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Identification of Diverse Alphacoronaviruses and Genomic Characterization of a Novel Severe Acute Respiratory Syndrome-Like Coronavirus from Bats in China

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ABSTRACT

Although many severe acute respiratory syndrome-like coronaviruses (SARS-like CoVs) have been identified in bats in China, Europe, and Africa, most have a genetic organization significantly distinct from human/civet SARS CoVs in the receptor-binding domain (RBD), which mediates receptor binding and determines the host spectrum, resulting in their failure to cause human infections and making them unlikely progenitors of human/civet SARS CoVs. Here, a viral metagenomic analysis of 268 bat rectal swabs collected from four counties in Yunnan Province has identified hundreds of sequences relating to alpha- and betacoronaviruses. Phylogenetic analysis based on a conserved region of the RNA-dependent RNA polymerase gene revealed that alphacoronaviruses had diversities with some obvious differences from those reported previously. Full genomic analysis of a new SARS-like CoV from Baoshan (LYRa11) showed that it was 29,805 nucleotides (nt) in length with 13 open reading frames (ORFs), sharing 91% nucleotide identity with human/civet SARS CoVs and the most recently reported SARS-like CoV Rs3367, while sharing 89% with other bat SARS-like CoVs. Notably, it showed the highest sequence identity with the S gene of SARS CoVs and Rs3367, especially in the RBD region. Antigenic analysis showed that the S1 domain of LYRa11 could be efficiently recognized by SARS-convalescent human serum, indicating that LYRa11 is a novel virus antigenically close to SARS CoV. Recombination analyses indicate that LYRa11 is likely a recombinant descended from parental lineages that had evolved into a number of bat SARS-like CoVs.

IMPORTANCE

Although many severe acute respiratory syndrome-like coronaviruses (SARS-like CoVs) have been discovered in bats worldwide, there are significant different genic structures, particularly in the S1 domain, which are responsible for host tropism determination, between bat SARS-like CoVs and human SARS CoVs, indicating that most reported bat SARS-like CoVs are not the progenitors of human SARS CoV. We have identified diverse alphacoronaviruses and a close relative (LYRa11) to SARS CoV in bats collected in Yunnan, China. Further analysis showed that alpha- and betacoronaviruses have different circulation and transmission dynamics in bat populations. Notably, full genomic sequencing and antigenic study demonstrated that LYRa11 is phylogenetically and antigenically closely related to SARS CoV. Recombination analyses indicate that LYRa11 is a recombinant from certain bat SARS-like CoVs circulating in Yunnan Province.

Coronaviruses (CoVs) in the subfamily *Coronavirinae* are important pathogens of mammalian and avian animals and currently compose four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (1). Members of *Alphacoronavirus* and *Betacoronavirus* are found exclusively in mammals, e.g., human CoV 229E, NL63, and OC43, and cause human respiratory diseases (2). A CoV is also the causative agent of severe acute respiratory syndrome (SARS), the first global human pandemic disease of the 21st century, which spread to 30 countries in five continents, resulting in >8,000 human cases with 774 deaths (3, 4). SARS CoV is a member of the *Betacoronavirus* genus and is largely distinct from previously known human CoVs OC43 and 229E (5–7). To identify the transmission source of SARS, large-scale animal screening was implemented in May 2003, and several strains of SARS CoVs were isolated from nasal and/or fecal swabs of six masked palm civets (*Paguma larvata*) and one raccoon dog (*Nyctereutes procyonoides*) collected from a wet market in Shenzhen retailing wild animals for exotic foods (8). Their full genome sequences were 99.8% identical to that of human SARS CoV, and therefore civets were deemed to be an animal reservoir of this virus (8). Further serological studies over a larger area revealed that only civets in the market were SARS seropositive, while farmed civets were seronegative, indicating that civets likely became infected from an unknown source in wet markets, not in the farming environment (9). Moreover, a comprehensive analysis of cross-host

