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Short Communication

Detection of diverse astroviruses from bats in China

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Astroviruses infect humans and many different animal species and are associated with gastroenteritis. Recent studies first detected the virus from bat species in Hong Kong. To understand astrovirus distribution in the wider region further, we examined the prevalence of this virus family in bat specimens collected from a large geographical region of mainland China. We collected 500 anal swabs from 20 bat species in 51 natural habitats from 11 provinces of China and tested these for astroviruses. Our study revealed a remarkably high genetic diversity of astroviruses; five monophyletic groups were identified in bats, including two novel groups. Evidence for varying degrees of host restriction for astroviruses from bats has been found. Phylogenetic analyses also provided insight into the inter-species transmission of Mamastrovirus.

The family *Astroviridae* is a large family of positive-sense single-stranded RNA viruses that have been identified in humans as well as a variety of animal species (Méndez & Arias, 2007). Genetic analysis of astrovirus sequences available from human, bat, cat, cheetah, mink, sheep, pig, chicken and turkey has classified the astroviridae into two genera that are found in avian and mammalian hosts, the genera *Avastrovirus* and *Mamastrovirus*, respectively (Strain *et al.*, 2008; Chu *et al.*, 2008). Astroviruses cause diarrhoea in infants and young children, and in animals they are also found in association with gastroenteritis (Herrmann *et al.*, 1991; Snodgrass & Gray, 1977; Shimizu *et al.*, 1990; Reynolds & Saif, 1986). However, the ecological inter-relationship between human astrovirus and viruses from other species has not been established, despite the zoonotic origin of this pathogen.

To identify the natural reservoirs of the severe acute respiratory syndrome coronavirus, extensive surveillance has been conducted in wild animal populations, leading to the detection of diverse novel bat coronaviruses and providing insights into the ecology of coronaviruses (Guan *et al.*, 2003; Tang *et al.*, 2006; Vijaykrishna *et al.*, 2007). Further investigation showed that novel groups of astroviruses were also detected in apparently healthy insectivorous bats from Hong Kong (Chu *et al.*, 2008). The findings suggested that bats possessed a high prevalence rate and high genetic diversity of astroviruses (Chu *et al.*, 2008). Herein, we report the detection and genetic characterization of astroviruses from bats collected throughout China.

In this study, 500 anal swabs from 20 different species of bats in 51 natural habitats collected from 11 provinces of China from November 2004 to June 2007 were tested (Table 1). Species identification of bats was conducted as described previously (Tang *et al.*, 2006). Swabs were taken and placed in transport medium with antibiotics, kept in liquid nitrogen for transportation to the laboratory and then stored at −80 °C.

Astrovirus detection and sequencing were carried out as previously described (Chu *et al.*, 2008; Tang *et al.*, 2006). Briefly, viral RNA was extracted from 140 µl swab material using QIAamp viral RNA mini kit (Qiagen) and cDNA was generated using Superscript II reverse transcriptase (Invitrogen). The random hexamer-generated cDNA was screened for astrovirus using hemi-nested PCR targeting the most conserved region of the RNA-dependent RNA polymerase (RdRp) gene (Chu *et al.*, 2008). The RdRp fragments were sequenced and analysed to determine the diversity of the detected astroviruses and to select representative strains for extended ORF1b and ORF2 sequencing using gene-specific and oligo(dT) primers. Sequences derived from this study were deposited in GenBank with accession numbers FJ571065–FJ571140.

The GenBank/EMBL/DDBJ accession numbers for the sequences derived in this study are FJ571065–FJ571140.

