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Identification and genetic characterization of five novel bat coronaviruses from Yunnan, China

Qian Li[1,2†], Yutong Hou[1†], Baoyang Huang[1], Xiang Le[1], Binghui Wang[1,3*] and Xueshan Xia[1,3*]

Abstract

Background Coronaviruses (CoVs) represent a serious threat to human health and have become a major transmissible, endemic, and causative pathogen in humans; they represent a major health concern, given their ability to cause infectious diseases. Bats are natural hosts for diverse viruses. Many transmission events of CoVs and identification of multiple novel CoVs in bats has increased attention towards their capacity to serve as hosts for zoonotic viruses.

Results In this study, 61 bats from Yunnan Province were analyzed, identifying seven CoVs, including three α- and two β-CoVs with full-genome sequences. Among the five identified alpha-CoVs, four belong to the *Decacovirus* subgenus and one to the *Minunacovirus* subgenus. Two beta-CoVs were also identified, both belonging to the *Sarbecovirus* subgenus.The genetic structures revealed similarities to known strains such as HKU10 and SARS-CoV-2, along with novel findings such as the *Minunacovirus* subgenus CoV YJ3c/f and unique ORF patterns. Our results demonstrated that strain JCC9 has a unique recombination pattern and shows a higher binding affinity to civet and pangolin ACE2 receptors, then the HpJC8xc strain transmits and recombines between hosts (bats), indicating a potential risk of crossing the interspecies barrier and infecting other animals.

Conclusions The CoVs detected in the bats studied in this research exhibit high diversity. Genomic analysis revealed that CoVs in bats undergo frequent recombination events. Furthermore, recombination patterns and evolutionary analyses suggest that alpha-CoVs are more prone to cross-species transmission across different bat families/genera, whereas beta-CoVs demonstrate host specificity and tend to co-evolve with their bat hosts.Our finding suggest that bats, as hosts of CoVs, be constantly monitored to prevent outbreaks of new infections caused by viruses passing across interspecies barriers, and consequently, viral diseases in humans or livestock.

Keywords Coronaviruses, Bats, Characterization, Transmission, Yunnan

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Background

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses characterized by large genomes, multiple hosts, frequent recombination, and cross-species transmission. These viruses are belong to the family *Coronaviridae*, the subfamily *Orthocoronavirinae*, and the genus Alpha-, Beta-, Gamma-, Deltacoronavirus, and classiy into the various subgenus such as Sarbecovirus [1]. Several severe transmission events of CoVs, including SARS, MERS, and COVID-19, which are thought originate from bats, have occurred recently [1–3]. Bats have since attracted increasing attention as natural hosts of multiple zoonotic viruses.

According to *China's List of National Key Wild Animals* in February 2021, bats belong to the order Chiroptera, the second largest order of mammals, are omnivorous group-living animals; they are also the only mammals that can fly [4]. The total count of extant Chiroptera is 18 families and 202 genera, including about 1,116 species, of which 7 families, 30 genera, and 120 species are found in China [5].

Bats are natural hosts for a wide range of viruses, carrying more than 130 viruses, half of which are virulent pathogens, such as the Hendra and Nipah viruses, which can cause major infectious diseases in humans [1, 6, 7]. Moreover, with the application of metavirome research, multiple novel CoVs have been identified in bats. Of the many viruses carried by bats, CoVs are among those of the greatest concern today. Among the α-CoVs, Rhinolophus bat CoV HKU-2, Miniopterus bat CoV HKU-8, and Rousettus bat CoV HKU-10 are directly derived from bats, while among the β-CoVs, Tylonycteris bat CoV HKU-4, Pipistrellus bat CoV HKU-5, Rousettus bat CoV HKU-9, SARS-CoV, and MERS-CoV also have bat origins. Additionally, other CoVs such as bat CoV HKU-6, bat CoV HKU-7 and other species have been identified in bats, though they are not included in the current classification by the International Committee on Taxonomy of Viruses (ICTV) [8–11]. Bats are considered to be the natural hosts of SARS-CoV, MERS-CoV, HCoV-229E and HCoV-NL63 [12].Although other human CoVs such as HCoV-OC43, HCoV-HKU1, and SARS-CoV-2 are not directly derived from bats, they are believed to have significant evolutionary links to bat CoVs, possibly through intermediate hosts. This highlights the critical role of bats in the broader CoV ecosystem.Given the significant impact of SARS-CoV-2 on global public health, understanding the similarities and differences between SARS-CoV-2 and other bat CoVs is crucial. Therefore, studying the S proteins of novel bat CoVs in comparison to SARS-CoV-2 provides insights into the evolutionary relationships and potential cross-species transmission risks, which are essential for preventing future pandemics.

Yunnan Province, with its diverse topography, complex natural conditions, favorable climate, and abundant resources, is suitable for the survival and reproduction of a wide variety of wildlife; it is an internationally renowned "animal kingdom." Yunnan, which is covered by dense forests and caves suitable for bats, has more than 14 bat species [13]. The richness of bat species and their large populations have contributed to the mutation and recombination of CoVs [14]. Multiple teams have identified a variety of novel viruses in bats [12, 15], and many close relatives of SARS-CoV-2 have been identified in neighboring countries [16]. Currently, numerous sequences closely related to SARS-CoV-2 have been reported in bats, but it remains unproven that bats are the natural hosts of SARS-CoV-2. Therefore, additional sequences are needed to further investigate its evolutionary origin and trends. A higher proportion of zoonotic viruses have been observed in bats, primates, and rodents compared to other mammals [1].

