

Short Communication

Coronaviruses in bent-winged bats (*Miniopterus* spp.)

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A novel group 1 coronavirus was previously identified in bent-winged bats (*Miniopterus* spp.). Here, results are described from our ongoing surveillance of these bats for coronaviruses. These findings show that group 1 coronaviruses are endemic in these bat populations in Hong Kong. Genetic analysis of these viruses indicates that there are at least four different, but closely related, group 1 coronaviruses (bat-CoV 1A, 1B, HKU7 and HKU8) circulating in bent-winged bats. Phylogenetic analysis revealed that these group 1 bat coronaviruses have descended from a common ancestor and that these viruses have been established in these bats for a long period of time. These data provide a better understanding of the emergence and evolution of coronaviruses. Bat-CoV 1A and 1B were detected in apparently healthy *Miniopterus magnater* and *Miniopterus pusillus*, respectively, on repeated sampling occasions at a single habitat, suggesting that these viruses have established a persistent infection in these populations.

Coronaviruses are a group of enveloped, single-stranded RNA, positive-strand viruses with a corona-like morphology (Gorbalenya *et al.*, 2006). Based on antigenic and genetic analyses, coronaviruses can be subdivided into three groups. Coronaviruses from groups 1 and 2 have been found to infect mammals. By contrast, avian species are believed to be the natural reservoir of group 3 coronaviruses. The discovery of a novel coronavirus (Guan *et al.*, 2003; Peiris *et al.*, 2003) as the cause of severe acute respiratory syndrome (SARS) has led to a resurgence in interest in these viruses and to the discovery of other novel coronaviruses in humans (Fouchier *et al.*, 2004; van der Hoek *et al.*, 2004; Woo *et al.*, 2005) and animals (Jonassen *et al.*, 2005; Wise *et al.*, 2006). Recent studies also indicated that bats might be an important reservoir for coronaviruses (Lau *et al.*, 2005; Li *et al.*, 2005; Poon *et al.*, 2005; Woo *et al.*, 2006).

Previously, we identified a novel group 1 coronavirus (hereafter called the bat-CoV 1) in bent-winged bats (*Miniopterus* spp.) (Poon *et al.*, 2005). This novel bat virus was predominantly found in enteric specimens (Poon *et al.*, 2005; Woo *et al.*, 2006), but some respiratory samples from these bats were also positive for the virus (Poon *et al.*, 2005). Following on from the discovery of this virus, here we

report a systematic surveillance for coronaviruses in *Miniopterus* spp. bats in Hong Kong. Surveillance was carried out in June, August and December of 2005 and March of 2006 in an abandoned mine cave located in a restricted area in Hong Kong SAR adjacent to the border with mainland China, which has become a habitat for *Miniopterus* spp. The majority of bats found in the cave under surveillance in 2005 were *Miniopterus* spp., but bats of other genera have also been occasionally observed in this location. On each surveillance trip, 20–50 bent-winged bats were caught in the cave using a net, and throat and faecal samples were collected. After sampling, the bats were released back into the cave.

A total of 114 *Miniopterus magnater* and 22 *Miniopterus pusillus* bats were captured in these four visits (Table 1). The samples were screened by an RT-PCR assay (forward: 5'-GGTTGGGACTATCCTAAGTGTGA-3' and reverse 5'-CCA-TCATCAGATAGAATCATCAT-3') specific for coronavirus RNA-dependent RNA polymerase (RdRp) sequence (Poon *et al.*, 2005). To enhance the sensitivity of the detection system, a second hemi-nested PCR (forward primer: 5'-GGTTGGGACTATCCTAAGTGTGA-3' and reverse primer 5'-ATCAGATAGAATCATCATAGAGA-3') was used to test those samples that were negative in the first PCR assay. Of these 136 bats, 24 were RT-PCR-positive for RdRp sequences of coronaviruses. Six of these positive bats had viruses detectable in both faecal and throat samples, whereas the others had virus only detectable in one or other of these samples. Sequence analysis of these PCR amplicons revealed

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Supplementary material is available in JGV Online.

