bat rehabilitation centres from the different geographic regions. Permits to investigate carcasses of deceased bats were granted by the respective local governmental authorities. Sampling for Hungarian bats was allowed by the Hungarian National Inspectorate for Environment, Nature and Water.

Origin of the samples, sample preparation

The German samples consisted of homogenised internal organ tissues (pool of heart, liver, intestine, kidney, brain, spleen, salivary gland). German bat

samples were extracted using NucleoSpin® Tissue Kit (Macherey-Nagel). The sample number ranged from 1 to 58 with an average of 11 per species (Table 1). Among the Hungarian specimens, 38 guano samples from roosting places of identified bat species were collected predominantly on the territory of the Aggtelek National Park and its surroundings (North-eastern Hungary), which is the region best mapped for bats in Hungary (Boldogh, 2006). More than one hundred individual rectal swabs, collected from bats captured transiently for faunistic surveys and migration studies with appropriate permits, were also screened. We purified the DNA from the samples with E.Z.N.A. DNA Stool Kit (OMEGA Bio-Tek) following the manufacturer's instructions. From the organs of the dead Hungarian bats, the DNA was extracted from the homogenised liver as described earlier (Doszpoly et al., 2014).

Polymerase Chain Reaction (PCR)

For the initial detection of adenoviral DNA, we used a general nested PCR with highly degenerate, consensus primers targeting the most conserved region of DNA-dependent DNA polymerase gene (Wellehan et al., 2004). The size of the PCR product of was 318−324 bp. We used genomic DNA of bovine adenovirus 1 as positive control (Evans et al., 1998).

To further characterise the identified AdVs, a second nested PCR was used. The highly degenerate primers were designed for the amplification of a 253-bp fragment from the gene of the IVa2 protein. This gene is also a very much conserved gene of AdVs. The name and sequence of the primers were as follow (using the IUPAC nucleotide ambiguity codes). Outer primers: *IVa2outfo*: 5'-CCN-NSN-CCN-GAR-CAN-GTN-TTY-TT-3' and *IVa2outre*: 5'-GG-RTT-CAT-RTT-RTG-NAR-NAC-NAC-3'. Inner primers: *IVa2info*: 5'-CCN-CAR-RTN-GAY-ATG-ATH-CCN-CC-3' and *IVa2inre*: 5'-TTN-SWN-GGR-AAN-GCR-TGR-AAR-AAY-TT-3'.

The PCR mixtures contained 0.25 µl Dream Taq DNA polymerase enzyme (Fermentas Life Sciences), 2 µl (25 mM) $MgCl_2$, 1 µl (50 pmol/µl) from each primer (outer and inner primers in the first and second circle of the reaction, respectively) and 2 µl target DNA. Milli-Q water was added up to 50 µl of final volume. In the second circle of the nested PCRs, we used 5 µl from the reaction mixture of the first circle as template.

For both PCRs, the program consisted of an initial denaturation step at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 sec, annealing at 46 °C for 1 min and elongation at 72 °C for 1 min. The final elongation step was at 72 °C for 3 min. The results of the PCRs were checked by agarose gel electrophoresis.

