

Szent István University
Postgraduate School of Veterinary Science

Search for novel bat species and bat viruses

Brief Summary of Doctoral Thesis

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Introduction and objectives

Recently there are about 1300 bat species, but the number is continuously increasing due to new collecting methods, molecular biological approaches and taxonomic revisions. Descriptions of new species are based not just on newly collected materials, but also on the revision of old museum specimens.

The need of the investigation of the type specimens in the taxonomic studies is inevitable. These specimens are distributed in many museums around the world even in the case of closely related taxa. This is the cause that taxa remain undescribed even for decades.

We often forget that the taxonomic background is fundamental for many supraindividual researches. If we cannot make difference between taxa, we cannot correctly interpret the results of the conservation biological, ecological and ethological etc. studies.

Vespertilionidae is the most species rich family of bats. The author studied three genera belonging to this group, namely: the *Hypsugo*, *Glischropus*, and *Myotis*.

Bats became one of the favourite research topics of virologists as they are hosts for many different viruses. Beside zoonotic viruses, many other are infecting bats, like adenoviruses (AdVs). AdVs were detected only from vertebrates and these viruses infect usually only one or a handful of closely related host species.

AdVs may cause serious illnesses in animals, and hence may be important from economical and veterinary aspects. Beside these, they are used as models of different molecular biological researches and can be used as vectors in human therapy. Their usually strict host specificity and large scale of host species make them ideal models of virus evolution.

The tropical expeditions to study bats are ideal opportunities to do various other investigations, which need the capture of the animals. During these trips, besides collecting voucher specimens and ectoparasites, virus samples are also collected to find new viruses and study their diversity in different parts of the world. AdVs are one of the best in this manner, as they remain stable even in warmer

conditions; hence, the samples do not need cooling, which is hard to organize in cutting-edge circumstances.

During the work, my goals were:

1. The definition of genus *Hypsugo* based on external, skull, dentition and bacular traits, to clearly differentiate the genus from *Pipistrellus*. The anatomical investigation of *Hypsugo pulveratus* and the description of a new *Hypsugo* species based on skull, dentition and genetic information.
2. The taxonomic investigation of a new *Glischropus* species based on a specimen from Sumatra (Indonesia). The phylogenetic analysis of the new species.
3. The taxonomic revision of the *Myotis montivagus* group, the elevation of subspecies to species rank. The collection of information for an amended diagnosis of the species. The taxonomic investigation of the new Vietnamese *Myotis* species.
4. Detection of new adenoviruses from Namibia, the Democratic Republic of the Congo, Vietnam and Mexico. The partial genetic characterization of the new viruses.
5. The phylogenetic investigation of the new viruses and the calculation of the phylogenetic tree of the known mastadenoviruses.

Materials and methods

Bat taxonomy

The investigated specimens were sent to the Mammal Collection of the Hungarian Natural History Museum in the frame of joint research projects. For comparisons, we investigated also specimens, which were collected by us on the field. The bats were collected with mist-nets and harp-traps.

The external measurements were taken from live bats or wet specimens with a digital calliper to the nearest 0.1 mm. The skull and dental measurements were taken with the help of a stereomicroscope and a digital calliper to the nearest 0.01 mm.

All statistical analyses were done in the R program. FactoMineR was used for the PCA and the party package for the conditional interference tree.

DNA was extracted with the help of the QIAGEN DNeasy Blood & Tissue Kit and the QIAGEN QIAamp DNA Micro Kit. Two mitochondrial genes, the cytochrome b (*cyt b*) and cytochrome c oxidase I (*COI*) was used for the phylogenetic comparisons.

The *cyt b* sequences of the *Glischropus* taxa were aligned with the representatives of other genera. The phylogenetic tree was calculated with maximum likelihood method and the pairwise sequence divergence with the Kimura-2 parameter model. All analyses were performed in the MEGA 6 program.

Virological research

We investigated samples from Namibia, the Democratic Republic of the Congo (hereafter as Congo), Vietnam and Mexico. In the field, the bats were put into sterile perforated paper bags for 10-15 minutes and the guano was collected with sterilized forceps.

The samples originated from Namibia and Congo were processed with E.Z.N.A. Stool DNA Kit, while the Vietnamese and Mexican samples with the GeneJET Viral DNA/RNA Purification Kit, which is good also for RNA extraction.