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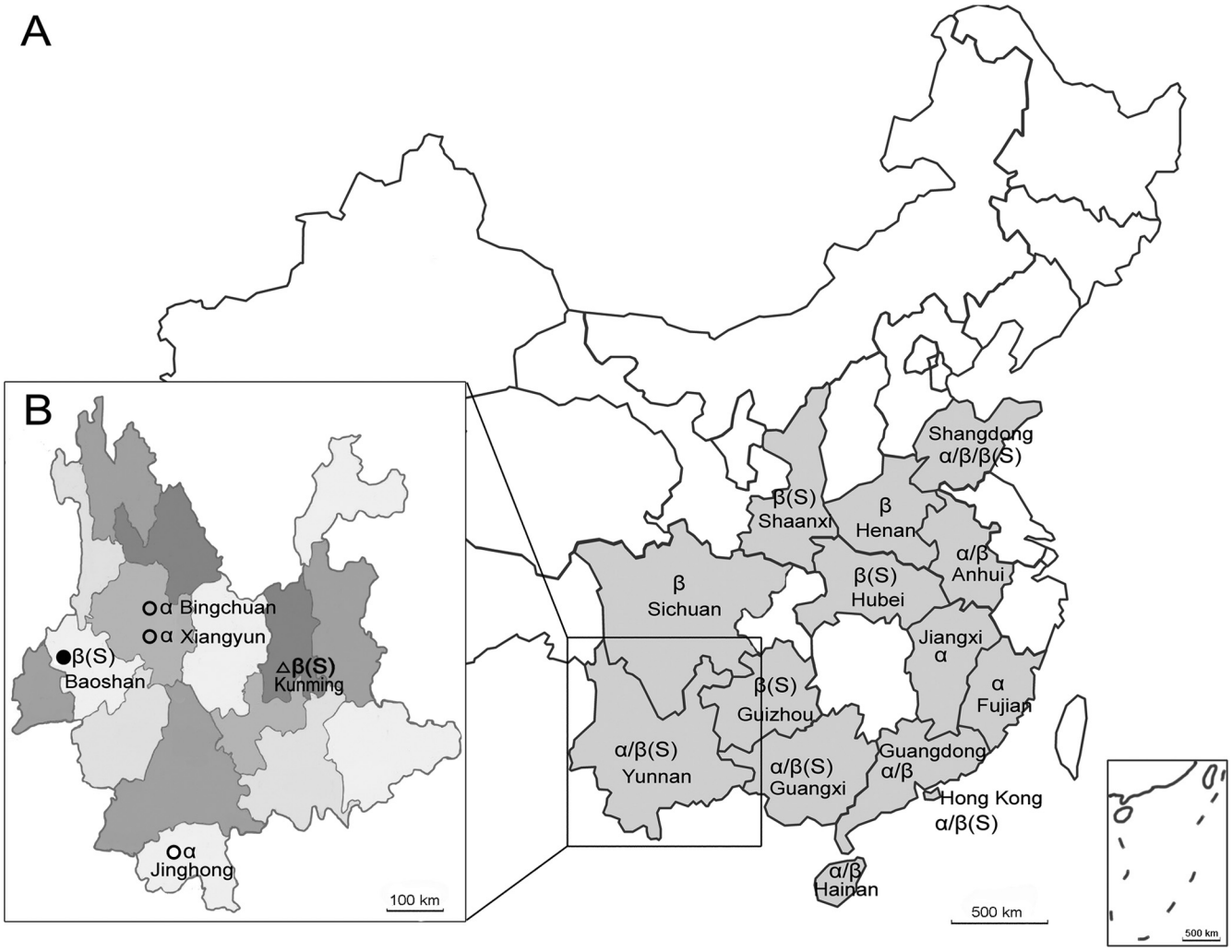

FIG 1 (A) Geo-distribution of bat CoVs in China (gray provinces); (B) locations of bat alphacoronaviruses (open circles) and SARS-like CoVs identified in our study (solid circle) and in Ge et al.'s study (open triangle) (29). α, alphacoronavirus; β, betacoronavirus; S, SARS-like CoV.

evolution between SARS CoVs in civets and humans indicated that civets might be spillover animals rather than the natural hosts of SARS CoV (10). In 2005, SARS-like CoVs sharing 87 to 92% nucleotide (nt) identity with SARS CoVs were identified in horseshoe bats (11, 12). These studies provided the first evidence that bats were the natural hosts of SARS CoVs. Since then, more SARS-like CoVs have been reported in several insect bat species in China, Europe, and Africa, but none have genomes identical to SARS CoVs (13–21). In particular, in these viruses, the key S1 domain of the S gene, responsible for receptor binding and determining host tropisms (22, 23), shared a sequence identity as low as 76 to 78% with SARS CoVs and had a deletion of 19 amino acids (aa) in the

