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# Extensive diversity of coronaviruses in bats from China

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ABSTRACT

To help reveal the diversity and evolution of bat coronaviruses we collected 1067 bats from 21 species in China. A total of 73 coronaviruses (32 alphacoronaviruses and 41 betacoronaviruses) were identified in these bats, with an overall prevalence of 6.84%. All newly-identified betacoronaviruses were SARS-related Rhinolophus bat coronaviruses (SARSr-Rh-BatCoV). Importantly, with the exception of the S gene, the genome sequences of the SARSr-Rh-BatCoVs sampled in Guizhou province were closely related to SARS-related human coronavirus. Additionally, the newly-identified alphacoronaviruses exhibited high genetic diversity and some may represent novel species. Our phylogenetic analyses also provided insights into the transmission of these viruses among bat species, revealing a general clustering by geographic location rather than by bat species. Inter-species transmission among bats from the same genus was also commonplace in both the alphacoronaviruses and betacoronaviruses. Overall, these data suggest that high contact rates among specific bat species enable the acquisition and spread of coronaviruses.

## 1. Introduction

Coronaviruses (CoVs; family *Coronaviridae*) are enveloped positive-sense, single-stranded RNA viruses with the largest genomes (25–31 kb) among known RNA viruses (de Groot et al., 2011). Based on genome-scale phylogenies the known CoVs are classified into 30 species within four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (ICTV, 2017). Coronaviruses can infect humans, other mammals, and birds, causing respiratory, enteric, hepatic, and neurological diseases of varying severity (Masters and Perlman, 2013). Coronaviruses are well known globally due to the emergence of severe acute respiratory syndrome (SARS) during 2002–2003 caused by a previously unknown CoV (Ksiazek et al., 2003; Peiris et al., 2003). Subsequently, other two human CoVs (NL63 and HKU1) causing respiratory disease were identified (van der Hoek et al., 2004; Woo et al., 2005). Strikingly, the Middle East respiratory syndrome (MERS) that emerged in 2012 and characterized by a higher mortality than SARS was also caused by a previously unknown CoV (Bermingham et al., 2012; Zaki et al., 2012). The ongoing emergence of these CoVs in humans means that CoVs will likely remain a key public health threat for the foreseeable future.

Since the discovery of SARS-CoV in Himalayan palm civets (Guan et al., 2003), intense effort has been directed toward identifying and characterizing coronaviruses from animals globally. Consequently, a number of CoVs have been identified in a diverse range of vertebrates including domestic and wild mammals, and birds (Poon et al., 2005;

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Wang et al., 2015; Woo et al., 2012). Bats (order Chiroptera), with more than 1240 species, have remarkable species diversity, and comprise more than 20% of living mammalian species (Nowak, 1994). The discovery of SARS-related CoV in Rhinolophus horseshoe bats in China in 2005 (Lau et al., 2005; Li et al., 2005) attracted the global attention to these mammals, such that diverse Alpha- and Beta-CoVs have now been identified in a variety of bats globally over the past decade (Corman et al., 2013, 2014, 2015; Drexler et al., 2014; He et al., 2014; Huang et al., 2016; Smith et al., 2016; Woo et al., 2012). More importantly, due to the close relationship between CoVs in bats and those causing human infections, it is believed that bats are the original source of human CoVs including SARS-CoV and MERS-CoV (Corman et al., 2015; Ge et al., 2013; Huynh et al., 2012; Ithete et al., 2013; Tao et al., 2017). Due to their high diversity and biological and ecological characteristics that potentially facilitate virus maintenance and transmission, bats likely harbor a large number of viruses, some of which may then jump to other species (Balboni et al., 2012). Hence, more effort is needed to identify and characterize the currently unrecognized CoVs that circulate in bats.

At least 120 bat species are found in China, mainly distributed in the eastern, central, and southern regions of that country (Zhang et al., 1997). Herein we report novel and diverse CoVs and SARS-related CoVs identified in *Rhinolophus*, *Miniopterus*, *Murina* and *Myotis spp*. bats sampled from several geographic regions of China. In addition, we inferred their genomic characteristics and evolutionary relationships with known viruses and their hosts.

## 2. Results

### 2.1. Bats collected and prevalence of CoVs

During 2012–2015 a total of 1067 bats were collected from five caves in five counties from Guizhou, Henan, and Zhejiang provinces (Fig. 1, Table 1 and S1). After morphological examination and sequence analysis of the mitochondrial cytochrome *b* (mt-*cyt b*) gene, these bats were assigned to 21 species. The species and their abundance varied among regions, with *Miniopterus schreibersii* (47%) in Guizhou, *Rhinolophus ferrumequinum* (45%) and *R. pusillus* (32%) in Henan, and *R. monoceros* (41%) and *R. sinicus* (50%) in Zhejiang as the predominant species. Notably, only *R. pusillus* bats were found in all five regions.

Using RT-PCR targeting a conserved fragment of the RdRp (RNA-dependent RNA polymerase) gene of CoV as described previously (Lau et al., 2005; Wang et al., 2015), viral RNA was identified in a total of 73 bat fecal samples, with an overall detection rate of 6.84% (Table 1). Phylogenetic analysis revealed that all these viral sequences were clearly closely related to coronaviruses. Among the predominant bat species, CoV prevalence was high in *R. monoceros* (28/119, 23.53%) and *M. schreibersii* (16/198, 8.08%), but was lower in *R. sinicus* (8/219, 3.65%), *R. ferrumequinum* (2/183, 1.09%) and *R. pusillus* (1/154, 0.64%).

Of the 73 newly-identified CoVs, 32 belong to alphacoronaviruses and 41 to betacoronaviruses. To better characterize these newly-identified bat CoVs, the complete viral RdRp gene sequence was obtained from 65 (89%) of the viral RNA positive bat samples. In addition, 5 complete and 4 near-complete viral genome sequences were successfully obtained from CoV positive samples.