A nested PCR method was used to screen the samples for AdVs. The method amplifies a part of the highly conserved DNA-dependent DNA polymerase (*pol*) gene. Two outer and two inner primers were used for the PCR. Based on the

results of other studies, this method can amplify all members of the *Adenoviridae* family.

PCR reactions were performed in 25 μ l, the template of the second round was the product of the first round. A 250 bp length sequence of the *I/Va2* gene was also determined in case of *pol*-positive samples. DNA was cleaned by the QIAquick Gel Extraction Kit. The nucleotide sequence was determined by the BigDye™ Terminator v3.1 Cycle Sequencing Kit. The sequencing PCR reaction was done with the inner primers of the nested PCR diluted to 4 pmol/ μ l.

The electrophoretograms were visualized and edited with the Staden program package. Primer sequences were cut; the two complement sequences were aligned and joined. A multiple amino acid alignment with other mastadenovirus *pol* sequences was performed in MEGA 6. Distance matrixes were calculated with the Protdist program (JTT model) of the PHYLIP package. The phylogenetic tree was calculated with the Fitch program (global rearrangement function). The tree was visualized with the Figtree program.

Results

The definition of *Hypsugo*

The ear is short and broadly rounded; the tragus is rectangular, short and wide. The lobe on the calcar (epiblema) is usually present, but narrow and elongated, without a central supporting cartilage. The baculum is long, distally thickened and roof-like in section. The dental formula is 2123/3123. The first upper premolar is displaced lingually (sometimes totally absent) from the toothrow. The lower molars are myotodont.

The description of *Hypsugo dolichodon* n. sp.

A medium-sized representative of *Hypsugo* with a relatively robust skull, almost flat cranial profile and well-developed upper canines which are almost twice as high as P4.

The species was recorded from Lao PDR and Vietnam.

The description of *Glischropus aquilus* n. sp.

A rather large representative of the genus, with dark brown fur, elevated frontal part, globose braincase and gradually narrowing interorbital region.

The analysed *Glischropus cyt b* sequences grouped into a monophyletic clade and *G. aquilus* n. sp. is clearly separated from other congeners. The genetic distance between *G. aquilus* n. sp. and other *Glischropus* species – including *G. bucephalus* paratypes and a *G. tylopus* specimen collected close to the type locality – is between 12.1-14.6%, which clearly supports that *G. aquilus* n. sp. is a separate species.

The only known specimen was caught along a trail in a secondary forest with a four-bank harp-trap, which was set near a bamboo stand and a small stream.

The revision of the *Myotis montivagus* group

The subspecies of *Myotis montivagus* can be separated by a combination of their external and craniodental features. The subspecies *borneoensis* (from Borneo) and *peytoni* (from south and east-central India) have the largest forearm while *montivagus* (from south China, north Myanmar) and *federatus* (from Peninsular Malaysia) are smaller externally. Cranially, Bornean specimens are the largest, whereas bats from south China and north Myanmar are the smallest. Cranial dimensions overlap in Peninsular Malaysian and Indian bats. Bornean specimens have the highest sagittal and lambdoid crests; in other subspecies these are poorly developed. The subspecies can be separated also according to the location and development of upper premolars. The PCA and conditional interference tree resulted in a good separation of most of the taxa.

As a result of the abovementioned differences, the former subspecies were elevated to species rank, hence their new names are *Myotis borneoensis*, *M. federatus*, *M. montivagus* and *M. peytoni*.

The description of *Myotis indochinensis* n. sp.

A medium-sized *Myotis*, with moderately long ears, four-lobed lower incisors, flat cranial profile, well developed sagittal and lambdoid crests, middle premolars intruded lingually in both the upper and the lower toothrows.

M. annectans, *M. borneoensis*, *M. federatus*, *M. peytoni* and *M. sicarius* overlap with *M. indochinensis* n. sp. in external and/or craniodental measurements. To compare these, we used a Principal Component Analysis in addition to morphological comparisons and univariate statistical analyses. The PCA on the 15 craniodental measurements showed good divergence between *M. indochinensis* and four of the other species, but not *M. sicarius*. However, *M. sicarius* can be readily distinguished from *M. indochinensis* by its measurements and skull shape.

M. indochinensis is most similar to *M. sicarius* in general skull shape and dental features (including the relative size and position of premolars in both toothrows), but is significantly smaller with no overlap in several measurements. The new species is further separable from *M. sicarius* by its even more flattened cranial profile and more developed sagittal and lambdoid crests.