Table 1

The results of PCR screening of bat samples for the presence of adenoviruses

	Host species	Common name	Number of samples		Positive samples ('types')			
					pol pos		IVa2 pos	
			Hun	Ger	Hun	Ger	Hun	Ger
	Suborder Yinpterochiroptera Family Rhinolophidae							
1	***Rhinolophus euryale***	Mediterranean horseshoe bat	3	–	1(4)		1	
2	*Rhinolophus ferrumequinum*[*]	greater horseshoe bat	6	–	1		1	
3	*Rhinolophus hipposideros*[*]	lesser horseshoe bat	10	–	1		1	
	Suborder Yangochiroptera Family Miniopteridae							
4	*Miniopterus schreibersii*	Schreibers' bat	10	–	–			
	Family Vespertilionidae							
5	*Barbastella barbastellus*	barbastelle	3	2	–	–	–	–
6	***Eptesicus nilssonii***	northern bat	–	5		1		1
7	***Eptesicus serotinus***	serotine bat	8	13	2(1)	1	1	1
8	*Hypsugo savii*	Savi's pipistrelle	1	–	–		–	
9	*Myotis alcathoe*	alcathoe whiskered bat	3	–	–		–	
10	*Myotis bechsteinii*	Bechstein's bat	19	–	–		–	
11	***Myotis blythii***	lesser mouse-eared bat	7	–	1		1	
12	*Myotis brandtii*	Brandt's bat	5	2	–	–		
13	***Myotis dasycneme***	pond bat	5	–	2(1)		1	
14	*Myotis daubentonii*	Daubenton's bat	11	4	–	–	–	–
15	***Myotis emarginatus***	Geoffroy's bat	4	–	1		1	
16	*Myotis myotis*	greater mouse-eared bat	12	1	4(1)	–	1	–
17	***Myotis mystacinus***	whiskered bat	2	17	–	1	–	–
18	*Myotis nattereri*	Natterer's bat	8	5	–	–	–	–
19	*Nyctalus lasiopterus*	greater noctule	4	–	–		–	
20	*Nyctalus leisleri*[*]	lesser noctule	1	3	–	1(4)		1
21	*Nyctalus noctula*[*]	common noctule	3	58	1	20(5)	1	4
22	***Pipistrellus kuhlii***	Kuhl's pipistrelle	3	4	–	1		–
23	***Pipistrellus nathusii***	Nathusius' pipistrelle	3	11	–	2(2)		–
24	*Pipistrellus pipistrellus*[*]	common pipistrelle	3	42	–	6(4)		2
25	***Pipistrellus pygmaeus***	soprano pipistrelle	8	3	1	1(3)	1	1
26	***Plecotus auritus***	brown long-eared bat	7	11	–	1		–
27	*Plecotus austriacus*[*]	grey long-eared bat	1	1	–	–		
28	***Vespertilio murinus***	parti-coloured bat	2	12	–	1		1
∑	**27 Hungarian species, 17 German species**		**152**	**194**	**15(13)**	**36(24)**	**10**	**11**

Abbreviation: [*], from the marked species, AdVs have been described before. Hun, Hungary; Ger, Germany; pol pos, DNA polymerase-based PCR positive; IVa2 pos, IVa2 based PCR positive. The presence of multiple AdV types (shown in brackets) was presumed if the deduced amino acid (aa) sequence of the cloned pol fragments from a single sample exhibited at least one aa divergence after molecular cloning. The names of bat species found to harbour AdV at the first time are in bold

DNA sequencing

The PCR products were excised from the gels and the DNA was purified by using the NucleoSpin® Extract II kit (Macherey-Nagel GmbH & Co. KG). Sequencing was done on both strands with the use of the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the inner primers of the nested PCRs. The electrophoresis was done on an ABI Prism® 3500 Genetic Analyzer by a commercial service.

Molecular cloning

In case of multiple sequence trace signals, the PCR products were cloned with the use of the CloneJET™ PCR Cloning Kit (Fermentas Life Sciences) according to the manufacturer's instructions. The ligated products were transformed into a chemically competent *Escherichia coli* strain TOP10 (iNtRON Biotechnology) with heat shock. Plasmid DNA was purified by the alkaline lysis miniprep method. For nucleotide sequence (nt) determination, the pJET1 Forward and Reverse Sequencing Primers were used.

Bioinformatics and phylogenetic calculations

The new sequences were identified by different BLAST algorithms at the NCBI website. The hitherto unknown sequences were also checked with a local BLAST run by the BioEdit programme package against our own database consisting of unpublished AdV sequences.

Multiple alignments were prepared by the use of MultAlin algorithm (hosted by the Plateforme Bioinformatique Genotoul, http://multalin.toulouse.inra.fr/multalin/multalin.html). Distance matrix analyses were performed with JTT model online at the Mobyle portal of the Pasteur Institute in Paris (http://mobyle.pasteur.fr) by Protdist of the Phylip program package followed the Fitch-Margoliash method (Fitch program) with global rearrangements as described earlier (Harrach and Benkő, 2007). The phylogeny tree reconstructions were visualised by the TreeView software.

Results

Prevalence of adenoviruses in German and Hungarian bats

Positive PCR results were obtained in 51 out of 346 samples (14.74%). The overall positivity rates in the two countries differed markedly, reaching almost 18.6% among the German and 9.9% among the Hungarian samples. It is noteworthy that out of 17 bat species that could be sampled in both countries, only three gave parallel positive results. Interestingly, however, the viral sequences were not identical in any of these cases in the two countries. On the

other hand, sequences, identical with those published earlier, were obtained sometimes from bats of rather different species.

The bats from which AdV-positive samples were obtained belonged to 18 bat species. As Table 1 shows, these included all the three species in the Rhinolophidae, but the majority of positive samples were from bats classified into the family Vespertilionidae. No AdV could be detected in samples of the remaining species. The ten samples, from the only examined species from the recently established family Miniopteridae, were also negative.