### 2.2. Newly-identified SARS-related Rhinolophus bat coronaviruses in bats

Genetic analysis of the conserved domains in the replicase polyprotein pp1ab – ADP-ribose 1-phosphatase (ADRP), nsp5 (3CLpro), nsp12 (RdRp), nsp13 (Hel), nsp14 (ExoN), nsp15 (NendoU) and nsp16 (O-MT) – revealed that newly-identified bat betacoronaviruses shared more than 90% amino acid sequence identity with *Severe acute respiratory syndrome-related coronavirus* (SARSr-CoV) (Table S2) and clustered together in a phylogenetic analysis (Fig. 2). Hence, these data suggest that these bat viruses belong to the species SARSr-CoV according to the criteria for species demarcation in the subfamily


**Fig. 1.** A map of China illustrating the location of trap sites in which bats (red circles) were captured.

**Table 1**
Prevalence of coronaviruses in the bats collected during 2012–2015 in Guizhou, Henan and Zhejiang provinces, China.

| Species | Guizhou | Henan | | | Zhejiang | Total |
|---|---|---|---|---|---|---|
| | Anlong | Neixiang | Lushi | Jiyuan | Longquan | |
| *Aselliscus stoliczkanus* | 0/2 | – | – | – | – | 0/2 |
| *Barbastella beijingensis* | – | – | – | 0/2 | – | 0/2 |
| *Hipposideros armiger* | 1/35 | – | – | – | – | 1/35 |
| *Hypsugo savii* | – | – | – | 0/1 | – | 0/1 |
| *Miniopterus ricketti* | – | – | 0/1 | – | – | 0/1 |
| *Miniopterus schreibersii* | 14/179 | 2/19 | – | – | – | 16/198 |
| *Murina leucogaster* | – | 3/21 | 2/19 | 0/2 | – | 5/42 |
| *Murina* sp. | – | – | – | 0/3 | – | 0/3 |
| *Myotis davidii* | 3/5 | 2/4 | 0/1 | 0/1 | – | 5/11 |
| *Myotis siligorensis* | 1/4 | – | – | – | – | 1/4 |
| *Plecotus auritus* | – | – | – | 0/4 | – | 0/4 |
| *Rhinolophus affinis* | – | 0/3 | – | – | – | 0/3 |
| *Rhinolophus ferrumequinum* | – | 0/8 | 0/18 | 2/157 | – | 2/183 |
| *Rhinolophus luctus* | – | – | 0/2 | – | 0/1 | 0/3 |
| *Rhinolophus macrotis* | 1/1 | – | – | – | – | 1/1 |
| *Rhinolophus pearsonii* | 2/30 | 0/7 | – | – | 1/21 | 3/58 |
| *Rhinolophus pusillus* | 1/22 | 0/45 | 0/57 | 0/29 | 0/1 | 1/154 |
| *Rhinolophus rex* | 1/23 | – | – | – | – | 1/23 |
| *Rhinolophus sinicus* | 5/83 | – | – | – | 3/136 | 8/219 |
| *Rhinolophus thomasi* | – | – | – | – | 1/1 | 1/1 |
| *Rhinolophus monoceros* | 0/1 | 0/4 | 0/2 | – | 28/112 | 28/119 |
| Total | 29/385 | 7/111 | 2/100 | 2/199 | 33/272 | 73/1067 |

Note: "-" no animals were captured.

*Coronavirinae* defined by the International Committeeon Taxomony of Viruses (ICTV) (de Groot et al., 2011). As these viruses were from *Rhinolophus* bats we therefore designed them as SARS-related Rhinolophus bat coronaviruses (SARSr-Rh-BatCoV). Among these, 33 SARSr-Rh-BatCoVs were identified in 28 *R. monoceros*, 1 *R. pearsonii*, 3 *R. sinicus* and 1 *R. thomasi* sampled from the city of Longquan, Zhejiang province. Similarly, 2 SARS-related CoVs were identified in *R. ferrumequinum* sampled from the city of Jiyuan, Henan province, while the remaining 6 viruses were identified in 5 *R. sinicus*, and 1 *R. rex* sampled from the county of Anlong, Guizhou province.

On the RdRp phylogeny (Fig. 2) all known SARSr-Rh-BatCoVs from China could be divided into four clusters, within which the newly-identified viruses fell into three clusters that reflect their geographic origins. Specifically: (i) the viruses identified in *Rhinolophus* bats sampled in Zhejiang province (denoted *Rhinolophus* bat Longquan-) were closely related to each other and clustered with *Rhinolophus* bat CoV HKU3 sampled from *R. sinicus* in Hong Kong (Lau et al., 2005); (ii) The viruses identified in *R. ferrumequinum* from Jiyuan in Henan province (Jiyuan-84 and Jiyuan-331) formed a cluster with those viruses identified in *R. ferrumequinum* from other regions of China. The viruses within the cluster were from central China (Henan, Hubei and Shaanxi provinces), with the exception of the lineage comprising