Table 1. Summary of the detection of group 1 bat coronaviruses, HKU7 and HKU8 in *Miniopterus* spp. from four surveillance visits in 2005 and 2006

Visit	Species	No. bats sampled	Bat positive for group 1 coronaviruses		
			Bat-CoV	Bat-CoV HKU7	Bat-CoV HKU8
June 2005	<i>M. magnater</i>	25	3 (12%)	0	0
	<i>M. pusillus</i>	0	–	–	–
August 2005	<i>M. magnater</i>	44*	9 (20%)†	0	1 (2%)
	<i>M. pusillus</i>	1	0	0	0
December 2005	<i>M. magnater</i>	25	3 (12%)†	0	0
	<i>M. pusillus</i>	11	2 (18%)	0	0
March 2006	<i>M. magnater</i>	20	0	0	0
	<i>M. pusillus</i>	10	2 (20%)	0	3 (30%)

*A *M. magnater* bat found to be positive with bat-SARS coronavirus was included.

†One of the faecal samples was only positive in the hemi-nested PCR assay.

13% (15/114) of sampled *M. magnater* and 18% (4/22) of *M. pusillus* bats were infected with bat-CoV 1. The bat-CoV 1 could be found in most of the sampling occasions and the detection rates of this group of virus in *M. magnater* bats were 3/25 (12%) in June, 9/44 (20%) in August, 3/25 (12%) in December 2005 and 0/20 (0%) in March 2006. *M. pusillus* bats were found mainly in the cool season, with bat-CoV 1 detection rates of 18% (2/11) in December 2005 and 20% (2/10) in March 2006. One *M. magnater* bat captured in August 2005 and three *M. pusillus* bats captured in March 2006 were found to be infected by a recently reported bat coronavirus, HKU8 (99% nucleotide sequence identity) (Woo *et al.*, 2006) (Table 1). In addition, one of the PCR amplicons was found to have its sequence similar to a previously described bat-SARS coronavirus identified in *Rhinolophus* spp. (98% nucleotide sequence identity; Lau *et al.*, 2005; Li *et al.* 2005). This particular sample was not analysed further in this study.

Based on the RdRp sequences deduced from the current ($n=30$) and our previous studies ($n=12$, Poon *et al.*, 2005), a phylogenetic tree was constructed (Fig. 1). From the topology of the tree constructed, the predominant bat-CoV 1 detected in *Miniopterus* spp. throughout the whole studied period could be further divided into two very close, but distinct lineages (Fig. 1, clusters 1A and 1B). Viruses of cluster 1A (bat-CoV 1A) were detected almost exclusively in *M. magnater* bats, while those of cluster 1B (bat-CoV 1B) were all detected in *M. pusillus* bats. The association of clusters 1A and 1B with different species of *Miniopterus* bat remains notable in the surveillance carried out in 2005 and 2006, even though both species of bat cohabited the same cave. However, sample AFCD100 collected from a *M. magnater* bat appears to be an exception (Fig. 1) and is discussed in more detail later. In addition, apart from HKU8, our studied samples also contained another novel bat coronavirus, HKU7 (Woo *et al.*, 2006). Of 42 samples analysed, one HKU7 sample was detected in *M. magnater* bats collected in 2004, and a total of six HKU8 samples were

detected in *M. magnater* (August 2005) and *M. pusillus* (May 2004 and March 2006) bats. Overall, our studies suggested that there are at least four distinct phylogenetically distinct coronaviruses (i.e. bat-CoV 1A, bat-CoV 1B, HKU7 and HKU8) circulating in *Miniopterus* spp.

To test whether the bat-CoV 1 could be subdivided into two sublineages, the above samples were subjected to other RT-PCR assays. Attempts to use consensus primers to deduce the whole viral genome of the above viruses were not successful, but we were able to amplify a 1.5 kb fragment of the Orf1b and a fragment at the 3' end of the genomic RNA with a size range from 1.6 to 2.1 kb, depending on the virus, from our specimens. At least one representative virus from the clusters of bat-CoV 1A (AFCD82), 1B (WCF6), HKU7 (WCF88) and HKU8 (AFCD77) was analysed.