In the present study, the CoVs were investigated with regard to three aspects: screening of CoVs in bats, characterization of bat CoV genomes, and exploration of the differences in S proteins between novel bat CoVs and SARS-CoV-2 and related viruses. This study aims to provide a preliminary understanding of the CoVs carried by bats in the Yunnan Province, characterize the evolution of the viruses in their natural hosts and the pattern of cross-species transmission, and contribute towards the prevention and control of emerging infectious diseases.

Methods

The samples were stored in virus preservation solution, transported on dry ice, and subsequently stored in a -80 °C refrigerator in the laboratory. They were then dissected, following which the lung and intestine tissues were removed and placed in virus preservation solution and stored at -80 °C until RNA extraction. This study was approved by the Institutional Ethical Committee of Kunming University of Science and Technology (protocol no. 16048). Rare and protected animals were not involved.

To further confirm the presence of CoVs in the sample, total RNA was extracted from 200 μL of the sample homogenates using a TIANamp virus RNA kit (TIANGEN, China) following the manufacturer's instructions. CoV infection in samples was detected based on the *RdRp* gene present in the α and β CoV genomes using nested PCR. The primers for the *RdRp* gene were designed and evaluated using Oligo 7 Primer Analysis Software (Molecular Biology Insights, Inc., https://www.oligo.net), targeting conserved regions of the viral genome to ensure specificity and amplification efficiency, modified bases were incorporated at the 5' ends of the primers. For a broader context on CoV genome detection methodologies, the work of Poon et al. was referenced [17]. Similarly,

bat species were identified using the *cytochrome b* gene (*Cytb*) through semi-nested PCR to distinguish between different mammals [18] (Table 1). The nested PCR involved two rounds of amplification to increase specificity and sensitivity: the first round used outer primers(10 μM), and the second round employed inner primerss(10 μM) designed to target the specific gene regions The first round PCR reaction contained a 25 μL mixture, including 12μL of PCR buffer (PrimeScript™ One Step RT-PCR Kit Ver.2, Takara, United States), 1 μL of template RNA, 1 μL of each forward, reverse primers(10 μM) and enzyme, then 9 μL of ddH2O.The second round PCR reaction contained a 25 μL mixture, including 12μL of PCR Mix (Takara, United States), 1 μL of template cDNA, 1 μL of each forward and reverse primer(10 μM), then 10 μL of ddH2O.The conditions set for the PCR machine were: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min. The amplified products were identified using 1% agarose gel electrophoresis; the samples with positive target bands were sent to a sequencing company (Kunming Qingke Biotechnology Co., Ltd.) for sequencing by the Sanger sequencing method.

The full-genome sequences were obtained by segmental sequencing and assembly using Sanger sequencing. In addition to following the basic principles of primer design, the amplification primers for the full-genome sequences were designed with at least 100 bp overlap between each segment. Nested primers were designed using all reference sequences of the same cluster by phylogenetic analyses as the target.

Sequencing results were first analyzed using the Chromas software to confirm the accuracy of the sequencing results in the peak plots. Preliminary editing and final splicing of the sequencing results was then performed using the SeqMan module of the DNAStar software (version 7.1). The reference strains of the *Cytb* gene and the *RdRp* gene were downloaded from the GenBank database, and then, sequence comparison and editing were performed using ClustalW (version 2.0) and BioEdit (version 7.1.9).

CoV genotypes were determined based on the nucleotide sequences of obtained bat CoVs compared with known representative CoV sequences.Neighbor-joining phylogenetic trees was run a Model Test as the best fit model by the MEGA 7.0 software. Neighbor-joining phylogenetic trees were constructed using the Kimura 2 parameter model, Gamma Distributed, and Transitions+Transversions in the MEGA 7.0 software. The construction of the evolutionary tree was repeated 1,000 times and the accuracy of typing was verified on the basis of bootstrap values>70%.

The full genome sequences were initially aligned using BLAST in NCBI(Basic Local Alignment Search Tool) to determine the homology and conserved regions between our sample sequences and the reference sequences. Based on the alignment results, we identified the conserved and variable regions within the genomes. For the conserved regions, we used known ORF sites as references to locate the corresponding ORFs in our sample sequences. Each identified ORF was then functionally annotated.

Coronavirus genome structure drawing uses the online tool Gene Structure Display Server (http://gsds.gao-lab.org/). In the recombination analysis, Simplot (v3.5.1) (http://sray.med.som.jhmi.edu/SCRoftware/simplot/) was used to draw the whole genome sequence similarity map, with a window size of 1000 bp and a step size of 100 bp, using RDP5(http://web.cbio.uct.ac.za/~darren/rdp.html) to conduct a statistical test on the recombination event that occurred in Yunnan_Hp_JC8xc_2020. The software BioAider (version 1.314) (http://www.mrdisease.com/bioaider/) was used to analyze the sequence identity of the CoVs. The three-dimensional structures of the S protein from Yunnan_Rp_JCC9_2020, PrC31, and SARS-CoV-2 were modeled using the Swiss-Model program(https://swissmodel.expasy.org/) employing PDB: 7sxs.1.A as the template. The structures were built using UCSF Chimera 1.14(https://www.cgl.ucsf.edu/chimera/).