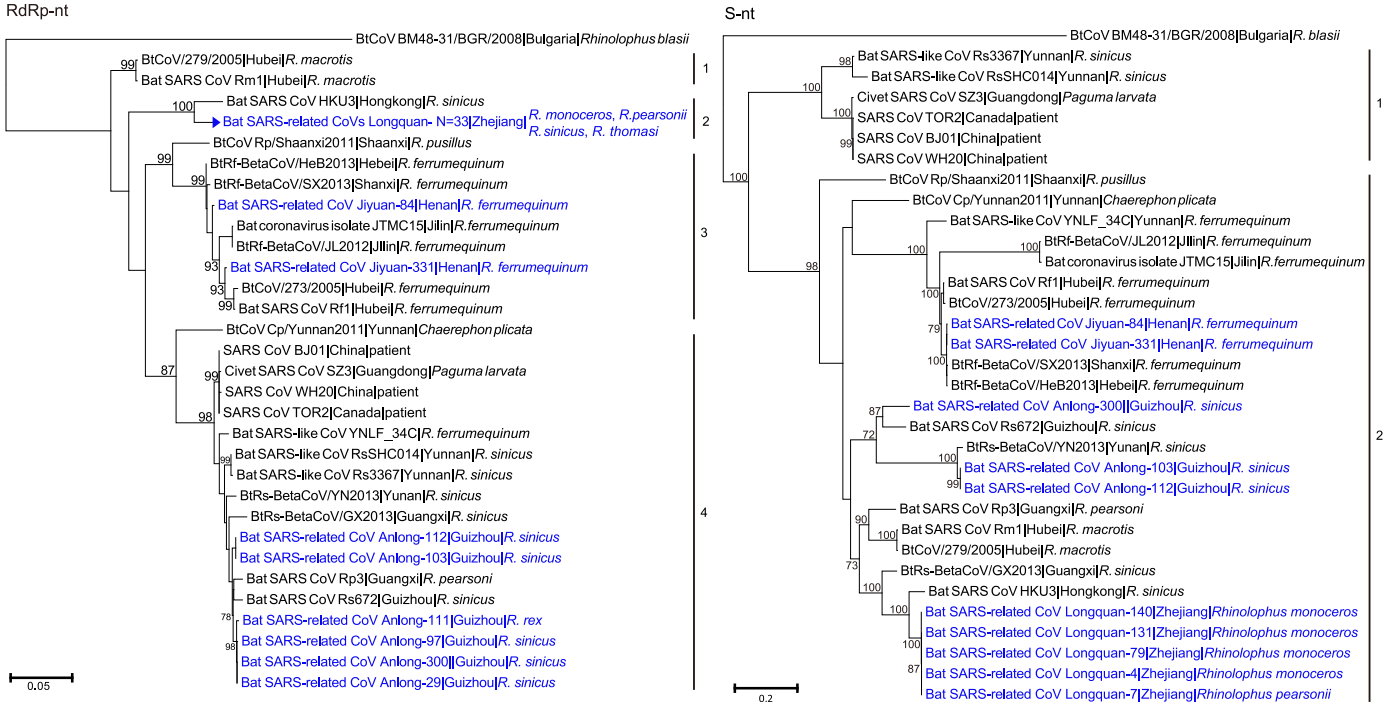


**Fig. 2.** Phylogenetic analysis of the nucleotide sequences of the RdRp and S genes including those CoVs obtained here. Bootstrap values ( > 70%) are shown at relevant nodes. The trees were mid-point rooted for clarity only. The scale bar depicts the number of nucleotide substitutions per site.

JMC15 and BtRf-BetaCoV/JL2012 identified in *R. ferrumequinum* sampled in northeastern China (Jilin province); (iii) The viruses identified in *R. sinicus* sampled from Anlong in Guizhou province clustered with those identified in *Rhinolophus* bats from southwestern China including Guangxi, Guizhou and Yunnan provinces (Ge et al., 2013; Li et al., 2005). Strikingly, only the bat SARSr-Rh-BatCoVs from southwestern China exhibited a close evolutionary relationship with SARS-related human coronavirus (SARS-CoV) and SARS-related palm civet coronavirus (SARSr-CiCoV) (Tor2 and SZ3), suggesting that SARS-CoV may have originated in this region. Finally, within each of these three clusters the SARS-related coronaviruses clustered according to their geographic origins.

Unlike the RdRp gene tree, all SARSr-Rh-BatCoVs and SARS-CoVs from China fell into two distant clades on the S gene phylogeny (Fig. 2). The first included the SARS-CoVs and two bat SARSr-Rh-BatCoVs (Rs3367 and RsSHC014), while the second comprised all the remaining bat SARSr-Rh-BatCoVs including the newly-identified CoVs, which could be further sub-divided into three clusters according to their geographic and/or host origins. Finally, additional analysis revealed that the S1 and S2 gene tree topologies differed from that of the S gene as a whole (Fig. S1), suggestive of potential recombination events (see below).

### 2.3. Characterization of the SARSr-Rh-BatCoV genome

To better characterize these newly-identified SARSr-Rh-BatCoVs we recovered the complete genome sequences from each of the lineages described. Their genome sizes varied from 29,665 to 29,693 nucleotides, and shared similar genome organizations with known SARS-related CoV viruses (Fig. 3), including the putative transcription regulatory sequence (TRS) motif, 5′-ACGAAC-3′, at the 3′ end of the 5′ leader sequence and preceding each ORF except ORF7b. Additionally, a single long ORF8 was observed in all newly-identified SARSr-Rh-BatCoVs.

The SARSr-Rh-BatCoVs from Longquan (Zhejiang) were closely related to the HKU3 strain with nucleotide identities ranging from 94.7% to 97.0% and amino acid identities of 98.2–99.1% in the RdRp. Notably, however, in the nsp2 gene they differed at up to 23% at the amino acid level. Additionally, the Longquan-140 virus was markedly different from SARSr-CiCoV at the amino acid level in the nsp2 (25.3%), S (20.3%), ORF3 (17.5%), and ORF8 (62.9%) gene sequences. The SARS-related viruses from Jiyuan (Henan) were closely related to BtRf-BetaCoV/HeB2013 and BtRf-BetaCoV/SX2013, with 99.0–99.2% nucleotide identities. Strikingly, the SARSr-Rh-BatCoVs sampled from Anlong (Guizhou) were closely related to SARS- and SARSr-CiCoVs, with > 94% nucleotide identities.