TABLE 1 Details of rectal swabs from bats and positive number of bats detected by nested RT-PCR

Organism	Xiangyun			Bingchuan			Jinghong			Baoshan			Total		
	No.	No. (%) positive[a]	Clade[b]	No.	No. (%) positive[a]	Clade[b]	No.	No. (%) positive[a]	Clade[b]	No.	No. (%) positive[a]	Clade[b]	No.	No. (%) positive[a]	Clade[b]
Rhinolophus ferrumequinum	15	0		32	2 (9)	αC	30	1 (3)	αC				77	3 (4)	αC
Rhinolophus affinis										11	2 (18)	β	11	2 (18)	β
Rhinolophus hipposideros				4			4	1 (25)	αC	3			11	1 (9)	αC
Myotis daubentonii	22	2 (9)	αA/E	64	5 (8)	αA/D/E							86	7 (8)	αA/D/E
Myotis davidii	83	11 (13)	αA/B/D/E										83	11 (13)	αA/B/D/E
Total	120	13 (11)	αA/B/D/E	100	7 (7)	αA/C/D/E	34	2 (6)	αC	14	2 (14)	β	268	24 (9)	αA/B/C/D/E/β

[a] By nested RT-PCR.
[b] Clade the amplicons clustered into. α, *Alphacoronavirus*; β, *Betacoronavirus*; A, myotis bat coronavirus 5; B, miniopterus bat coronavirus 1; C, hipposideros bat coronavirus HKU10-like; D, myotis bat coronavirus HKU6-like; E, myotis bat coronavirus 4.