Table 1. Astrovirus distribution in different bat species and locations

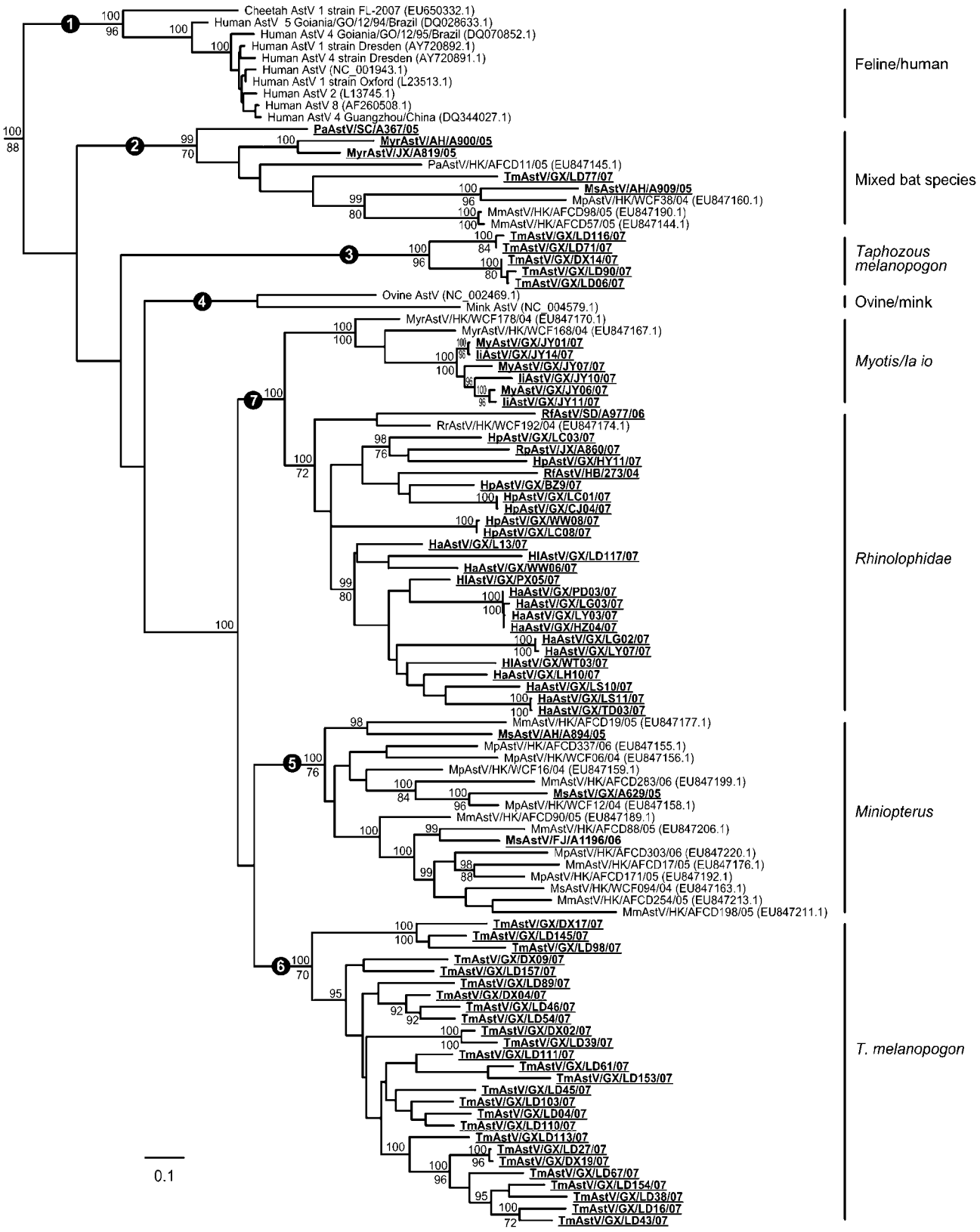
NA, Not applicable.