The deduced 3' ends contain the open reading frames (ORFs) of the M (partial 34 aa) and the N (full-length) genes (see Supplementary Fig. S1, available in JGV Online). The lengths of the 3'-untranslated region (UTR) of virus in clusters 1A, 1B, HKU7 and HKU8 are 270, 270, 789 and 719 nt, respectively. The octanucleotide motif that is conserved in all coronavirus genomes can be found near the 3' end of these sequences. We have also identified the putative sequences for the highly conserved pseudoknots in these 3' UTRs (Williams *et al.*, 1999; Goebel *et al.*, 2004). As the N gene from different coronaviruses is genetically very diverse (Gonzalez *et al.*, 2003), we selected the N protein encoding sequences for further analysis. The nucleotide sequence identities between these bat viruses were 49–90% [AFCD82 (bat-CoV 1A) vs WCF6 (bat-CoV 1B), 90.0%; AFCD82 (bat-CoV 1A) vs WCF88 (HKU7), 49.3%; AFCD82 (bat-CoV 1A) vs AFCD77 (HKU8), 58.6%; WCF88 (HKU7) vs AFCD77 (HKU8), 49.7%]. The bat viruses had the highest protein sequence identities among the studied N protein sequences from different group 1 coronaviruses (see Supplementary Table S1, available in JGV Online), indicating that these viruses are related