Diversity and emerging host range of the newly detected bat adenoviruses

In the 51 positive samples, we revealed the presence of 34 distinct AdV sequences, of which 28 were novel while six ones (listed separately in Table 2) had been described previously. The GenBank accession numbers of the novel pol sequences are KM043079 to KM043112. As a tentative name for the putative bat AdVs, detected by PCR only, we used the English common name of the host species with ascending numbers. If identical sequences were found in several animals belonging to different bat species, then a more comprehensive name, referring to the host's family, was chosen. As shown in Table 2, four such viruses, designated vespertilionid AdV-1 to 4, were identified. For example, identical sequences (of the putative virus labelled as vespertilionid AdV-1) were found in the samples of four common noctules, as well as in each of a whiskered bat and a Nathusius' pipistrelle from Germany (Table 2). Similarly, identical novel pol sequences were identified in a common noctule and a common pipistrelle, both collected in Germany. The pol sequence of vespertilionid AdV-3 and -4 have previously been detected in a greater noctule in Spain and in a great evening bat in China, respectively (Casas et al., 2010; Li et al., 2010*b*). We found these viruses in the German samples of a common noctule and a serotine bat, respectively.

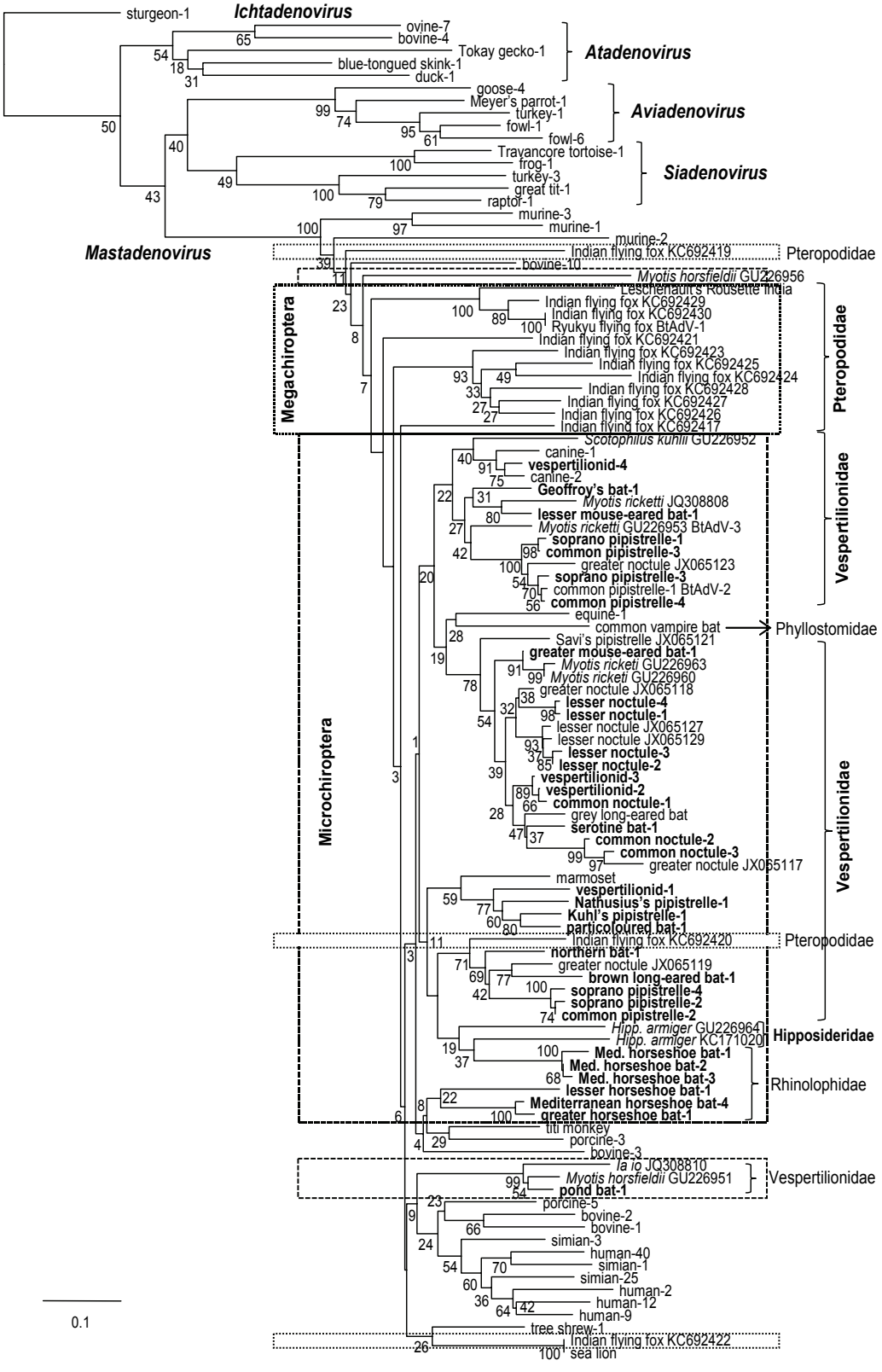
Table 2

Designation and origin of the 28 novel and 6 earlier detected bat AdVs based on partial DNA polymerase sequences