### 2.4. Recombination of SARSr-Rh-BatCoVs

We next conducted an analysis of possible recombination events using available genome sequence of SARSr-Rh-BatCoVs and other betacoronaviruses, including those discovered in this study and SARS- and SARSr-CiCoVs (Tor2 and SZ3). As noted in Table S2, the Longquan-140 virus was markedly different from HKU3 in the nsp2 gene, suggestive of possible recombination. When the Longquan-140 sequence was used as the query for a sliding widow analysis with HKU3 as a potential parent, two recombination breakpoints, located at the nsp2 (nucleotide 1727) and nsp3 (nucleotide 3055) genes, were detected in Longquan-140 with strong p-values ($< 10^{-25}$), and supported by a similarity plot (Fig. 4). Indeed, there was an abrupt change in the topological position of Longquan-140 and HKU3 downstream of the first breakpoint (position 1727) and upstream of the second breakpoint (position 3055). Accordingly, the Longquan-140 genome can be divided into three regions with different evolutionary histories: regions A (nucleotides 1–1726), B 1727–3055) and C (3056 to the end of the genome sequence). In regions A and C, Longquan-140 was most closely related to HKU3 with 96.0% nucleotide similarities, while in region B it occupied basal position, suggesting the recombination event originated from a currently un-sampled parental virus. No other putative recombination events received significant statistical support.