Table 1 Primer sequences

Gene	Name	Sequence (5′-3′)[a]	Target fragment size
RdRp	Cov-*RdRp*-WF	ATGGGWTGGGAYTAYCC(dI)AARTG[b]	440 bp
	Cov-*RdRp*-WR	TGYTG(dI)GARCAAAAYTCRTG	
	Cov-*RdRp*-NF	GG(dI)TGGGAYTAYCC(dI)AARTGYGA	
	Cov-*RdRp*-NR	CCRTCATCWGA(dI)ARWATCATCAT	
Cytochrome b	F-14,724	CGAAGCTTGATATGAAAAACCATCGTT	753 bp
	F-15,162	GCAAGCTTCTACCATGAGGACAAATATC	
	R-15,915	GGAATTCATCTCTCCGGTTTACAAGA	

[a]Concatenated base: Y=C, T; W=A, T; R=A, G

[b]Modifying bases: (dI) denotes a 5′ modification

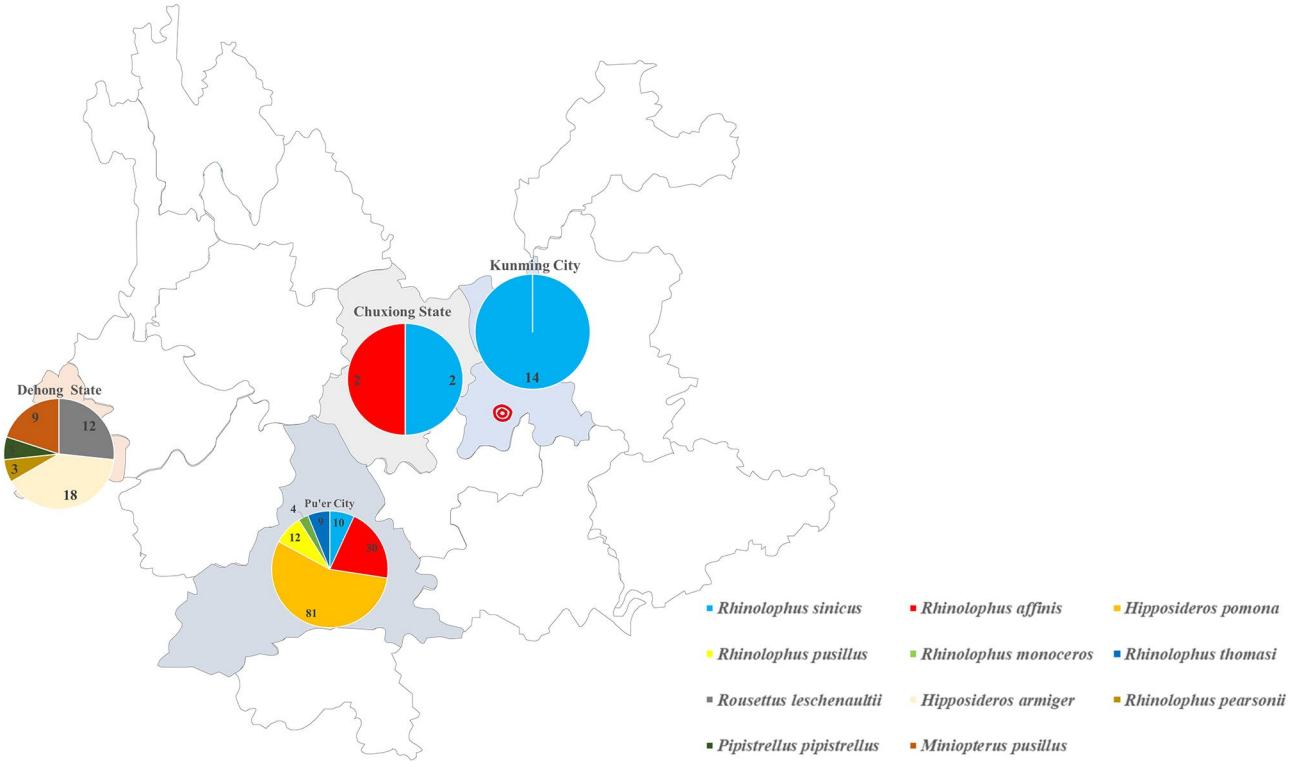

Fig. 1 Geographic distribution map of bat species in Yunnan. The gray background represents the state/city where the sampling originated from, and the different colors in the sector map indicate different bat species

Table 2 Sample information of full-genome sequences

Number	Bat species	Sample number	Collected place	Collected time
Yunnan_Hp_JC8xc_2020	*Hipposideros pomona*	JC8xc	Yunnan	2020
Yunnan_Rs_CX18c_2020	*Rhinolophus sinicus*	CX18c	Yunnan	2020
Yunnan_Rp_JCC9_2020	*Rhinolophus pusillus*	JCC9	Yunnan	2020
Yunnan_Rs_KMC6_2020	*Rhinolophus sinicus*	KMC6	Yunnan	2020
Yunnan_Mp_YJ3c/f_2020	*Miniopterus pusillus*	YJ3c/f	Yunnan	2020

The last letter in the sample ID indicates the tissue type: "c" represents the intestine, and "f" represents the lung

Results

In this study, a total of 61 bats were captured from wild environments or suburbs in four states/cities of the Yunnan Province between March 2020 and August 2021, from Kunming, Chuxiong, Pu'er, and Dehong states (Fig. 1). The following species were studied: *Rhinolophus sinicus*, *Rhinolophus affinis*, *Hipposideros pomona*, *Rhinolophus pusillus*, *Rhinolophus monoceros*, *Rhinolophus thomasi*, *Rousettus leschenaultii*, *Hipposideros armiger*, *Rhinolophus pearsonii*, *Pipistrellus pipistrellus*, and *Miniopterus pusillus*, as identified using host the *Cytb* gene (Fig. 1).