Nr.	Name of the AdV	Host Strain name is shown if more than one AdV detected in the species	New GenBank acc. no.	Previously found + GenBank acc. no.
1	brown long-eared bat AdV-1	brown long-eared bat (1G)	KM043094	
2	common noctule AdV-1	common noctule (1H, BS24)	–	common noctule (H) (HUN/2007) GU198877; 0 nt differences
3	common noctule AdV-2	common noctule (7G, 119/08)	KM043110	
4	common noctule AdV-3	common noctule (1G, 150/08)	KM043111	
5	common pipistrelle AdV-2	common pipistrelle (2G, 141/07)	KM043096	
6	common pipistrelle AdV-3	common pipistrelle (1G, 202/09)	KM043108	

Table 2 continued

7	common pipistrelle AdV-4	common pipistrelle (2G, 199/09)	KM043109	
8	Geoffroy's bat AdV-1	Geoffroy's bat (1H)	KM043084	
9	greater horseshoe bat AdV-1	greater horseshoe bat (1H)	–	greater horseshoe bat (H) (HUN/2009) GU289918; 0 nt differences
10	greater mouse-eared bat AdV-1	greater mouse-eared bat (4H)	KM043106	
11	Kuhl's pipistrelle AdV-1	Kuhl's pipistrelle (1G)	KM043100	
12	lesser horseshoe bat AdV-1	lesser horseshoe bat (1H)	–	lesser horseshoe bat (H) (Teresztenye/2010) JN167522
13	lesser mouse-eared bat AdV-1	lesser mouse-eared bat (1H)	KM043086	
14	lesser noctule AdV-1	lesser noctule (1G, 239/08 clone 1)	KM043102	
15	lesser noctule AdV-2	lesser noctule (1G, 239/08 clone 3)	KM043104	
16	lesser noctule AdV-3	lesser noctule (1G, 239/08 clone 4)	KM043105	
17	lesser noctule AdV-4	lesser noctule (1G, 239/08 clone 6)	KM043103	lesser noctule (Sp) JX065124
18	Med. horseshoe bat AdV-1	Med. horseshoe bat (1H, BS12 clone 1)	KM043079	
19	Med. horseshoe bat AdV-2	Med. horseshoe bat (1H, BS12 clone 2)	KM043080	
20	Med. horseshoe bat AdV-3	Med. horseshoe bat (1H, BS12 clone 5)	KM043081	
21	Med. horseshoe bat AdV-4	Med. horseshoe bat (1H, BS12 clone 12)	KM043082	
22	Nathusius' pipistrelle AdV-1	Nathusius' pipistrelle (1G, 140/07)	KM043095	
23	northern bat AdV-1	northern bat (1G)	KM043101	
24	parti-coloured bat AdV-1	parti-coloured bat (1G)	KM043099	
25	pond bat AdV-1	pond bat (2H)	KM043083	
26	serotine bat AdV-1	serotine bat (2H, BS14)	KM043085	
27	soprano pipistrelle AdV-1	soprano pipistrelle (1H)	KM043090	
28	soprano pipistrelle AdV-2	soprano pipistrelle (1G, 176/09 clone 4)	KM043091	
29	soprano pipistrelle AdV-3	soprano pipistrelle (1G, 176/09 clone 5)	KM043092	
30	soprano pipistrelle AdV-4	soprano pipistrelle (1G, 176/09 clone 7)	KM043093	
31	vespertilionid AdV-1	common noctule (4G, 191/07) whiskered bat (1G); 0 nt differences Nathusius' pip. (1G, 154/09); 0 nt diff.	KM043087 KM043088 KM043089	
32	vespertilionid AdV-2	common pipistrelle (1G, 157/08) common noctule (1G, 121/08); 6 nt diff.	KM043097 KM043098	
33	vespertilionid AdV-3	common noctule (7G, 162/09), 2 nt diff.	KM043112	greater noctule (Sp) JX065126
34	vespertilionid AdV-4	serotine bat (1G, 492/08); 10 nt diff.	KM043107	great evening bat (Ch) JQ308807