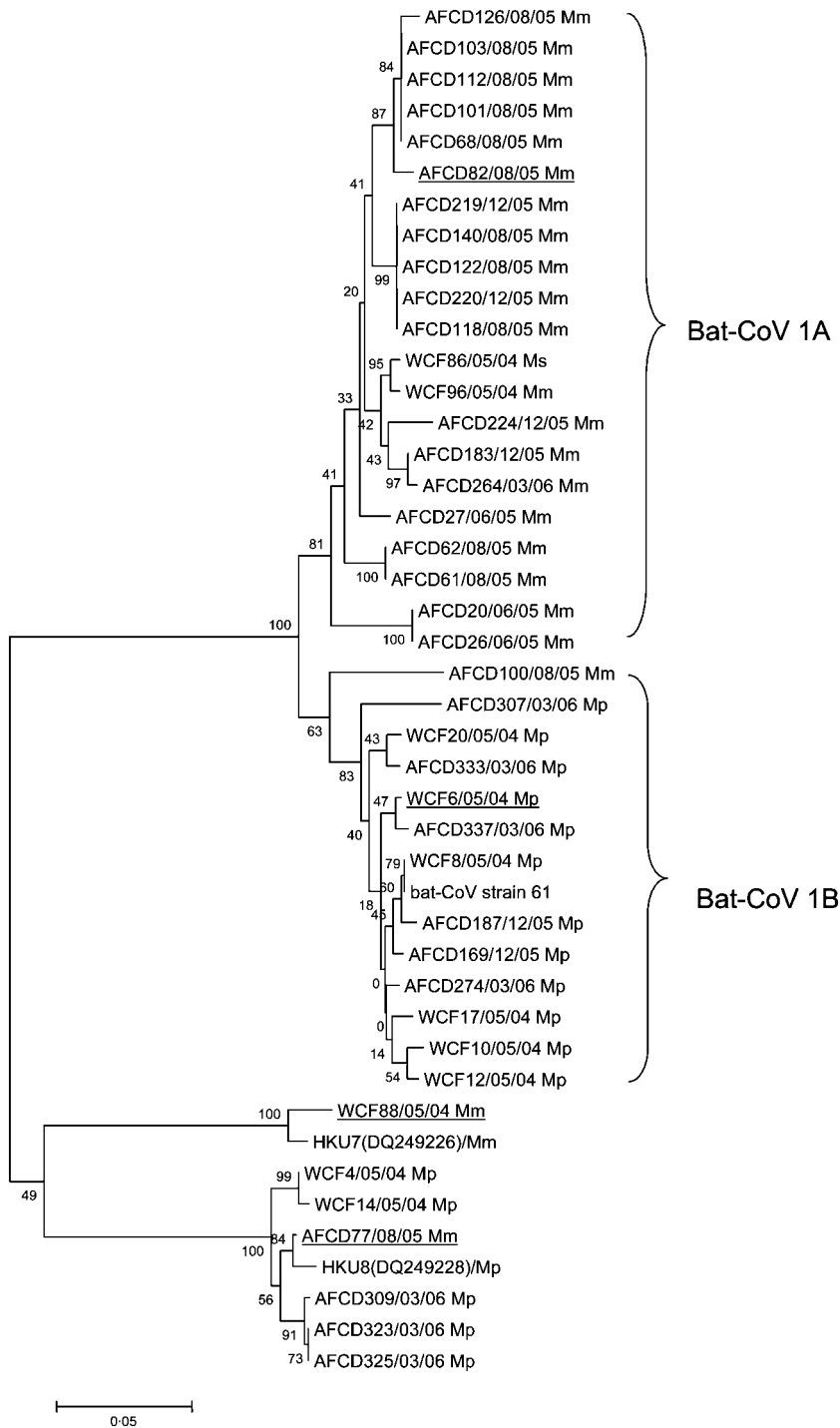


Fig. 1. Phylogenetic analysis of partial RdRp sequences (440 bp) of group 1 bat coronaviruses. Viruses labelled AFCD were samples collected during the current study. Viruses labelled with WCF were samples collected from our previous investigation in May 2004 (Poon *et al.*, 2005). The sequences were named as follows: sample designation/mm/yy, where 'mm/yy' represents the month and year of the sampling date. Mm, Mp and Ms represent the host of that particular virus (i.e. *M. magnater*, *M. pusillus* or *Miniopterus schreibersii*). A partial RdRp sequence derived from other group 1 coronaviruses, HKU7 (GenBank accession no. DQ249226) and HKU8 (GenBank accession no. DQ249228), was used as reference. Numbers at nodes indicate bootstrap values as a percentage. The scale bar shows the estimated genetic distance of these viruses. Representative viruses selected for further investigations are underlined.