It was found in Vietnam.

Detection of new adenoviruses

Altogether 142 guano and 14 swab samples were investigated during this study. The host species belonged to 9 bat families and 32 bat genera. Samples were collected from four countries of three continents. No swab sample was positive for adenovirus, but 15 guano samples contained 14 novel AdVs.

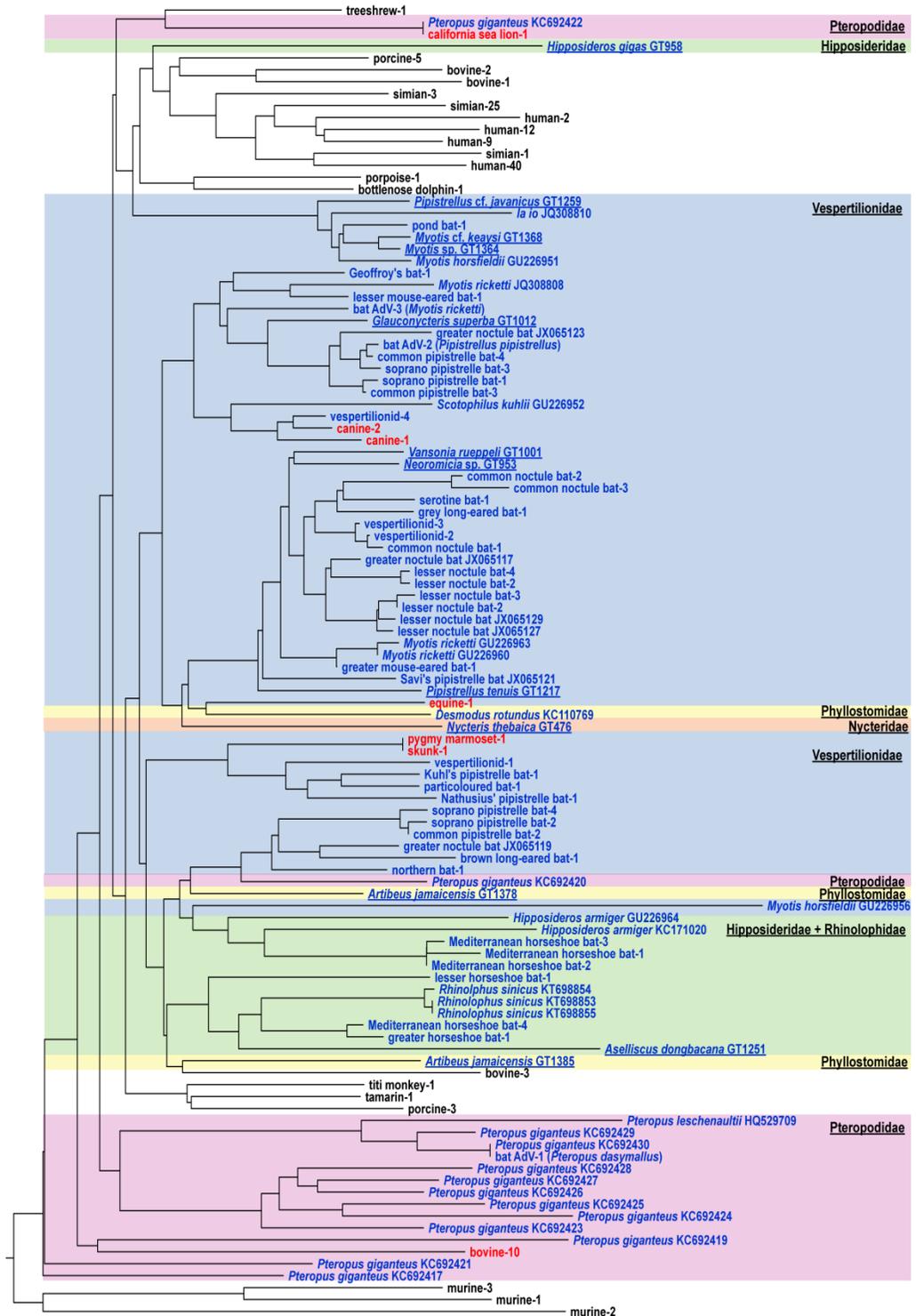
All the AdVs belong to genus *Mastadenovirus* and are novel, different from the published ones. Altogether 12 host species were found, belonging to 8 genera and 4 bat families. Two virus types were found in different *Hipposideros fuliginosus* and *Artibeus jamaicensis* specimens, while the same AdV was found in two specimens from the same *Neoromicia* species. We screened a high number of samples from the families Pteropodidae and Rhinolophidae, however, no positives were found.