sturgeon-1
Ichtadenovirus
ovine-7
bovine-4
Tokay gecko-1
blue-tongued skink-1
duck-1
Atadenovirus
goose-4
Meyer's parrot-1
turkey-1
fowl-1
fowl-6
Aviadenovirus
Travancore tortoise-1
frog-1
turkey-3
great tit-1
raptor-1
Siadenovirus
murine-3
murine-1
murine-2
Mastadenovirus
Indian flying fox KC692419
Pteropodidae
bovine-10
Myotis horsfieldii GU226956
Leschenault's Rousette India
Megachiroptera
Indian flying fox KC692429
Indian flying fox KC692430
Ryukyu flying fox BtAdV-1
Indian flying fox KC692421
Indian flying fox KC692423
Indian flying fox KC692425
Indian flying fox KC692424
Indian flying fox KC692428
Indian flying fox KC692427
Indian flying fox KC692426
Indian flying fox KC692417
Pteropodidae
Scotophilus kuhlii GU226952
canine-1
vespertilionid-4
canine-2
Geoffroy's bat-1
Myotis ricketti JQ308808
lesser mouse-eared bat-1
Myotis ricketti GU226953 BtAdV-3
soprano pipistrelle-1
common pipistrelle-3
greater noctule JX065123
soprano pipistrelle-3
common pipistrelle-1 BtAdV-2
common pipistrelle-4
Vespertilionidae
equine-1
common vampire bat
Phyllostomidae
Savi's pipistrelle JX065121
greater mouse-eared bat-1
Myotis ricketi GU226963
Myotis ricketi GU226960
greater noctule JX065118
lesser noctule-4
lesser noctule-1
lesser noctule JX065127
lesser noctule JX065129
lesser noctule-3
lesser noctule-2
vespertilionid-3
vespertilionid-2
common noctule-1
grey long-eared bat
serotine bat-1
common noctule-2
common noctule-3
greater noctule JX065117
Microchiroptera
Vespertilionidae
marmoset
vespertilionid-1
Nathusius's pipistrelle-1
Kuhl's pipistrelle-1
particoloured bat-1
Indian flying fox KC692420
Pteropodidae
northern bat-1
greater noctule JX065119
brown long-eared bat-1
soprano pipistrelle-4
soprano pipistrelle-2
common pipistrelle-2
Vespertilionidae
Hipp. armiger GU226964
Hipp. armiger KC171020
Hipposideridae
Med. horseshoe bat-1
Med. horseshoe bat-2
Med. horseshoe bat-3
lesser horseshoe bat-1
Mediterranean horseshoe bat-4
greater horseshoe bat-1
Rhinolophidae
titi monkey
porcine-3
bovine-3
Ia io JQ308810
Myotis horsfieldii GU226951
pond bat-1
Vespertilionidae
porcine-5
bovine-2
bovine-1
simian-3
human-40
simian-1
simian-25
human-2
human-12
human-9
tree shrew-1
Indian flying fox KC692422
sea lion
0.1

←

Fig. 1. Phylogenetic tree based on distance matrix analysis of the partial DNA polymerase sequences. From every genus other than *Mastadenovirus*, five AdV types were included at the most. Each primate AdV species is represented by one type only. The word 'adenovirus' was omitted from the virus type names. Bat AdVs are in dashed frames. The narrower frames contain the AdVs from bats that belong to the Microchiroptera suborder, the wider the AdVs from Megachiroptera bats. The newly found BtAdVs are printed in bold. The hosts' vernacular names are used except for the bat AdVs described in China which are shown with the Latin name of the host species

Amplification of a fragment from an additional gene, namely that of the multifunctional, encapsidation and transcriptional activator protein IVa2 (Pardo-Mateos and Young, 2004), was attempted from every positive sample. This PCR was successful from 21 samples only, including 11 German and 10 Hungarian ones from bats of 14 species (Table 1). We also PCR-amplified and sequenced this gene fragment from four putative bat AdVs that had been detected and described in our laboratory before (Jánoska et al., 2011; Vidovszky and Boldogh, 2011). All IVa2 sequences seemed to be homogeneous, even those which were obtained from three samples that contained multiple, albeit just slightly diverging, pol sequences. The IVa2 sequences were assigned to accession numbers KM043057 to KM043078 in the GenBank.