The positive bat samples screened in this study were named on the basis of the province, the bat species and sample number. For example, sample JC8xc, *Hipposideros pomona* which was collected in May 2020 and was positive for CoV, was named Yunnan_Hp_JC8xc_2020. Other samples were named as follows: (in order) Yunnan_Rs_CX18c_2020, Yunnan_Rp_JCC9_2020, Yunnan_Rs_KMC6_2020, and Yunnan_Mp_YJ3c/f_2020 (Table 2).

Seven CoVs were identified using partial *RdRp* gene amplification, among which five were classified as α-CoVs (four *Decacoviruses* and one *Minunacovirus*) and two as *Sarbecoviruses* of β-CoVs, with a positive rate of 11.5% as per the phylogenetic tree (Fig. 2).The full-genome sequences of three α- and two β-CoV strains were obtained and their genetic structures with compared with those of representative CoV strains. Yunnan_Hp_JC8xc_2020 and Yunnan_Rs_CX18c_2020 were identified as *Decacoviruses*. Two other open reading frames (ORFs) downstream of the N gene at the 3′ end, namely, ORF7 and ORF8, were identified in the strain JC8xc. No ORF8b was identified in ORF8. Meanwhile, Yunan_MP_YJ3c/f_2020 belonged to the *Minunacovirus* subgenus of α-CoVs, with an additional ORF3 in the basic skeleton of

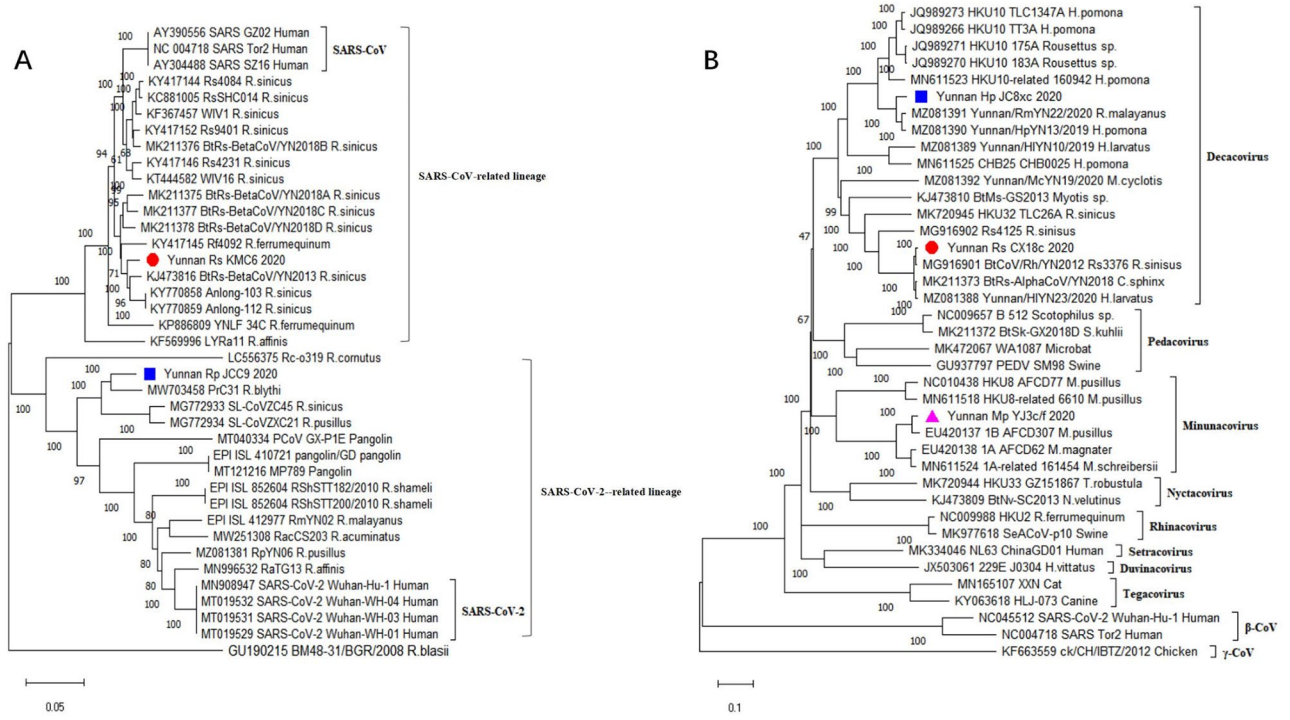

Fig. 2 Neighbor-joining phylogenetic tree showing the homologous relationships of five CoVs strains. Phylogenetic tree of (**A**) α- and (**B**) β-CoVs constructed based on the complete nucleotide sequences of CoVs. Each reference strain is represented by serial number, strain name, and species origin. Each group is annotated. The red, pink, and blue markers refer to the CoVs detected in this study

the CoV genomic structure (Fig. 2B). The structures of the two β-CoV strains are broadly similar to those of the representative strains in the SARS-CoV-related lineage, except for Yunnan_Rs_KMC6_2020 not having ORF8b and ORF9b. Interestingly, Yunnan_Rp_JCC9_2020 has an ORF10 downstream of the N gene, also present in SARS-CoV-2 (Fig. 3).

Recombination analysis of the viral strains showed that the entire S protein region of HpJC8xc recombined during the infection of different bat families. We reanalyzed the full-length sequences using RDP5 and confirmed that the recombination event in Yunnan_Hp_JC8xc_2020, the only recombination event identified in this study with a *P*-value of 3.533×10^{-248}. Most genes of the viral strain were very similar to Yunnan/HpYN13/2019, and the S gene region showed 85.26/92.96 (nt/aa%) similarity to HKU10_175A [13].This strain is recombinant of HKU10_175A and HKU10_183A found in *R. leschenaultii*, together with Yunan/RmYN22/2020 found in *Rhinolophus* and Yunnan/HpYN13/2019 found in *H. pomona*. This was also confirmed by the homologic evolutionary tree of the sequences of different gene fragments (Fig. 4).