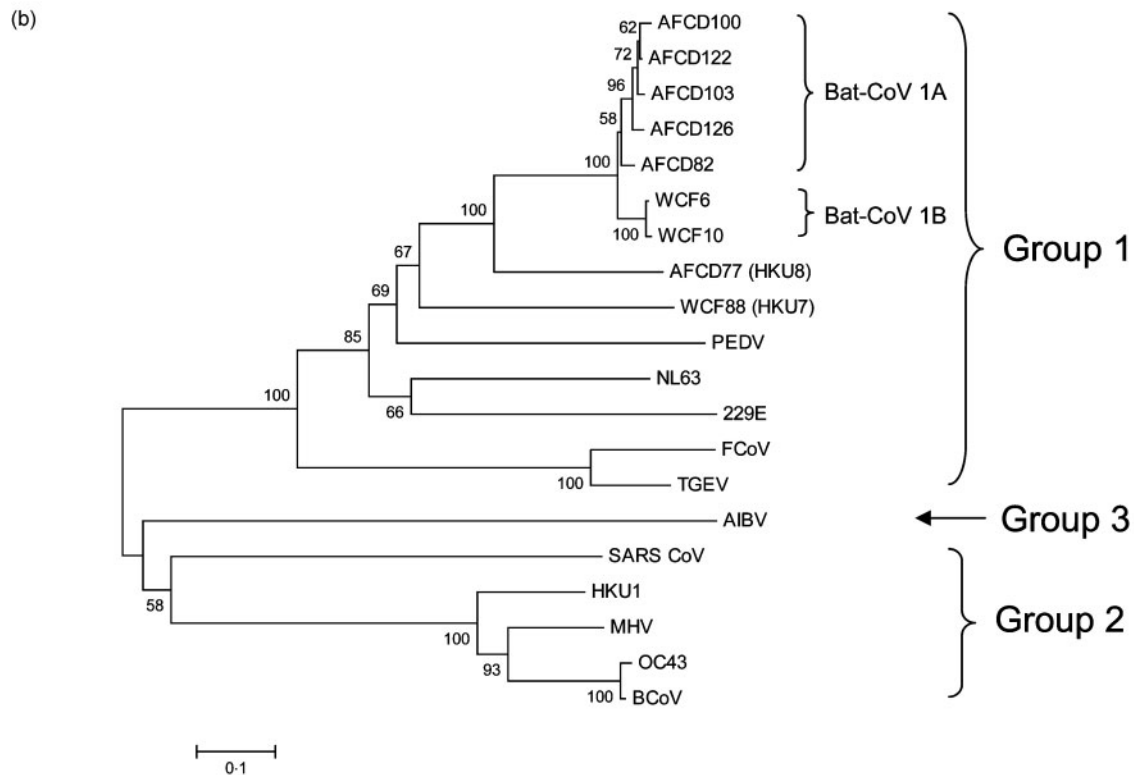
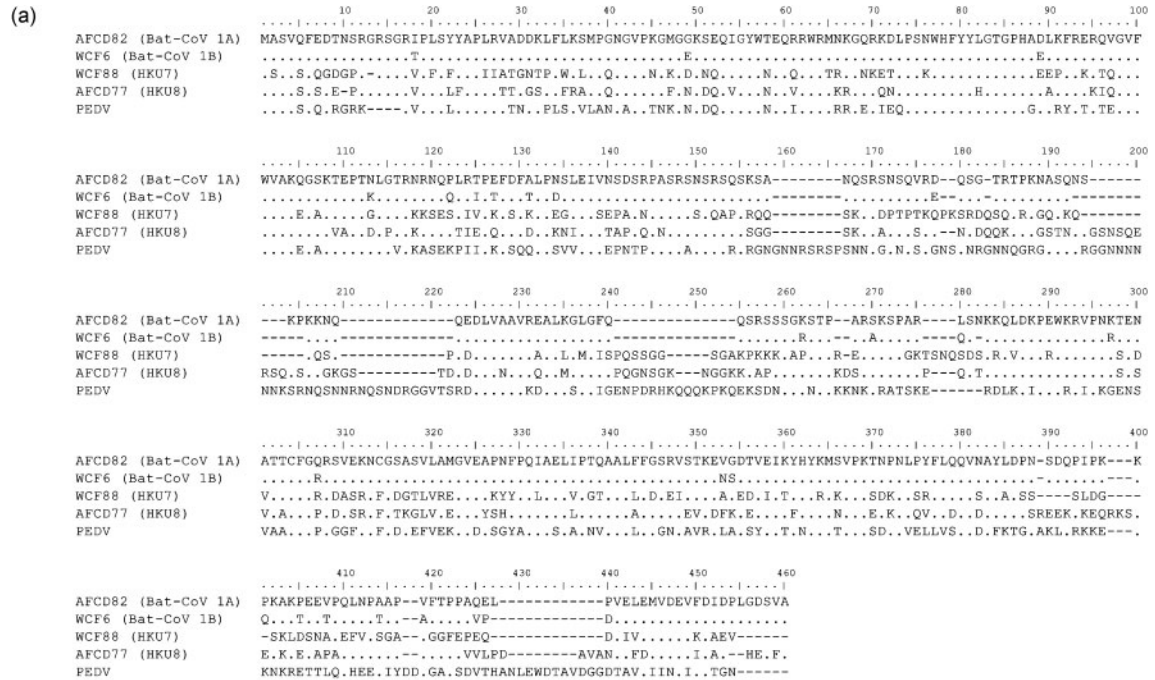
viruses. Nonetheless, that the N protein sequences of these bat viruses were clearly distinct from each other (Fig. 2a). Both the topology of the phylogenetic tree and the bootstrap values indicated that bat-CoV 1A and bat-CoV 1B viruses are genetically different (Fig. 2b). Besides, the amino acid sequence identity between clusters 1A and 1B (e.g. AFCD82 vs WCF10) was slightly less than that within cluster 1A or 1B (e.g. WCF10 vs WCF6) (see Supplementary Table S1,