Phylogeny of bat adenoviruses

A phylogeny tree reconstruction, obtained by protein distance matrix analysis on the partial pol sequences, is presented in Fig. 1. The sequence of this gene fragment is now available from AdVs of a large variety of vertebrate hosts, including the majority of bat AdVs. As expected, every bat AdV clustered clearly with other members of the genus *Mastadenovirus*. The highest number of sequences was obtained from the common or evening bats belonging to the family Vespertilionidae. Their AdVs formed one small and two large monophyletic clades, in addition to a third large, but paraphyletic one (Fig. 1). The AdVs of the horseshoe bats (Rhinolophidae) and the flying foxes (Pteropodidae) were also quite close to each other, respectively. There were only few exceptions, such as a sequence derived from an Indian flying fox, which appeared in the clade of the vesper bat AdV sequences, whereas the sequence from a Chinese *Myotis horsfieldii* clustered closer to AdVs found in members of the Pteropodidae than to those derived from bats belonging to the Vespertilionidae family.

Phylogeny was also inferred from the IVa2 partial sequences. Unfortunately in this analysis, we could not include as many viruses. Consequently, the simpler topology was not readily comparable to that of the pol tree (Fig. 2). However, the AdVs from the Vespertilionidae separated well from those of the Rhinolophidae.

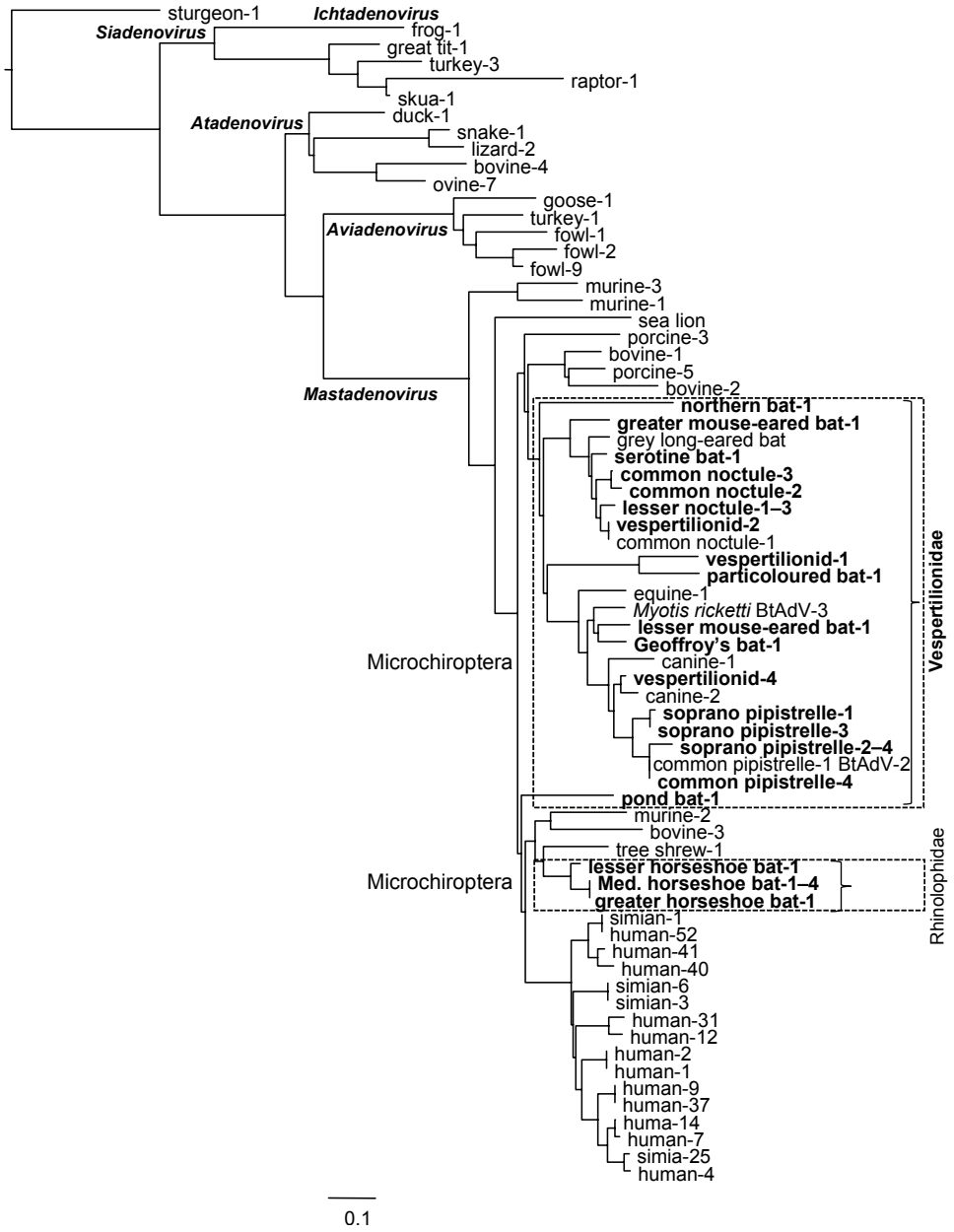


Fig. 2. Distance matrix tree based on the partial IVa2 sequences. Names and signs are as in Fig. 1

Discussion

Bats have become a hot topic in virology since the discovery of the important reservoir role they play in the maintenance of lyssaviruses and other serious viral pathogens (van der Poel et al., 2006). The distinguished attention facilitated the discovery of less harmful or non-pathogenic viruses in bats as well. In many countries, the continuous shrinkage and gradual disappearance of the natural resting and roosting places force the bats closer to human habitats, to the attics of houses and the towers of churches. Therefore it is very important to get knowledge about all the viruses that may occur in bats.