The SARS-CoV-2 spike protein contains a unique S1/S2 furin cleavage site that can be cleaved by furin and other proprotein convertases. The cleavage site of the Flynn protease also lacked the signature component of a pandemic virus (Fig. 5A). The signal structures of the S protein subunits of JCC9, PrC31, and SARS-CoV-2 were predicted using a homology model. Similar to PrC31, the two rings near the receptor-binding domain (RBD) are shorter in JCC9 than in SARS-CoV-2. This region may affect the binding of the RBD to the ACE2 receptor of JCC9. In addition, the conformational rings of JCC9 and PrC31 are significantly different from those of SARS-CoV-2 near the S1/S2 cleavage site [18] (Fig. 5B), suggesting that this viral strain is highly unlikely to use ACE2 as a receptor to infect human cells, despite its close affinity to SARS-CoV-2. From the binding affinity of the RBD of Yunnan_Rp_JCC9_2020 S protein with the ACE2 of the four species, we found that the RBD cannot bind to the ACE2 of the human receptor (Fig. 6), with a binding energy of only −8.3 kcal/mol, which is in accordance with the analysis above. However, it may bind to the civet (-17.4 kcal/mol) and pangolin (-15.6 kcal/mol) ACE2. Overall, there is a potential risk of JCC9 crossing the interspecies barrier and infecting other animals.

Discussion

Bats have always been regarded as natural reservoir of many viruses, especially coronavirus. Due to geological and climatic reasons, such as many natural caves and ripe fruits all year round, the species and quantity of bats are very huge in Yunnan Province. A phylogenetic tree

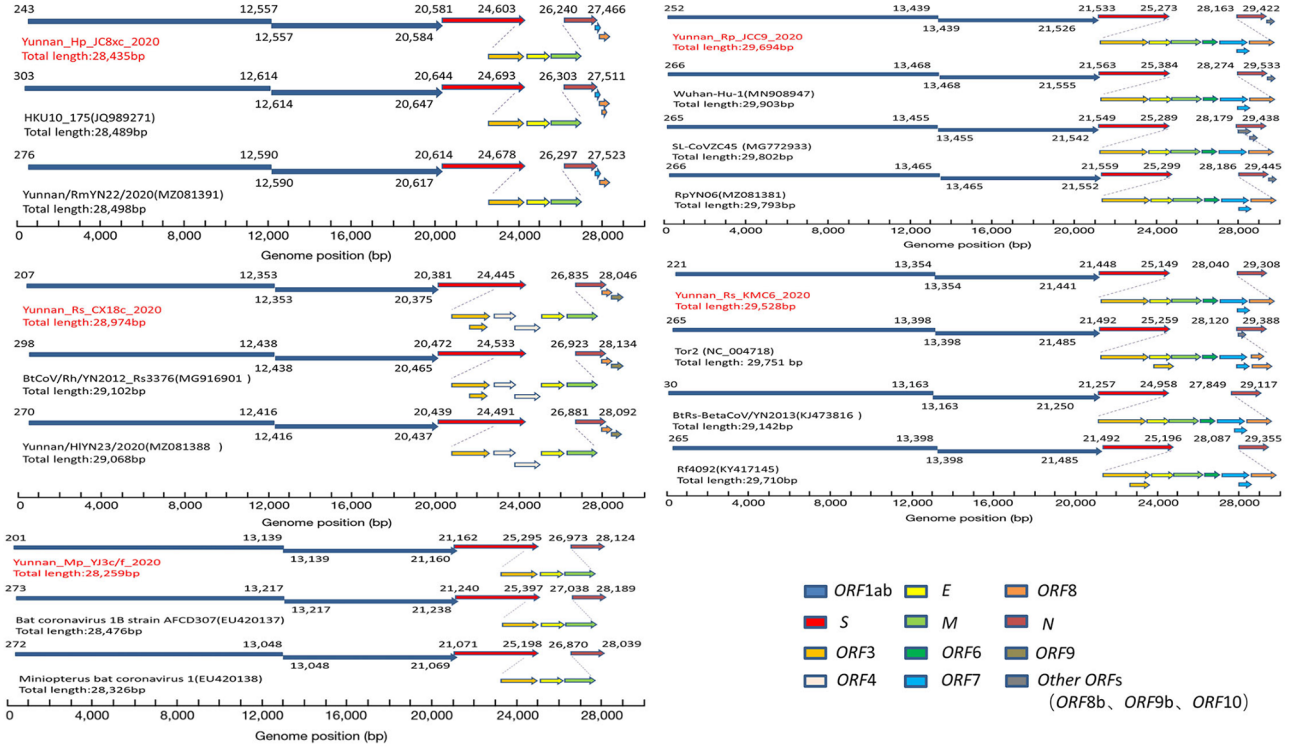

Fig. 3 Full-length genomic sequence analysis of bats. The individual open reading frames of CoVs are indicated by different colors, and the respective lengths of the open reading frames correspond to the scale below. Login numbers for related viruses are detailed in the phylogenetic tree

constructed based on complete nucleotide sequences five alpha-CoVs and two beta-CoVs were identified, with unique genetic characteristics and recombination, could be inferred that bats in Yunnan harbor a highly diverse range of CoVs. The Phylogenetic analysis revealed that JCC9, SL-COV ZC55, SL-CoVZXC21, and PrC31 form a single evolutionary branch. The strains found in this study and PrC31 were from the same species in the same city and had a nucleotide homology of 95.16% [19]. In addition, SL-COV ZC55 and SL-CoVZXC21 discovered in *Rhinolophus sinicus* from Zhoushan City, Zhejiang Province, also had similar genetic distances [12]. This lineage has a large genetic distance from human SARS-CoV-2 (89.19% nucleotide homology with Wuhan-Hu-1), suggesting a limited potential for direct human infection.