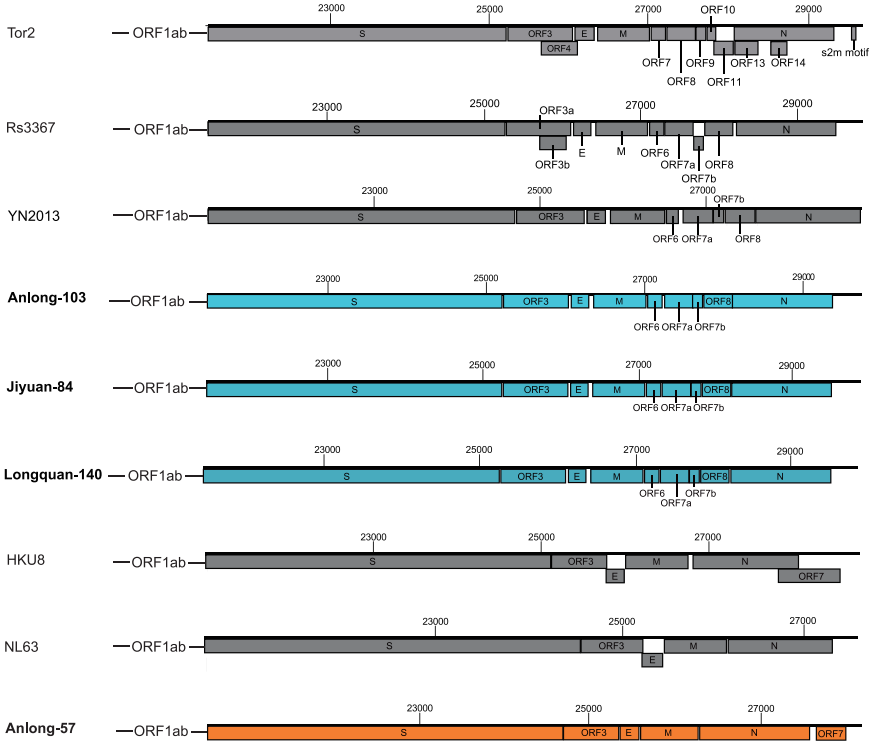


**Fig. 3.** Genome organization of coronaviruses. The four CoVs discovered in this study are shown in bold.

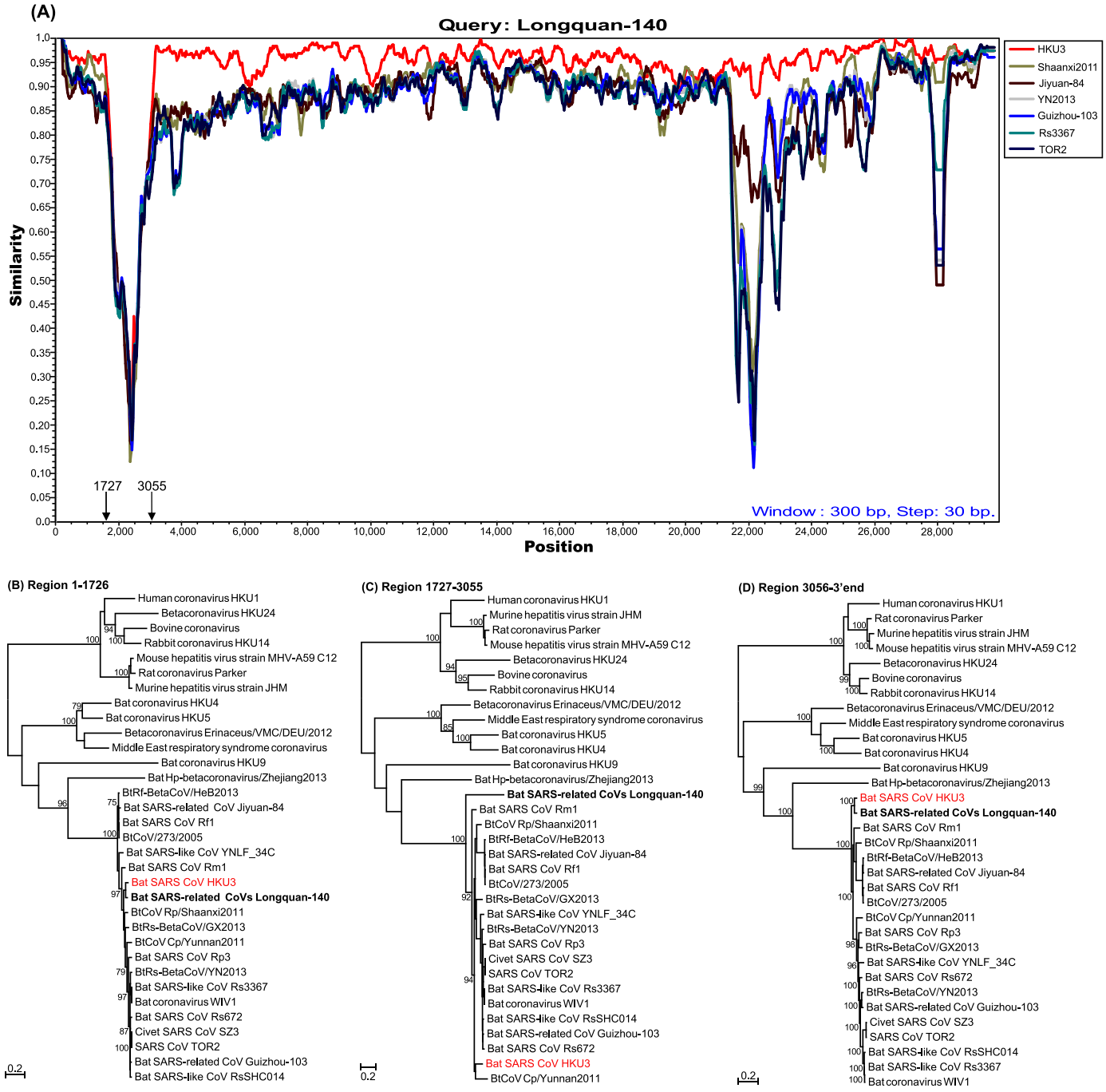


**Fig. 4.** Recombination within the genome of the Longquan-140 virus. A sequence similarity plot (A) reveals two recombination breakpoints shown by black arrows with their locations. The plot shows genome-scale similarity comparisons of the Longquan-140 (query) against HKU3 and other selected SARS-related CoVs. Phylogenies of regions A, B and C are shown below the similarity plot. Numbers ( > 70%) above or below branches indicate percentage bootstrap values.

### 2.5. *Newly-identified alphacoronaviruses in bats*

All remaining bat viruses discovered here belong to genus *Alphacoronavirus*, and had genome organizations similar to those of known alphacoronaviruses (Fig. 3). In the RdRp phylogeny these viruses fell into seven distinct lineages within three clusters (Fig. 5). The first cluster included the two newly-identified lineages, comprising the virus (Neixiang-31) identified in one *Myotis davidii* bat from Neixiang (Henan) and those identified in *Hipposideros armiger*, *M. davidii*, *M. siligorensis*, *R. macrotis*, *R. pearsonii*, *R. pusillus* bats from Anlong (Guizhou). Further comparison of the CoV replicase domains (ADRP, 3CLpro, RdRp, Hel, ExoN, NendoU and O-MT) revealed that these newly-identified bat viruses, as well as the bat virus BtMr-AlphaCoV/SAX2011 (NC_028811.1), exhibited more than 10% amino acid difference in all seven replicase domains (Table S3). These viruses also formed a distinct cluster in all gene trees, and exhibited a clear cluster according to their geographic origins (Fig. 5 and S2). Together, these data suggest that these viruses satisfy the species demarcation criterion defined by ICTV and should therefore be considered as a novel member of the genus *Alphacoronavirus*, which we denote as Neixiang Md bat coronavirus (NMBV).

Within the second cluster, the viruses discovered in bats from both Anlong (Guizhou province) and Neixiang (Henan province) formed a distinct lineage that was closely related to *Scotophilus bat coronavirus 512* (Sc-BatCoV 512) (Tang et al., 2006). As these viruses exhibited 10% amino acid difference from members of the genus