On the phylogenetic tree, the bat AdVs were clustered mainly according to the host families, but were not always monophyletic. The viruses of the Vespertilionidae and Pteropodidae families constituted larger groups.

Adenoviruses found during the screening

AdV	host species/taxon	family	country	pol	IVa2
hipposiderid AdV-1	<i>Hipposideros fuliginosus</i> (GT937_1)	H	Congo	double	+
hipposiderid AdV-2	<i>Hipposideros fuliginosus</i> (GT937_2)	H	Congo	double	+
hipposiderid AdV-3	<i>Hipposideros gigas</i> (GT958)	H	Congo	+	+
hipposiderid AdV-4	<i>Aselliscus dongbacana</i> (GT1251)	H	Vietnam	+	+
nycterid AdV-1	<i>Nycteris thebaica</i> (GT476)	N	Namibia	+	+
phyllostomid AdV-1	<i>Artibeus jamaicensis</i> (GT1378)	P	Mexico	+	+
phyllostomid AdV-2	<i>Artibeus jamaicensis</i> (GT1385)	P	Mexico	+	+
vespertilionid AdV-5	<i>Neoromicia</i> sp. (GT953)	V	Congo	+	+
	<i>Neoromicia</i> sp. (GT971)			+	+
vespertilionid AdV-6	<i>Vansonia rueppeli</i> (GT1001)	V	Congo	+	+
vespertilionid AdV-7	<i>Glauconycteris superba</i> (GT1012)	V	Congo	+	+
vespertilionid AdV-8	<i>Pipistrellus tenuis</i> (GT1217)	V	Vietnam	+	+
vespertilionid AdV-9	<i>Pipistrellus</i> cf. <i>javanicus</i> (GT1259)	V	Vietnam	+	
vespertilionid AdV-10	<i>Myotis</i> sp. (GT1364)	V	Mexico	+	+
vespertilionid AdV-11	<i>Myotis</i> cf. <i>keaysi</i> (GT1368)	V	Mexico	+	+

Abbreviations: + – successful amplification, N – Nycteridae, H – Hipposideridae, V – Vespertilionidae, P - Phyllostomidae



Discussion

The diversity of *Hypsugo*

Thanks to intensive research efforts in recent years, the number of bat species recorded from Vietnam continuously increases. In the latest compilation of the mammals of Vietnam published in 2008, 111 bat species were listed and since then, within a few years, 8 more species new to science have been reported from the country, although one other species was synonymized. Presently the following *Hypsugo* species are known from Vietnam: *H. cadornae*, *H. pulveratus*, *Hypsugo* cf. *joffrei*, and *H. dolichodon* n. sp.

The bat fauna of Lao PDR was summarized recently by Thomas and colleagues, who listed 90 species of seven families. Vespertilionidae is the most numerous with 47 species, and now three *Hypsugo* species are currently known from the country: *H. cadornae*, *H. pulveratus* and *H. dolichodon* n. sp.

Unfortunately, there are no DNA data for most of the Southeast Asian *Hypsugo* species, because their rarity makes it hard to get appropriate samples for molecular analysis. Further work needs to be done to resolve phylogenetic relationships and evaluate the validity of the established “groups” within the genus.

The diversity of *Glischropus*

We confirmed the unidentified *Glischropus* bat reported by Huang and colleagues as a species distinct from its congeners, representing a new species of the genus. Previously, only *G. tylopus* has been confirmed from Sumatra with records known exclusively from the province of North Sumatra, hence, according to our present knowledge, the two species are allopatric on the island. In Indonesia, *G. tylopus* is also reported from Kalimantan and Maluku. A third species of thick-thumbed bats, *G. javanus*, is also recorded from Indonesia with only one specimen known from a single locality in West Java.

Based on a survey in southwestern Sumatra and a literature review, Huang and colleagues compiled a list of 87 bat species for the island of Sumatra. Our finding of *G. aquilus* n. sp. not only brings the count to 88 species from Sumatra, but also to 222 species from the whole Indonesian archipelago.

To help the differentiation of *Glischropus* species from each other, we provide an identification key in the dissertation.