Bats have an astonishing biodiversity seemingly accompanied by a similarly great diversity of their pathogens. In a quest for the biodiversity represented by adenoviruses, we started to examine bats hoping for the discovery of numerous novel types and variants. Indeed, the results of our very first surveys, demonstrating a rather high (28.5%) prevalence of AdVs in bat samples in Hungary, have verified this expectation (Jánoska et al., 2011; Vidovszky and Boldogh, 2011). During the short period of half a decade, more than two dozens of novel AdV sequences have been reported from representatives of different bat species from Europe, Asia and America. In the present work, we almost doubled the number of different putative bat AdVs detected worldwide.

The overall proportion of positive samples in the present study (14.73%) was markedly higher than that usually found when screening other mammalian hosts randomly (usually less than 5%; our unpublished results). The outcome of the survey appears to be influenced greatly by the sample collection method. The individual faecal or rectal swab samples, taken from mist-netted or harp-trapped live specimens that were presumably healthy, gave the lowest positivity. On the other hand, the combined faecal samples, collected from large colonies, had likely been 'contaminated' with AdVs through urination by the actively infected members of the given population (Barr et al., 2015). Hence the overall AdV prevalence in the guano samples was found to be high (31.48%). Building-dweller bat species generally roost separately from each other, thus the host species could be determined reliably also in case of such samples. The reason for the similarly high positivity rate among samples derived from dead or moribund and subsequently euthanised bats in Germany could be the eventual reactivation of persistent AdV infections by subacute stress or disease in these animals.

The number of samples, examined from each species in this study, mirrors roughly the prevalence of the given bat species in the two countries. Accordingly, some rare species were not well, if at all, represented. We succeeded to sample 17 out of the 25 species recorded in Germany. All the 28 but one bat species known to occur in Hungary were also sampled. The only missed species, the northern bat *(Eptesicus nilssonii)*, is an extremely rare visitor in Hungary. Interestingly, from the 10 bat species found negative for the presence of AdV, some

were represented by numerous (up to 19) individuals. On the other hand, in the bats of certain species a very high positivity rate (> 15%) could also be revealed. The species, identified as being frequently infected by AdVs, included the pond bat, the common noctule, the soprano pipistrelle, the greater mouse-eared bat, and a couple of additional (Kuhl's, Nathusius' and common) pipistrelles (Table 1).

Just as human and many other vertebrate hosts, representatives of a given bat species may also harbour more than one AdV type, even simultaneously. On the other hand, it seems that multiple bat species might be infected by the same type of AdV. A number of samples were found to contain AdV gene fragments with slightly divergent nucleotide, or even amino acid (aa), sequences. When naming the newly found AdVs, we presumed a new type (and gave a provisional type number) if at least 1 aa difference (non-synonymous mutation) was seen in the partial pol sequence. If only silent mutations were present (not altering the aa sequence), the putative viruses were considered as genetic variants of the same type.

In concordance with the present practice of nomenclature of the family *Adenoviridae*, we propose to name the isolated virus strains as 'bat adenovirus' with ascending numbers. For the putative AdVs, detected by PCR and sequencing only, the primary designation consists of the English vernacular name of the host, also with ascending numbers. If (previously described or newly recognised) identical AdV sequences are revealed in the samples of multiple bat species, we propose using the bat family name instead of the species name in the virus tags.

The identical partial pol and IVa2 sequences are a strong hint for identical or closely related virus types belonging to a common AdV species (Kaján et al., 2011). Nonetheless, the appearance of recombinant virus types, a frequent finding among human AdVs (Kajon et al., 2010), cannot be excluded until the full genome sequence is available for analysis. Such data or at least the hexon sequences would also be essential for the unambiguous confirmation of the new BtAdV types.