Family and species of bat	Common name	No. sampled (no. positive)		Group(s)
		Sites	Bats	
Rhinolophidae				
Aselliscus stoliczkanus	Stoliczka's Asian trident bat	1 (0)	1 (0)	NA
Hipposideros armiger	Great leaf-nosed bat	13 (11)	109 (21)	7
Hipposideros larvatus	Intermediate leaf-nosed bat	5 (2)	29 (4)	7
Hipposideros pomona	Pomona leaf-nosed bat	8 (6)	95 (13)	7
Rhinolophus affinis	Intermediate horseshoe bat	2 (0)	2 (0)	NA
Rhinolophus ferrumequinum	Greater horseshoe bat	3 (2)	4 (2)	7
Rhinolophus macrotis	Big-eared horseshoe bat	1 (0)	1 (0)	NA
Rhinolophus pearsonii	Pearson's horseshoe bat	1 (1)	1 (1)	7
Rhinolophus sinicus	Chinese horseshoe bat	1 (0)	1 (0)	NA
Vespertilionidae				
Ia io	Great evening bat	1 (1)	11 (4)	7
Miniopterus schreibersi	Schreiber's long-fingered bat	6 (5)	19 (12)	2, 5
Myotis ricketti	Rickett's big-footed bat	5 (2)	16 (2)	2
Myotis spp.		1 (1)	5 (3)	7
Nyctalus velutinus	Villus noctule	1 (0)	1 (0)	NA
Pipistrellus abramus	Japanese pipistrelle	6 (1)	20 (1)	2
Pipistrellus spp.		1 (0)	5 (0)	NA
Scotophilus kuhlii	Lesser Asiatic yellow house bat	2 (0)	5 (0)	NA
Tylonycteris pachypus	Lesser bamboo bat	1 (0)	2 (0)	NA
Emballonuridae				
Taphozous melanopogon	Black-bearded tomb bat	2 (2)	172 (160)	2, 3, 6
Megadermatidae				
Megaderma lyra	Greater false vampire bat	1 (1)	1 (1)	7
Total	**⩾20**	**51 (32)**	**500 (224)**	**2, 3, 5–7**

Sequences were assembled and edited with LASERGENE version 7.2 (DNASTAR) then aligned with previously published astrovirus sequences using TransAlign (Bininda-Emonds, 2005) with CLUSTAL W (Thompson *et al.*, 1994) to conserve codon positions. Alignments were manually optimized wherever necessary using Se-Al, version 2.0 (http://evolve.zoo.ox.ac.uk/), and all ambiguously aligned regions were removed prior to phylogenetic analysis. The best-fit nucleotide substitution model was selected using the MODELTEST programme, version 3.7, and used in all subsequent analyses (Posada & Crandall, 1998). Maximum-likelihood (ML) trees were constructed using GARLI, version 0.951 (Zwickl, 2006), and Bayesian analysis was conducted with MrBayes, version 3.1.2 (Huelsenbeck & Ronquist, 2001), using two replicates of 1×10^6 generations with six chains, sampling every 100 generations. The convergence of chains and estimation of burn-in were assessed using TRACER, version 1.4 (http://beast.bio.ed.ac.uk/). Estimates of the phylogenies were calculated by performing 100 ML bootstrap replicates and Bayesian posterior probabilities were calculated from the consensus of 18 000 trees after excluding the first 2000 trees as burn-in and ensuring that a sufficient number of samples (effective sample size >500) were achieved for tree likelihoods, tree length and nucleotide base frequencies.

A total of 224 of 500 (44.8 %) samples were tested as positive in 32 of the 51 sites located in seven provinces (Table 1), with no significant difference in detection between sites (Pearson's χ^2, $P>0.05$). Twelve of the 20 bat species from four families tested were found to harbour astrovirus with a significant difference in detection rates between bat species (Pearson's χ^2, $P<0.001$). The highest positive rates were observed in black-bearded tomb bat