For beta-CoVs, KMC6 is classified as SARS-CoV-related, with short distances to BtRs-BetaCoV/YN2013 and Anlong-103 (Fig. 2A), the latter of which is found in R. sinicus [12].In addition, the genetic structures of JC8xc and CX18c were similar to that of HKU10. The genome structure of JC8xc was the most similar to that of the strain Yunnan/RmYN22/2020 as same as lack of ORF8b. The other α-CoV, CX18c, was related to the BtCoV/Rh/YN2012_Rs3376, BtRs-AlphaCoV/YN2018, and Yunnan/HlYN23/2020 strains [14]. These unclassified strains were most closely related to HKU32. The novel *Minunacovirus* subgenus CoV YJ3c/f is the second full-genome α-CoV 1B-related CoV discovered to date.

Notably, JCC9 is closest to that responsible for the recent SARS-CoV-2 epidemic; the key site of the S protein is similar to that of most bat SARS-CoV-2, such as SL-CoVZC45, RsYN06, RmYN02, RacCS203, and PrC31, with one long (14 aa) deletion and one short (5 aa) deletion [12, 14, 19, 20]. Only 2 of the 20 amino acid residues required for the interaction between JCC9 and ACE2 are identical, indicating a low potential for human ACE2 binding. This is the first report of CoVs infecting bats of a different family, in which recombination of the entire S-protein region has occurred to form a novel strain. This supports the theory of cross-species transmission from R. le*schenaultii* to *H. pomona*, suggesting that interspecies CoV transmission and recombination between *Rhinolophus*, *Hipposideros*, and *R. leschenaultii* occurred.

However, this study has certain limitations. The geographical scope and sample size may limit the generalizability of the findings. Additionally, the quality and completeness of reference sequences from public databases may influence the interpretation of our findings. Future studies should expand the sample range and employ more advanced techniques to enhance the reliability of the results.