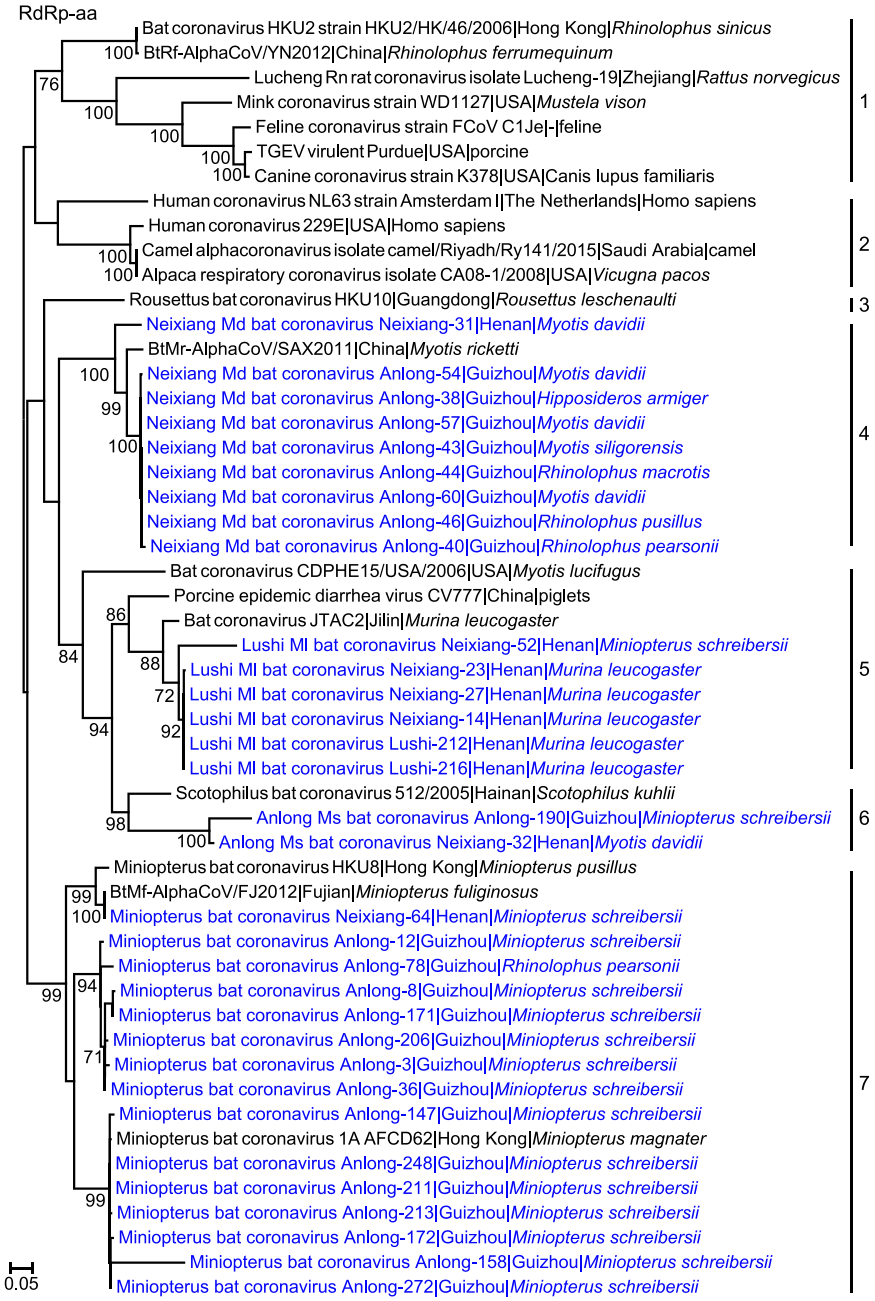


**Fig. 5.** Phylogenetic analyses of the amino acid sequences of the RdRp including those CoVs obtained here. Numbers ( > 70%) above or below branches indicate percentage bootstrap values. The trees were mid-point rooted for clarity only. The scale bar represents the number of amino acid substitutions per site.

*Alphacoronavirus* in the conserved replicase domains (with the exception of O-MT to Sc-BatCoV 512 (9.3%; Table S2)) it is possible that Anlong-190 and Neixiang-32 represent a novel CoV species which we denote as Anlong Ms bat coronavirus. However, further studies are clearly needed to determine whether this virus indeed represents a novel coronavirus. The remaining viruses, which were discovered in bats sampled from Lushi and Neixiang and designated as Lushi Ml bat coronaviruses, formed another distinct lineage that showed a close evolutionary relationship with the bat virus JTAC2 (KU182966). Remarkably, these viruses were most closely related to *porcine epidemic diarrhea virus* (PEDV) in all five gene trees (Fig. 5 and S2) and exhibited > 10% difference in six conserved replicase domains from known members of the genus *Alphacoronavirus* in other genes (Table S3). However, the O-MT gene of Lushi Ml bat coronaviruses was most closely related to that of PEDV with 7.3% amino acid difference, indicating that it is a novel variant of PEDV (designed as Lushi Ml bat coronavirus). Clearly, these data support the evolutionary origin of PEDV from bats (Huang et al., 2013).

Within the third cluster, the newly-identified bat viruses fell into three lineages. The newly-identified virus (Neixiang-64) in *Miniopterus schreibersii* from Neixiang formed a lineage with viruses in *M. fuliginosus* (KJ473799.1), and showed a close relationship with the HKU8 virus (Chu et al., 2008). Notably, the viruses from Anlong were grouped into two lineages, one of which contained *Miniopterus* bat coronavirus 1 A AFCD62 (Chu et al., 2008). In sum, our data reveal a high genetic diversity of alphacoronaviruses from bats in China.

### 2.6. Phylogenetic relationships between newly-identified viruses and their bat hosts

Comparison of the tree topologies of SARSr-Rh-BatCoVs and their bat hosts revealed strong incongruence between the SARSr-Rh-