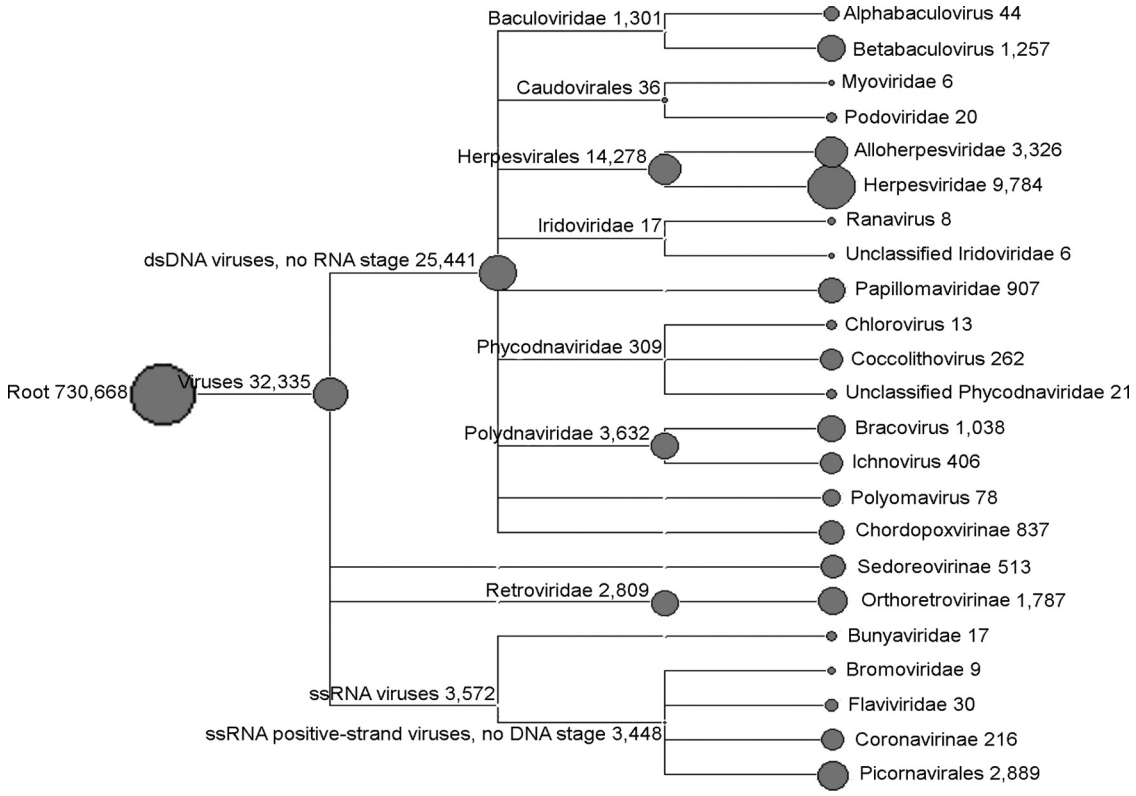

FIG 2 Taxonomic summary of viral reads with BLASTn ($E < 10^{-5}$) results exhibited in MEGAN 4. The number of reads in each taxonomic level is shown after the level name.

S gene receptor binding domain (RBD), which mediates human infection via binding to human angiotensin-converting enzyme 2 (ACE2) (11, 12, 24). Such key differences in the S gene between bat SARS-like CoVs and SARS CoVs determined their different host spectrums and made them unable to infect human and civets (25–27). Clearly, these known bat SARS-like CoVs are not the progenitors of human/civet SARS CoVs, and there remains to be identified an intermediate virus to bridge bat to human/civet transmission (24, 28). Recently, however, a novel SARS-like CoV (strain Rs3367) has been described which, so far, is more closely related to SARS CoVs than any previously reported bat SARS-like CoVs. Most importantly, it has been shown to use ACE2 receptor for cell entry, suggesting that it can cause direct human infection without an intermediate host (29). Here, we report another novel SARS-like CoV (LYRa11) identified from *Rhinolophus affinis* collected in Yunnan Province of China, which has high nucleotide and amino acid identities in its genome, similar to those of Rs3367, particularly in the RBD region. In addition, several clades of new alphacoronaviruses have been identified in *Rhinolophus* and *Myotis* spp.

MATERIALS AND METHODS

Ethics statement. The procedures for sampling of bats in this study were reviewed and approved by the Administrative Committee on Animal Welfare of the Institute of Military Veterinary, Academy of Military Medical Sciences, China (Laboratory Animal Care and Use Committee Authorization, permit number JSY-DW-2010-02). All live bats were maintained and handled according to the Principles and Guidelines for Laboratory Animal Medicine (2006), Ministry of Science and Technology, China.

Sample collection and preparation. In total, 268 adult bats were live captured with nets in 2011 in 4 counties/prefectures of Yunnan Province (Fig. 1). Within each county there was either a single sampling location or two adjacent sites. Bat details are shown in Table 1. All specimens were collected rectally using sterile swabs and immediately transferred to viral transport medium (Earle's balanced salt solution, 0.2% sodium bicarbonate, 0.5% bovine serum albumin, 18 μg/liter amikacin, 200 μg/liter vancomycin, 160 U/liter nystatin) and stored in liquid nitrogen prior to transportation to the laboratory, where they were stored at −80°C. All captured bats were released after sample collection.

Metagenomic analysis and RT-PCR screening. All specimens were pooled and subjected to viral metagenomic analysis as per our published method, using barcode primers for differentiation of sample species and locations (30). All sequences generated in one lane by Solexa sequencing (BGI) were subjected to BLASTn searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nonredundant database of GenBank, and all sequences with an E value of $<10^{-5}$ were imported into MetaGenome Analyzer v.4 (MEGAN) to determine their taxonomic classification (30). Sequences assigned to CoVs were used for further analysis. Nested reverse transcription (RT)-PCR primers targeting a 440-bp fragment of the RNA-dependent RNA polymerase (RdRp) gene were synthesized based on previous publications (31, 32). Total RNA of each rectal swab was extracted automatically using the RNeasy minikit (Qiagen) in a QIAcube (Qiagen). Reverse transcription was effected with the 1st cDNA synthesis kit (TaKaRa) according to the manufacturer's protocol. The cDNA was amplified using the PCR master mix (Tiangen) with the following PCR programs: 30 cycles (outer PCR) or 35 cycles (inner PCR) of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extending at 72°C for 40 s, with double-distilled water (ddH_2O) as a negative control. Positive PCR amplicons were ligated into pMD18T vector (TaKaRa) and used to transfect DH5α competent *Escherichia coli* (Tiangen). Six clones of each am-