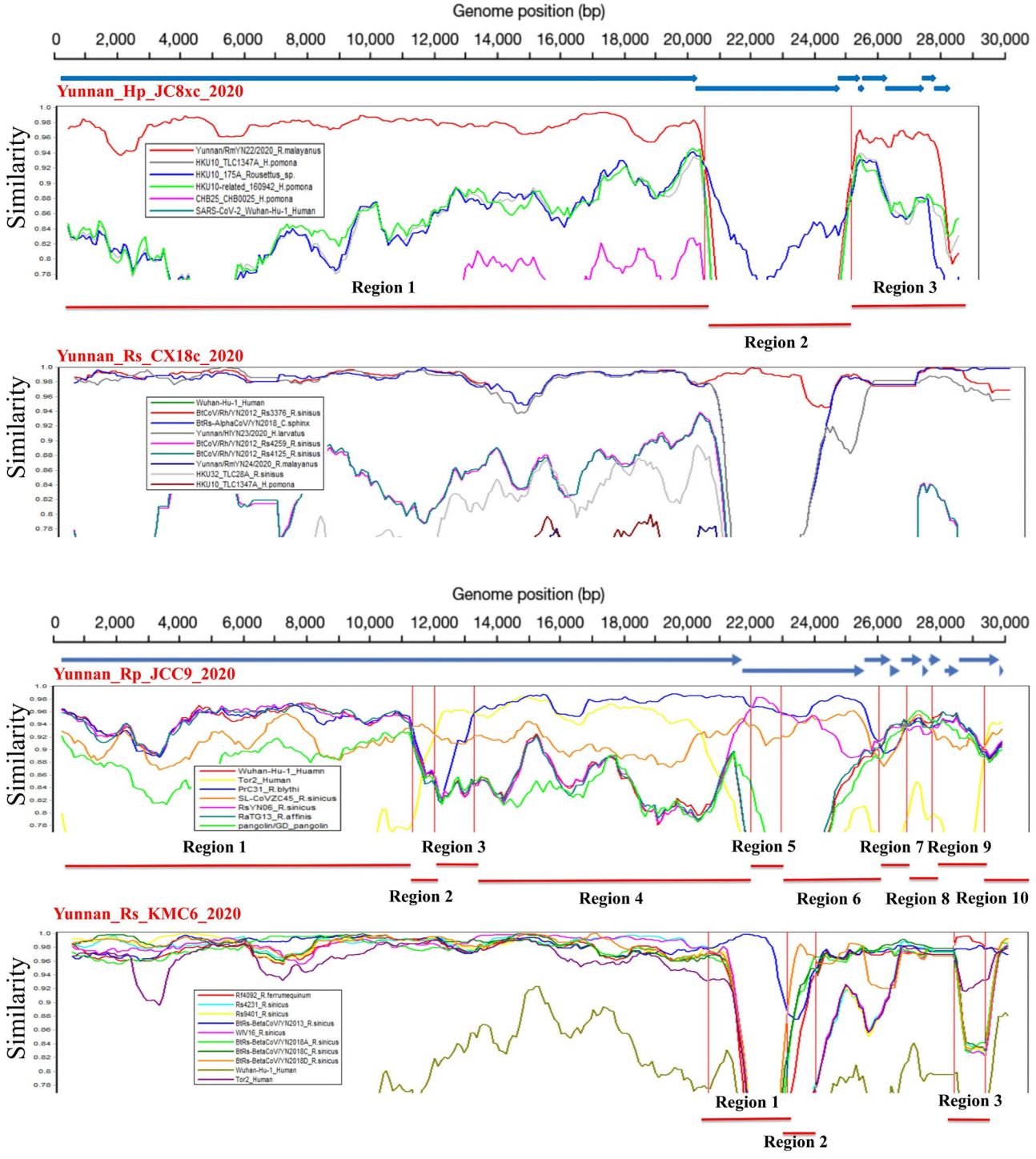

Fig. 4 Recombination analysis in CoVs. Sequence similarity plots with a window size of 1000 bp and a step size of 100 bp. Reference strains are indicated using different colors, and the blue arrow at the top indicates the position of the ORF at the time of alignment. Potential restructuring breakpoints are marked below with a solid red line. The login numbers of related viruses are detailed in the phylogenetic tree

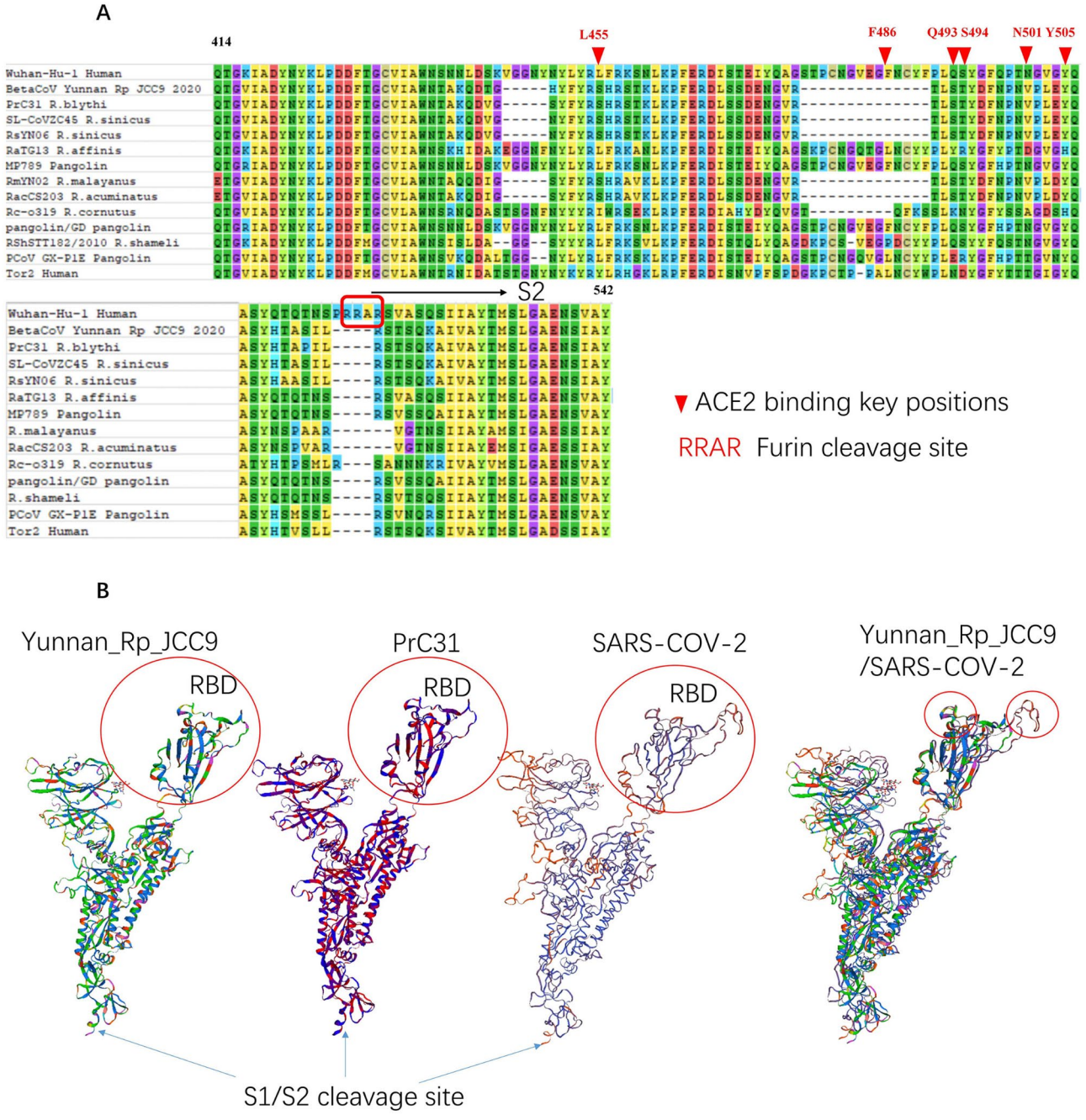

Fig. 5 Comparison of S protein. (**A**) Comparison of RBD Key Loci in Yunnan_Rp_JCC9_2020. Compared to the RBD and Flynn protease cleavage site regions, the RBD region corresponds to amino acid sequences 414–505 of the SARS-CoV-2 S protein, and the Flynn protease cleavage site region corresponds to 67–-707. The red triangles indicate the key amino acid residues and the red circles indicate the Flynn protease cleavage sites. (**B**) Structure and characterization of S protein subunit homology model in Yunnan_Rp_JCC9 and Yunnan_MP_YJ3C/F_2020 from β CoVs. The JCC9 homology to a representative β CoVs S protein subunit modeling structure and characterization. RBD has been marked with a large red circle, the two deletion loops are marked with small red circles, and the Flynn protease cleavage site is marked with a blue arrow