Fig. 1. Phylogenetic relationships of the RdRp genes of astroviruses detected from bats in China. The tree was generated based on 381 nt, by using the ML method in GARLI. Numbers above and below branches indicate Bayesian posterior probability and ML bootstrap values, respectively. The tree was rooted to avian astroviruses. Bar, 0.1 substitutions per site. Bold underlined text indicates viruses characterized in this study. Monophyletic groups 1–7 are indicated in circles on the branches. Abbreviations: AstV, astrovirus; AH, Anhui; FJ, Fujian; GX, Guangxi; Ha, *H. armiger*; HB, Hubei; HK, Hong Kong; Hl, *H. larvatus*; Hp, *H. pomona*; Ii, *I. io*; JX, Jiangxi; Mm, *Min. magnater*; Mp, *Min. pusillus*; Ms, *Min. schreibersi*; My, *Myotis* spp.; Myr, *My. ricketti*; Pa, *P. abramus*; Rf, *R. ferrumequinum*; Rp, *R. pearsonii*; Rr, *Rhinolophus rouxi*; SC, Sichuan; SD, Shandong; Tm, *T. melanopogon.*


Cheetah AstV 1 strain FL-2007 (EU650332.1)
Human AstV 5 Goiania/GO/12/94/Brazil (DQ028633.1)
Human AstV 4 Goiania/GO/12/95/Brazil (DQ070852.1)
Human AstV 1 strain Dresden (AY720892.1)
Human AstV 4 strain Dresden (AY720891.1)
Human AstV (NC_001943.1)
Human AstV 1 strain Oxford (L23513.1)
Human AstV 2 (L13745.1)
Human AstV 8 (AF260508.1)
Human AstV 4 Guangzhou/China (DQ344027.1)
PaAstV/SC/A367/05
MyrAstV/AH/A900/05
MyrAstV/JX/A819/05
PaAstV/HK/AFCD11/05 (EU847145.1)
TmAstV/GX/LD77/07
MsAstV/AH/A909/05
MpAstV/HK/WCF38/04 (EU847160.1)
MmAstV/HK/AFCD98/05 (EU847190.1)
MmAstV/HK/AFCD57/05 (EU847144.1)
TmAstV/GX/LD116/07
TmAstV/GX/LD71/07
TmAstV/GX/DX14/07
TmAstV/GX/LD90/07
TmAstV/GX/LD06/07
Ovine AstV (NC_002469.1)
Mink AstV (NC_004579.1)
MyrAstV/HK/WCF178/04 (EU847170.1)
MyrAstV/HK/WCF168/04 (EU847167.1)
MyAstV/GX/JY01/07
IiAstV/GX/JY14/07
MyAstV/GX/JY07/07
IiAstV/GX/JY10/07
MyAstV/GX/JY06/07
IiAstV/GX/JY11/07
RfAstV/SD/A977/06
RrAstV/HK/WCF192/04 (EU847174.1)
HpAstV/GX/LC03/07
RpAstV/JX/A860/07
HpAstV/GX/HY11/07
RfAstV/HB/273/04
HpAstV/GX/BZ9/07
HpAstV/GX/LC01/07
HpAstV/GX/CJ04/07
HpAstV/GX/WW08/07
HpAstV/GX/LC08/07
HaAstV/GX/L13/07
HlAstV/GX/LD117/07
HaAstV/GX/WW06/07
HlAstV/GX/PX05/07
HaAstV/GX/PD03/07
HaAstV/GX/LG03/07
HaAstV/GX/LY03/07
HaAstV/GX/HZ04/07
HaAstV/GX/LG02/07
HaAstV/GX/LY07/07
HlAstV/GX/WT03/07
HaAstV/GX/LH10/07
HaAstV/GX/LS10/07
HaAstV/GX/LS11/07
HaAstV/GX/TD03/07
MmAstV/HK/AFCD19/05 (EU847177.1)
MsAstV/AH/A894/05
MpAstV/HK/AFCD337/06 (EU847155.1)
MpAstV/HK/WCF06/04 (EU847156.1)
MpAstV/HK/WCF16/04 (EU847159.1)
MmAstV/HK/AFCD283/06 (EU847199.1)
MsAstV/GX/A629/05
MpAstV/HK/WCF12/04 (EU847158.1)
MmAstV/HK/AFCD90/05 (EU847189.1)
MmAstV/HK/AFCD88/05 (EU847206.1)
MsAstV/FJ/A1196/06
MpAstV/HK/AFCD303/06 (EU847220.1)
MmAstV/HK/AFCD17/05 (EU847176.1)
MpAstV/HK/AFCD171/05 (EU847192.1)
MsAstV/HK/WCF094/04 (EU847163.1)
MmAstV/HK/AFCD254/05 (EU847213.1)
MmAstV/HK/AFCD198/05 (EU847211.1)
TmAstV/GX/DX17/07
TmAstV/GX/LD145/07
TmAstV/GX/LD98/07
TmAstV/GX/DX09/07
TmAstV/GX/LD157/07
TmAstV/GX/LD89/07
TmAstV/GX/DX04/07
TmAstV/GX/LD46/07
TmAstV/GX/LD54/07
TmAstV/GX/DX02/07
TmAstV/GX/LD39/07
TmAstV/GX/LD111/07
TmAstV/GX/LD61/07
TmAstV/GX/LD153/07
TmAstV/GX/LD45/07
TmAstV/GX/LD103/07
TmAstV/GX/LD04/07
TmAstV/GX/LD110/07
TmAstV/GXLD113/07
TmAstV/GX/LD27/07
TmAstV/GX/DX19/07
TmAstV/GX/LD67/07
TmAstV/GX/LD154/07
TmAstV/GX/LD38/07
TmAstV/GX/LD16/07
TmAstV/GX/LD43/07
0.1
Feline/human
Mixed bat species
Taphozous melanopogon
Ovine/mink
Myotis/la io
Rhinolophidae
Miniopterus
T. melanopogon