available in JGV Online), supporting the above findings that viruses in clusters 1A and 1B are closely related, but distinct viruses.

Results from the analysis of the deduced partial Orf1b sequences of these bat viruses were similar to those described above. The partial ORF sequences encoded for RdRp were genetically distinct from each other (see

Supplementary Fig. S2a, available in JGV Online). The nucleotide sequence identities between these bat viruses were diverse [AFCD82 (bat-CoV 1A) vs WCF6 (bat-CoV 1B), 93.3%; AFCD82 (bat-CoV 1A) vs WCF88 (HKU7), 80.0%; AFCD82 (bat-CoV 1A) vs AFCD77 (HKU8),

78.7%; WCF88 (HKU7) vs AFCD77 (HKU8), 81.2%]. Besides, phylogenetic analyses of these sequences resulted in a consensus tree (see Supplementary Fig. S2b, available in JGV Online) with a topology similar to the one deduced from the N proteins (Fig. 2b).



We attempted to isolate infectious viruses from the specimens. A total of 25 specimens positive for coronavirus RNA by RT-PCR, which included seven specimens from our previous study (Poon *et al.*, 2005), were inoculated into FRhK-4 and TB 1 LU continuous cells from the ATCC, and also into primary kidney epithelium and lung fibroblast cells derived from a *M. magnater* bat that was accidentally killed. Virus replication was monitored by observing for cytopathic effects and by RT-PCR for viral RNA. No virus isolate was obtained.

In this study, bat-CoV 1 could be consistently detected in oropharyngeal or faecal samples collected from *Miniopterus* bats in Hong Kong. This virus could be further divided into two sublineages (clusters 1A and 1B). Viruses from cluster 1A were almost exclusively and repeatedly (three surveillance sampling occasions during 2005 at the same habitat) detected in *M. magnater* bats with detection rates of 12, 20 and 20% during June, August and December 2005, respectively. However, there was no detection of this virus in March 2006, probably reflecting the limited number of *M. magnater* bats collected on this occasion. Viruses from cluster 1B were found in *M. pusillus* bats in May 2004, December 2005 as well as in March 2006. There were few *M. pusillus* bats detected in June and August 2005. These findings implied that, even within a single location where these two species of bats co-mingle, viruses from these two sublineages of cluster 1 have different host tropisms. Furthermore, as bats are usually relatively long-lived (e.g. *M. schreibersii* bat, 22 years) (Wilkinson & South, 2002) and as there was no unusual mortality or illness detected in them during the surveillance visits, the high rates of detection of cluster A viruses from apparently healthy *M. magnater* bats on each sampling occasion from the same location in 2005 suggest that this virus establishes persistent infection in these bats. This hypothesis is supported by the genetic heterogeneity between viruses detected from the same location on a single sampling occasion, e.g. AFCD 27/06/05 versus AFCD20/06/05 (Fig. 1), suggesting that there were multiple genetic variants co-circulating within bats at a single location.

In contrast with bat-CoV 1, we were only able to identify bat-CoV HKU7 and HKU8 in a few samples. These findings may indicate that bat-CoV HKU7 and HKU8 are not persistent in these bat populations or that they may be endemic in another species (bat or other) and are

occasionally acquired by *Miniopterus* bats. HKU8 was previously reported to be detected in *M. pusillus* bats (Woo *et al.*, 2006) but we found this virus in both *M. pusillus* and *M. magnater* bats. More comprehensive viral surveillance studies in local bat populations in the future might help to address these questions.