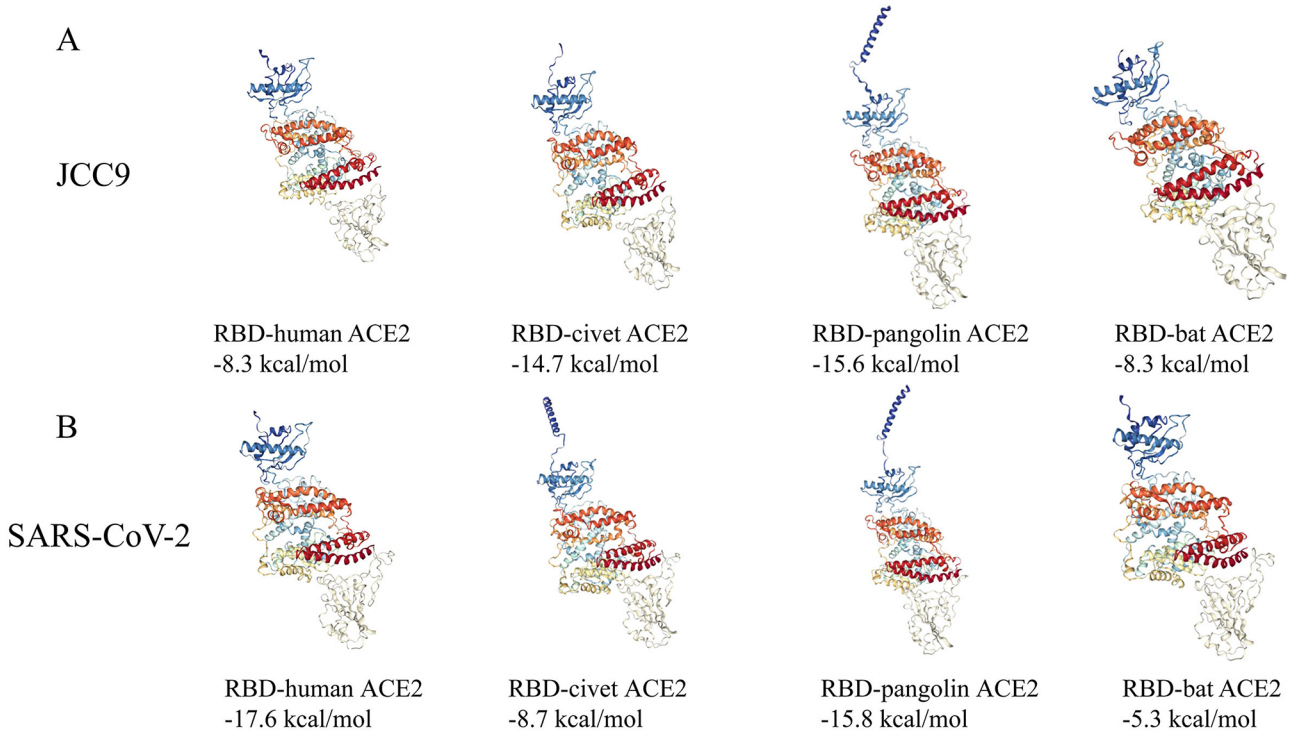

Fig. 6 Docking diagram of RBD, S protein, and related ACE2 protein. (**A**) Binding affinity of RBD of Yunnan_Rp_JCC9_2020 S protein with ACE2 of four species; (**B**) Binding affinity of RBD of SARS-CoV-2 S protein with ACE2 of four species

Conclusion

This study reveals that bats in Yunnan harbor a highly diverse array of CoVs, with unique recombination patterns and the potential for cross-species transmission. The S proteins of these CoVs are significantly different from that of SARS-CoV-2 and are unable to bind human ACE2, indicating a low likelihood of zoonotic transmission to humans. The discovery of these CoV strains suggests a spillover risk of cross-species transmission, which has important implications for public safety and health.

Although only 53 CoVs have been recorded and named by the International Committee on Taxonomy of Viruses, the genetic diversity of these viruses and recent research suggest that more undetected CoVs may exist in nature, and the vast majority have not been named and classified. This diversity in CoVs may lead to mutations and genome recombination during intraspecific transmission, which may lead to the emergence of CoVs capable of cross-species transmission. Bats and the CoVs they carry need monitoring to ensure the safety of people and livestock in Yunnan and the surrounding areas to prevent new and recurrent viral infectious diseases.

Abbreviations

CoV Coronavirus
RBD Receptor-binding domain
ICTV Committee on Taxonomy of Viruses

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04310-6.

Supplementary Material 1

Acknowledgements

Not applicable.

Author contributions

QL, YTH and BYH designed the research. XL participate in the field work. XSX lead the lab work. QL and BHW performed date analysis. BHW and QL performed final editing and revision. XSX and BHW performed as a laboratory supervisor, funding acquisition, and as corresponding author. All authors read and approved the final manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request. All the nucleotide sequences generated from this study have been deposited and are available in the GenBank database.The GenBank accession numbers for the genome sequence are OK287354.1 (BetaCoV_Yunnan_Rs_KMC6_2020), OK287355.1 (BetaCoV_Yunnan_Rp_JCC9_2020), OK287352.1 (Yunnan/HpJC8xc/2020_Bat), and OK287353.1 (Yunnan_Rs_CX18c_2020_Bat).

Declarations

Ethics approval and consent to participate

This study was written approved by the Institutional Ethical Committee of Kunming University of Science and Technology (Permit Number: 16048), Yunnan, China. Rare and protected animals were not involved.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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