(*T. melanopogon*, 93 %) and Schreiber's long-fingered bat (*Min. schreibersi*, 63.2 %). Some species, e.g. *R. ferrumequinm*, *R. pearsonii*, *Meg. lyra* and *Myotis* spp., were positive, even though their sampling number was small ($n<6$), while other species with limited sample sizes tested negative (Table 1). As many astroviruses were identified in different bat species from different regions, the host name, geographical location, sampling number (including code for sampling site) and year have been included in the nomenclature of astroviruses characterized in this study to avoid confusion, e.g. *Min. schreibersi* astrovirus/Anhui/A894/2005 is abbreviated to MsAstV/AH/A894/05.

Phylogenetic analysis of the RdRp gene sequences revealed that all bat astroviruses tested clustered with the genus *Mamastrovirus* (Fig. 1). Within mamastrovirus, seven monophyletic groups could be identified. Group 1 mostly contained viruses isolated from humans and formed a sister-group to all other mammalian astroviruses (Fig. 1). Astroviruses found in different species of bats showed high genetic diversity, as five of the six remaining groups consisted exclusively of viruses detected from bats. The one exception was group 4 which contained astrovirus from sheep and mink (Fig. 1). Five groups of astroviruses showed varying degrees of host restriction within the bat populations sampled. For example, the divergent groups 3 and 6 contained viruses detected from just a single species, *T. melanopogon*. However, viruses from group 5 were detected from three species of the genus *Miniopterus*. In contrast, groups 2 and 7 showed relatively little host restriction and contained viruses detected from multiple bat families (Fig. 1 and Table 1).