Identical virus sequences in individuals belonging to the same bat species were repeatedly detected in Hungary in common noctules (common noctule AdV-1) as well as in greater and lesser horseshoe bats (Table 2). Interestingly, in German samples of the common noctule, two additional, somewhat divergent pol sequences were found (common noctule AdV-2 and -3). The putative AdVs, named lesser noctule AdV-4 and vespertilionid AdV-3, were detected in samples of the same or different bat species, respectively, in Spain as well as in Germany. A reason for these findings could be that the yearly migration of the European bats typically has a north-east to south-west direction (Hutterer et al., 2005), giving possibility for sharing viruses in the countries involved only.

A sequence variant of vespertilionid AdV-4, found in a German serotine bat, had been reported from another host species, namely the great evening bat *(Ia io)* in China (Chen et al., 2012). These sequences shared 95% nt and 100% aa identity. This could be an example for an AdV that is capable of infecting differ-

ent host species. In this case a recent host switch of the virus can be hypothesised. Although the serotine and the great evening bats live on faraway territories that do not overlap, they are genetically closely related. Furthermore, there might be an intermediate species, *Eptesicus serotinus pahyomus,* whose distribution area partly overlaps with that of *Ia io* (Juste et al., 2013).

The results of the phylogenetic analyses further supported the hypothesis on the co-evolution of AdVs with their hosts (Benkő and Harrach, 2003) by the basic separation of the AdVs according to bat families. The viruses, detected in bats belonging to families Vespertilionidae, Rhinolophidae, Hipposideridae and Pteropodidae, appeared generally in separate but not necessarily monophyletic clusters. The group of Mediterranean horseshoe bat AdV-1, -2 and -3 (found in the same sample) unexpectedly appeared in the cluster of the AdVs of the Hipposideridae instead of the Rhinolophidae family. In this case, we hypothesise a host-switch event. Another example for a likely host switch is the AdV described from a New World vampire bat (common vampire bat *Desmodus rotundus,* family Phyllostomidae) recently (Lima et al., 2013), which appeared among the vespertilionid AdVs (Fig. 1).

In a recent study aiming at the assessment of viral diversity of mammals, numerous novel viruses including AdVs were found in the Indian flying fox *(Pteropus giganteus)* (Anthony et al., 2013). The authors described an AdV almost identical to BtAdV-1 (AB303301) (with a single silent mutation on the short pol fragment), which was detected in the closely related species (Ryukyu flying fox, *Pteropus dasymallus yayeyamae*, Maeda et al., 2008). Based on our phylogeny inference, another AdV sequence derived from the Indian flying fox is most closely related (with two silent mutations) to the sea lion AdV sequence (GU979536) released most recently (Fig. 1). Such astonishing findings would require further studies.

In our previous study, the striking similarity between the genomic organisation of canine adenoviruses (CAdVs) and BtAdV-2 (isolated from common pipistrelles in Germany) was noticed (Kohl et al., 2012). These viruses share several homologous genes in their E3 and E4 regions that are usually type specific in AdVs (Davison et al., 2003; Ursu et al., 2004). The phylogenetic clustering further supported the presumptive close common ancestry of CAdVs and certain vespertilionid BtAdVs (Jánoska et al., 2011; Benkő et al., 2014). In the present work, a newly found sequence from a putative bat AdV, named tentatively as vespertilionid AdV-4, appeared right between CAdV-1 and -2 implying its affiliation to the virus species *Canine mastadenovirus A* (Fig. 1). CAdVs belong to the group of the relatively few AdVs that are known to have a wide host range and to cross the host barrier readily. The presence of CAdVs has been documented in a large variety of animals classified in the suborder Caniformia. Numerous serological and PCR surveys, as well as occasional virus isolations indicated that, besides dogs, CAdVs may infect bears, foxes, golden jackals, wolves

and skunks as well (Dandár et al., 2010). Interestingly, equine adenovirus 1 (EAdV-1) and a recently described New World monkey AdV also appeared in the vicinity of other vesper bat AdVs, clearly suggesting a bat AdV origin for these viruses. The phylogenetic relatedness of EAdV-1 could be confirmed also by the similarity of its E3 and E4 genes (Cavanagh et al., 2012). However, the corresponding sequence data from the putative marmoset AdV are not available yet (Gál et al., 2013). It is tempting to speculate that certain bat AdVs might have changed hosts in the past. To challenge this hypothesis, sequence analysis of the E3 and E4 regions of respective BtAdVs would be of interest.

Compared to the amazingly great abundance and diversity of BtAdVs recognised to date, reports about the successful isolation and *in vitro* propagation of such viruses are scarce. For the detailed characterisation and ultimate classification of BtAdVs, as well as for a deeper investigation of the hypothesised host-switch scenarios, comparative analyses of full genome sequences of a typical representative from each larger BtAdV lineage will be indispensable.

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