The revision of *Myotis montivagus* group

Molecular investigations frequently present un-named lineages on phylogenetic trees for different chiropteran families because of questionable taxonomic assignment of studied specimens due to the lack of thorough revisions. Needless to say, the results of such studies must be harmonised with existing scientific names, which is hardly possible without re-examination – and frequently re-description – of type materials for less known taxa. The present work re-evaluated the taxonomic status of four formerly accepted subspecies of *M. montivagus* and elevated them to species rank using cranial and dental characters and multivariate statistical analyses. To assist determination of species within the *montivagus*-group and the morphologically similar *M. annectans*, a character matrix is provided.

M. montivagus (*sensu lato*, including all its former subspecies) is currently considered Least Concern in the IUCN Red List of Threatened Species. However, since the taxa elevated to species rank herein have significantly smaller distribution ranges, this categorisation should be reconsidered.

The description of *Myotis indochinensis* n. sp.

The large series of specimens of *Myotis indochinensis* sp. n. allowed a detailed morphological and statistical analysis and provided a solid base for taxonomic conclusions. The available DNA evidence supports the morphological conclusions, although DNA data are not yet available for some of the potentially most closely related species.

To facilitate research in the wider geographical area, an identification key is provided for the medium and large-sized *Myotis* species with small feet from the Indomalayan Region which includes the new species.

Detection of new adenoviruses

We investigated viruses from 9 families of the world's 18 bat families. This was the most comprehensive sampling among the bat AdV studies. We have found positive samples from four families. We investigated the Mormoopidae, Nycteridae and Natalidae for the first time. The prevalence in case of guano samples was 9.86%, which is comparable with the Hungarian samples. There was no geographic difference in prevalence.

All the viruses found were novel, which indicates that we have to count with many new bat AdVs in the future. All of them belong to the genus *Mastadenovirus*, which further supports the mammalian origin of mastadenoviruses.

On the phylogenetic tree, most of the viruses of the Pteropodidae bat family were monophyletic and comprise an older lineage than the other bat AdVs. Both of the accepted bat suborders' (Yinpterochiroptera and Yangochiroptera) viruses clustered more or less. There were found no geographic differences among the viruses. On the phylogenetic tree, the bat AdVs clustered mainly according to the host families, but were not always monophyletic. One reason for this could be the possible host change; the other may be the inaccuracy of our calculations, because the studied sequences are very short. However, according to earlier experiences, this shorter *pol* fragment can represent well the results generated from the whole *pol* sequence.

The canine AdV-1 and -2, and also the equine AdV-1 were monophyletic with certain bat AdV groups. Interestingly, the equine AdV-1 was monophyletic with the AdV of *Desmodus rotundus*, which may be the result of a host change from the bat, which feeds on the blood of larger bovines and horses. The bat AdV origin of the canine AdVs was supposed by previous studies based on their place on the phylogenetic trees and their genome organization. The large host spectrum and high pathogenicity of these viruses in dogs and foxes further corroborates with a recent host switch. We can observe similar host change in case of equine AdV-1, which differs significantly in the *hexon* gene from the equine AdV-2. Also, it's much more pathogenic than the latter one. Common bat ancestor was supposed in case of the skunk (*Mephitis mephitis*) AdV. Our newest observation is the bat AdV origin of the bovine AdV-10. Beside the phylogenetic results, it is supported by the higher pathogenicity and the fact that it can be isolated only on primate cell lines and has highly diverse fibre genes.

New scientific results

1. The definition of genus *Hypsugo* based on external, craniodental and bacular traits. The anatomical investigation of *Hypsugo pulveratus* and the description of a new *Hypsugo* species from Lao PDR and Vietnam based on skull, dentition and genetic information.
2. The taxonomic investigation of a new *Glischropus* species based on a specimen from Sumatra (Indonesia). The phylogenetic analysis of the new species.
3. The taxonomic revision of the *Myotis montivagus* group, the elevation of the subspecies to species level. The collection of information for an amended diagnosis of the species. The taxonomic investigation of the new *Myotis* species from Vietnam based on skull and dentition morphology.
4. Detection of new adenoviruses from Namibia, the Democratic Republic of the Congo, Vietnam and Mexico. The partial genetic characterization of the new viruses.
5. The phylogenetic investigation proved the ancestral origin of the flying fox AdVs, the more or less good clustering of viruses of different bat families, and the bat AdV origin of viruses of some other hosts, including the bovine AdV-10.

Publications this work was based on

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