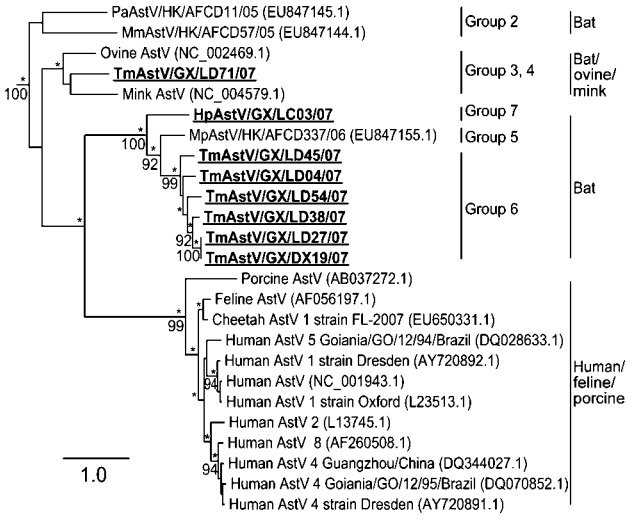


Fig. 2. Phylogenetic relationships of the capsid protein genes (ORF2) of astroviruses detected from bats in China. The tree was generated based on 1500 nt using the ML method in GARLI. Bayesian posterior probability of >95 % is indicated by an asterisk (*) above the branches; numbers below the branches indicate ML bootstrap values. The tree was rooted to avian astroviruses. Monophyletic groups and abbreviations of virus names are as shown in Fig. 1. Bar, 1.0 substitutions per site. Bold underlined text indicates viruses characterized in this study.

Viruses belonging to groups 2, 3 and 6 were detected from black-bearded tomb bats sampled on a single day (8th June, 2007) in the Lian-Dong cave (Guangxi province) (Fig. 1). This suggests that bat species host a larger genetic diversity of astroviruses than has been previously identified in other hosts. On the other hand, viruses detected from the same bat species sampled in different provinces still clustered within a host-specific group. For example, viruses detected in *Miniopterus* species from Anhui (MsAstV/AH/A894/05), Guangxi (MsAstV/GX/A629/05), Fujian (MsAstV/FJ/A1196/06) and Hong Kong (Chu *et al.*, 2008) all formed the monophyletic group 5 (Fig. 1). The differences in viral genetic diversities in different bat species may be associated with the geographical distribution and migratory patterns of individual bat species.

Further phylogenetic analysis of the capsid protein gene (ORF2) sequence of representative viruses revealed three distinguishable virus lineages from mammalian hosts, including human/feline/porcine, 'bats-only' and one 'mixed' that contained viruses from both bats and other animals (Fig. 2). The 'bats-only' virus lineage corresponds to groups 2, 5, 6 and 7 identified in the RdRp phylogeny (Figs 1 and 2). The 'mixed' lineage contains a bat virus from group 3, plus the ovine and mink astroviruses in group 4, indicating possible interspecies transmission between these hosts (Figs 1 and 2). It was noted that two viruses from Felidae spp. fell within the human/porcine astrovirus lineage and that porcine astrovirus had a sister-group relationship to human and feline astroviruses, also reflecting potential interspecies transmission events (Fig. 2). These phylogenetic relationships suggest the possible emergence and evolutionary pathways of the viruses detected from different hosts, even though it is not possible to determine donor/recipient relationships with the currently available data.

To evaluate whether the different gene phylogenies observed for RdRp and capsid protein gene segments were due to recombination, we sequenced the regions covering ORF1b and ORF2 genes of four representative bat astroviruses from groups 2, 3, 6 and 7 and compared these with data from GenBank. We tested for recombination in our data by identifying breakpoints using the genetic algorithm for recombination detection in the HYPHY program (Kosakovsky Pond *et al.*, 2006). Results of this analysis identified four breakpoints (nt 577, 724, 1511 and 2728) with statistical support for recombination. However, inspection of phylogenetic trees generated for each of the recombinant sections showed that the inter-host relationship of bat astroviruses with those isolated from other species was consistent. Furthermore, as each of these recombinant breakpoints were in highly divergent areas of the alignment, the differences in the phylogenies were therefore likely to be due to variation in the rates of

substitution between astroviruses (Holmes & Rambaut, 2004; Moya *et al.*, 2004).

The findings of the present study revealed that the bat species tested had a remarkably high prevalence of genetically diverse astroviruses. Phylogenetic analysis demonstrated that bat astroviruses exhibit varying degrees of host restriction that have also been observed for bat coronaviruses (Tang *et al.*, 2006). Furthermore, these analyses imply that there is interspecies transmission between different mammalian hosts and suggest that bat species are a primary host of mamastrovirus.

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