The viruses bat-CoV 1A, bat-CoV 1B, HKU7 and HKU8 found in *Miniopterus* bats are all branched from the same root, suggesting that they are derived from the same ancestor virus. The coexistence of genetically related, yet distinct group 1 coronaviruses in bent-winged bats in such a small geographical region is of interest. From the evolutionary point of view, this might indicate that the ancestor of this group of viruses has circulated in *Miniopterus* bats for a long time, a period long enough for the precursors to spin out several genetically distinct viruses. Our study also implies that there is a diversity of hitherto unrecognized coronaviruses that circulate in bats. Further surveillance of bat coronaviruses in different geographical regions may reveal a more comprehensive picture of how these coronaviruses, and even group 1 coronaviruses in general, have evolved.

It is interesting to note that the specimen AFCD100 from *M. magnater* bats, which falls into lineage 1B in RdRp sequence phylogenetic analysis (Fig. 1), clustered with lineage 1A when N protein was used for the analysis (Fig. 2b). A co-infection of 1A and 1B viruses in the sampled animal was excluded by sequencing six individual clones derived from the same PCR reaction (data not shown). As the viral sequences between 1A and 1B viruses were highly similar and the RdRp of AFCD100 was an outgroup within the cluster 1B (Fig. 1), the studied RdRp sequences used in the phylogenetic analysis (Fig. 1) might be too short (440 nt) to draw a definite conclusion on the classification of this particular virus. It is therefore premature to conclude that AFCD100 is a recombinant of 1A and 1B viruses. Further work is required to understand better the sequence diversity of these groups of viruses. However, previous studies on other coronaviruses indicated that recombination of different coronaviruses was not a rare event (Herrewegh *et al.*, 1998). Given the remarkably high prevalence of coronaviruses in these bat populations that cohabit with each other, there was ample opportunity for co-infections and recombination of these viruses. One should note that the putative precursor virus of the SARS coronavirus was detected in *M. magnater* bats in

Fig. 2. Sequence analysis of N proteins from bat coronaviruses. (a) Sequence alignment of N proteins in representative viruses bat-CoV 1A (AFCD82), bat-CoV 1B (WCF6), HKU7 (WCF88) and HKU8 (AFCD77). Homologous sequence from *Porcine epidemic diarrhea virus* (PEDV) was used as a reference. (b) Phylogenetic analysis of N proteins. Phylogenetic trees were constructed by using the neighbour-joining method and bootstrap values were determined by 1000 replicates. The scale bar shows the estimated genetic distance of these viruses. GenBank accession numbers of the reference sequences are as follows: PEDV (NP_598314); NL63, *Human coronavirus NL63* (YP_003771); 229E, *Human coronavirus 229E* (NP_073556); FCoV, *Feline coronavirus* (YP_239358); TGEV, *Transmissible gastroenteritis virus* (NP_058428); AIBV, *avian infectious bronchitis virus* (NP_040838); SARS-CoV, *severe acute respiratory syndrome-associated coronavirus* (AAU81611); HKU1, *Human coronavirus HKU1* (YP_173242); MHV, *Murine hepatitis virus* (AAF05706); OC43, *Human coronavirus OC43* (NP_937954); and BCoV, *Bovine coronavirus* (NP_150083).

this single bat cave, highlighting the possibility for recombination and the possible emergence of novel human pathogens (Dobson, 2005).

In summary, we have detected four distinct group 1 coronaviruses in bent-winged bats. Our results also provide further genetic sequence data on the recently reported bat coronaviruses, HKU7 and HKU8. More importantly, our data suggest that our previously reported bat virus (Poon *et al.*, 2005) comprises two genetically similar viruses with different host specificities. Bat-CoV 1A and 1B are found to circulate in *M. magnater* and *M. pusillus* bats, respectively. Viruses from these two clusters are frequently and repeatedly isolated from bats without overt evidence of illness and the evidence suggests that these are likely to represent persistent infections.

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