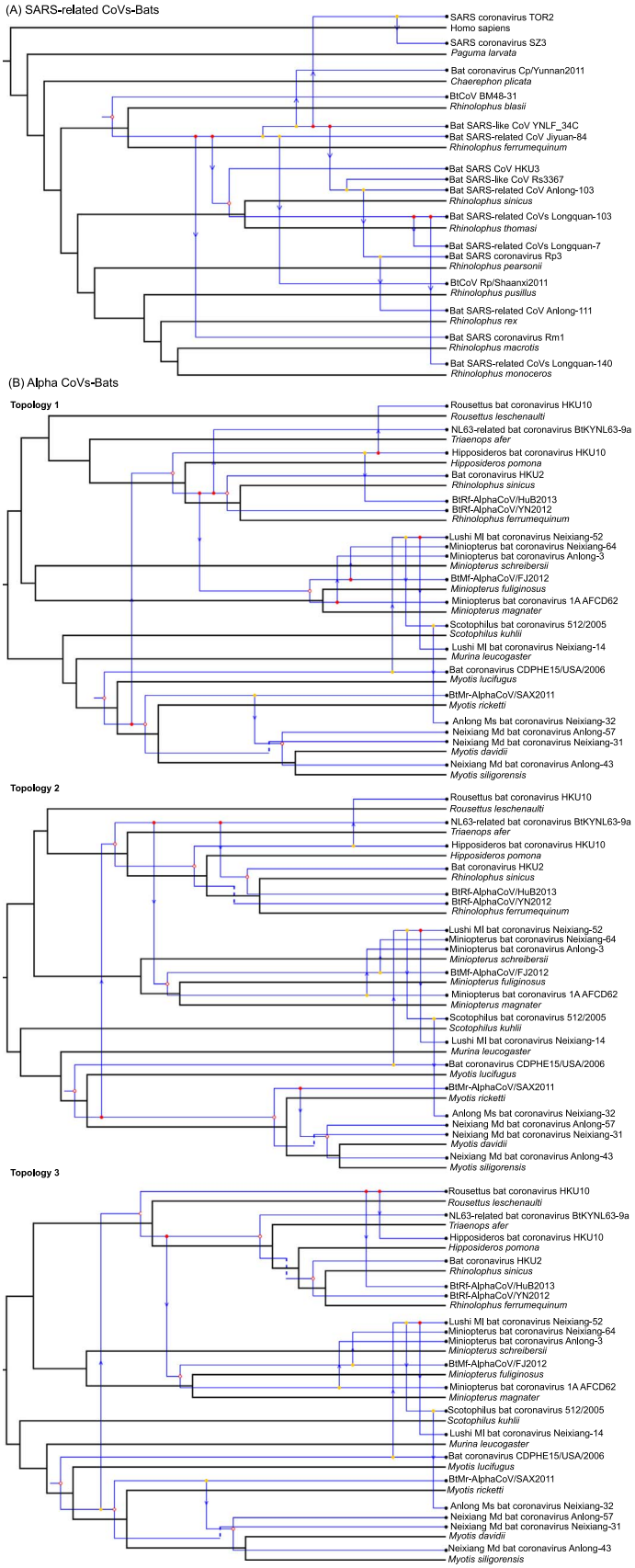


**Fig. 6.** Co-phylogenetic analyses of bats hosts and their associated coronaviruses. (A) SARS-related coronaviruses from bats, (B) alphacoronaviruses from bats. The coronavirus tree is shown in blue while the host phylogeny is shown in black. The host tree was based on mitochondrial cytochrome b gene sequences, and the coronavirus trees were based on the RdRp gene. Filled circles at the nodes indicate co-divergence events, empty circles indicate lineage duplication events, arrows indicate host-switching events, while dotted lines indicate loss events.

BatCoVs and their *Rhinolophus* bats hosts at the species level, with only two likely co-divergence events (Fig. 6A). Similarly, our co-phylogenetic analysis of alphacoronaviruses and their bat hosts provided evidence for 6–7 co-divergence events, 11–12 host switching events, 0 lineage duplication, 1–2 losses and 0 failure to diverge events (Fig. 6B). Overall, our co-phylogenetic analysis did not identify significant congruence between the phylogenetic trees of viruses and their bat hosts ($P$=0.04).

Clearly, more SARSr-Rh-BatCoVs were identified in *R. sinicus* and *R. ferrumequinum* bats sampled from a variety of locations (Fig. 2), as well as in *R. monoceros* sampled from Zhejiang province. In addition, the same virus could be identified in several species from the same geographic locality. For example, SARSr-Rh-BatCoVs were identified in four species of *Rhinolophus* bats from Longquan (Zhejiang) and in two bat species from Anlong (Guizhou). Similarly, Neixiang Md bat coronavirus (alphacoronavirus) was identified in six bat species sampled in Anlong (Guizhou). Hence, it is possible that high contact rates among some animal species may enable the acquisition and spread of CoVs.

## 3. Discussion

Since the discovery of SARSr-Rh-BatCoVs in bats in 2005 (Lau et al., 2005; Li et al., 2005), intense effort has focused on characterizing additional CoVs in bats, leading to the identification of diverse SARS-like viruses and other bat CoVs worldwide (Drexler et al., 2014; Hu et al., 2015; Huang et a, 2016; Woo et al., 2006). More importantly, recent studies provide more evidence that human CoVs including MERS and SARS viruses may have their ultimate ancestry in bats (Annan et al., 2013; Corman et al., 2014, 2015; Ge et al., 2013; Huynh et al., 2012; Hu et al., 2015; Ithete et al., 2013; Tao et al., 2017). These results highlight the potential significance of detecting and characterizing of CoVs in bats before their emergence in humans. Herein we describe diverse SARSr-Rh-BatCoVs and alphacoronaviruses (including novel species) in 13 species of bats sampled from three provinces in China, with an overall prevalence of 6.84%. As such, these data reveal both the high genetic diversity and the wide geographic distribution of CoVs in diverse bats in China.

Among the known SARSr-Rh-BatCoVs, those discovered by Yang et al. (2015) are most closely related to human SARS CoVs in the S gene ( > 90% amino acid similarities), but distant in the ORF8 gene ( < 43% amino acid similarities). Interestingly, other CoVs have been described that are closely related to SARS-CoVs in the ORF8 gene, but relatively distant in the S, nsp2, nsp11, and ORF3 genes (Table S2) (Lau et al., 2015; Wu et al., 2016). In this study, with the exception of the S and ORF3 genes, the viruses discovered in bats sampled in Guizhou province were most closely related to SARS-CoVs including the ORF8 gene. That all these SARSr-Rh-BatCoVs were sampled in southwestern China provides evidence for the origin of SARS-CoVs from bats in this region.

To date, SARSr-Rh-BatCoVs have been detected in bats sampled from diverse geographic regions in China as well as in Europe (Drexler et al., 2010). Notably, the majority of SARSr-Rh-BatCoVs have been discovered in Chinese *Rhinolophus* bats, while only one study reported the detection of SARS-related CoVs in *Chaerephon plicata* bats sampled from Yunnan province (Yang et al., 2013). In our study a total of 1067 bats from 21 bat species were captured, with SARSr-Rh-BatCoVs identified in 5 species of *Rhinolophus* bats. Although previous studies indicated that *R. sinicus* and *R. ferrumequinum* bats exhibited a higher prevalence of SARS-related viruses (Lau et al., 2005; Li et al., 2005; Tang et al., 2006), more SARSr-Rh-BatCoVs were identified in *R. monoceros* bats (Table S1). In sum, these data suggest that SARSr-Rh-BatCoVs may be specifically associated with *Rhinolophus* bats.

It is commonly stated that all the alphacoronaviruses originate from bat species (Woo et al., 2012), as may also be the case for the human CoVs NL63 and 229E (Corman et al., 2015; Tao et al., 2017). In addition, it is believed that PEDV might have originated from bats, although direct evidence is lacking (Huang et al., 2013). In this study, Lushi MI bat coronaviruses discovered in bats (*M. leucogaster*, *M. schreibersii*) from Lushi and Neixiang exhibited a close relationship to PEDV, especially in the O-MT domain (92.7% amino acid identity). In addition, all other viruses within the cluster were sampled from bats. Hence, these data provide more compelling evidence that PEDV may have originated from bats.

The evolutionary history of RNA viruses is characterized by both host switching and co-divergence (Li et al., 2015; Shi et al., 2016), which also appears to be true of coronaviruses (Annan et al., 2013; Cui et al., 2007; Drexler et al., 2014; Lau et al., 2010, 2012, 2013; Tang et al., 2006; Vijaykrishna et al., 2007; Wertheim et al., 2013; Woo et al., 2009, 2012). Similarly to previous studies (Drexler et al., 2010; Ge et al., 2013; Lau et al., 2005; Li et al., 2005; Wu et al., 2016; Yang et al., 2013), SARSr-Rh-BatCoVs were mainly identified in *Rhinolophus* bats, but not in other bats even when sampled in the same locality (e.g. in Guizhou and Henan). In contrast, alphacoronaviruses were mainly identified in non-*Rhinolophus* bats and no alphacoronaviruses were discovered in the colony that only comprised *Rhinolophus* bats. Finally, it was noteworthy that a SARSr-Rh-BatCoVs and Neixiang Md bat coronavirus (alphacoronavirus) were identified in several bat species (from up to three families) in the localities of Anlong and Longquan, indicative of local inter-species transmission. Hence, these data suggest that high contact rates among specific animal species enable the acquisition and spread of CoVs.

## 4. Material and methods

### 4.1. Bat trapping and specimen collection

Bats were captured alive with mist nets or harp traps in caves of natural roosts in Guizhou, Henan, and Zhejiang provinces during 2012–2015 (Fig. 1). Bat species were initially identified by morphological examination and further confirmed by sequence analysis of the mt-*cyt b* gene (Guo et al., 2013). All bats were anesthetized before surgery with every effort made to minimize suffering. Tissue samples, including those from the rectum, were collected from bats for coronavirus detection.

### 4.2. DNA and RNA extraction, PCR and sequencing

We extracted total DNA from bat tissue samples using the DNeasy Blood & Tissue kit (QIAGEN, Valencia, USA) according to protocols suggested by the manufacturer. Total RNA was extracted from fecal or tissue samples using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Coronavirus RNA was detected by RT-PCR as described previously (Lau et al., 2005; Wang et al., 2015). Other coronavirus gene sequences were amplified using the primers designed based on the conserved regions of known genome sequences.

RT-PCR amplicons were purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, USA) according to the manufacturer's recommendations. Purified DNA < 700 bp was subjected to a direct sequencing protocol, while purified DNA > 700 bp was cloned into pMD18-T vector (TaKaRa, Dalian, China), which was subsequently transformed into JM109-143 competent cells. DNA sequencing was performed with Applied Biosystems 377 gene sequencers.

### 4.3. Complete genome sequencing

Four representatives of CoV in bats were selected for full-genome sequencing. The initial PCR primer sets for PCR were designed from each pan-CoV amplicon sequence and/or from a conserved region in the CoV RdRp. As required, walking primers were designed for further PCR and sequencing. All primer sets used in this study are available upon request.

All sequences generated in this study have been deposited in GenBank and assigned accession numbers KF294268-KF294282, KF294373-KF294378, KF294381-KF294383, KF294420-KF294457, KY770850-KY770860.

### 4.4. Phylogenetic analysis of CoV sequences

In addition to the sequences recovered here, reference sequences that cover the phylogenetic diversity of CoVs were compiled for evolutionary analyses. Accordingly, sequence alignments were performed using the MAFFT algorithm (Katoh and Standley, 2013). After alignment, gaps and ambiguously aligned regions were removed using Gblocks (v0.91b) (Talavera and Castresana, 2007). Phylogenetic trees were estimated using the maximum likelihood (ML) method implemented in PhyML v3.0 (Guindon et al., 2010) with bootstrap support values calculated from 1000 replicate trees. The best-fit amino acid substitution models were determined using MEGA version 5 (Tamura et al., 2011).

### 4.5. Recombination analysis

Full-length genomic sequences of the SL-CoVs Longquan-140, Anlong-103 and Jiyuan-84 viruses were aligned with those of bat SARSr-Rh-BatCoVs and other betacoronaviruses using MEGA 5.0. The aligned sequences were preliminarily scanned for recombination events using the Recombination Detection Program 4.0 (RDP4), employing the RDP, GENECONV, and Bootscan methods (with default parameters) (Martin et al., 2015). The potential recombination events suggested by RDP (i.e. those with strong *P* values) were investigated further by similarity plots implemented in Simplot 3.5.1 (Lole et al., 1999). For each of the putative recombinant regions, phylogenies were estimated using the maximum likelihood method available in PhyML v3.0 (Guindon et al., 2010).

### 4.6. Analysis of CoV and host co-phylogenies

Co-phylogenetic analyses of bats hosts and their associated coronaviruses were conducted using the heuristic event-based method available in the Jane software package (Conow et al., 2010). We reconstructed patterns co-divergence (and hence cross-species transmission) using a weight of 0 for co-divergence and a weight of 1 for duplication, host switching, lineage loss and failure to diverge. We used Jane with 100 generations and a population size of 100 as parameters for the genetic algorithm. To test the probability of observing the inferred co-divergence number by chance, we employed the random tip mapping method with the generation number =100, population size =100, and sample size =50.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2017.03.019.

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