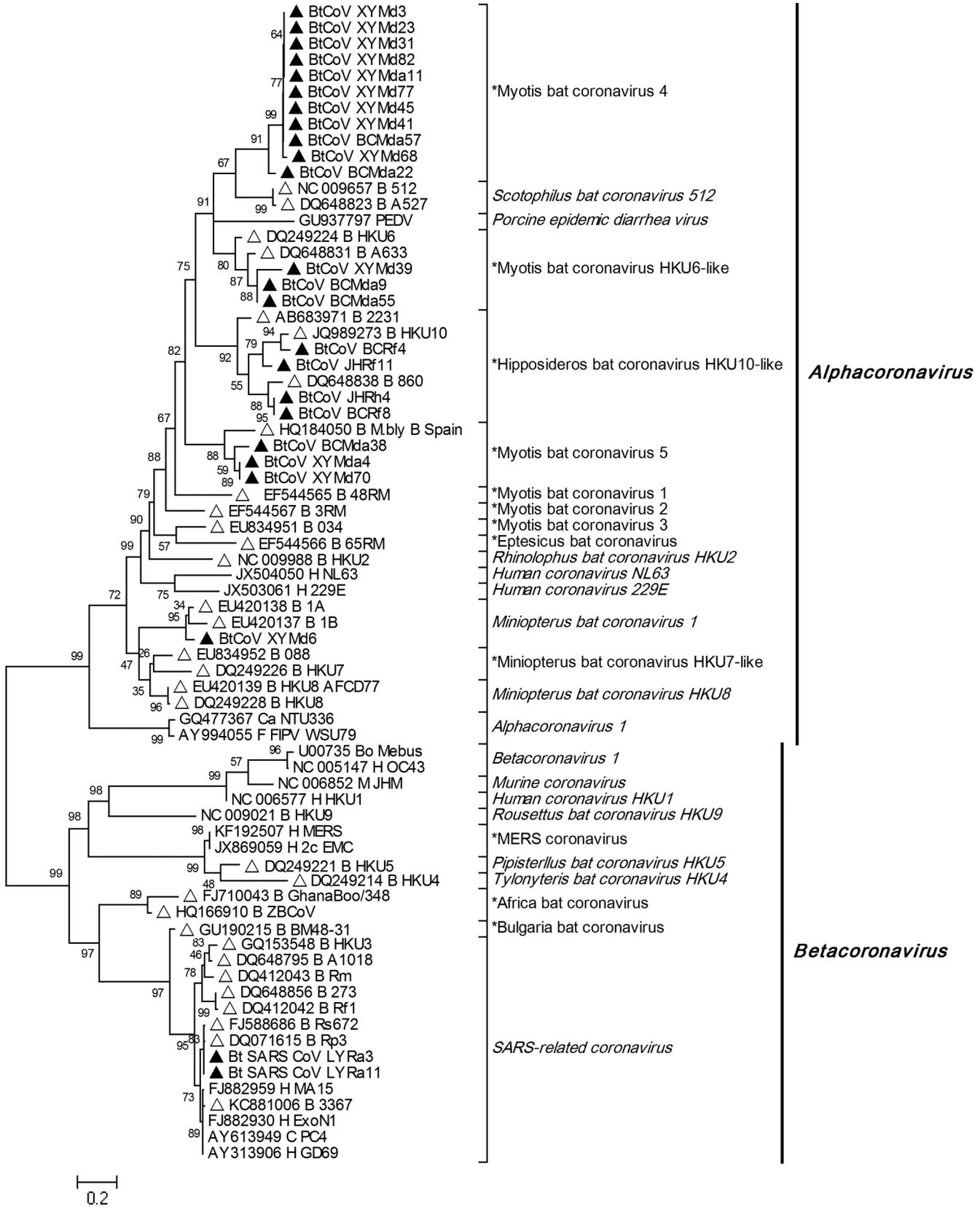

FIG 3 Phylogenetic analysis of RdRp amplicons obtained in this study and representatives of species in genera *Alphacoronavirus* and *Betacoronavirus* based on the maximum likelihood method. All sequences were classified into two groups: group *Alphacoronavirus* comprising 17 clades, and group *Betacoronavirus* comprising 10 clades. Clades containing approved species are in italics; clades containing unapproved novel species are marked with an asterisk. All amplicons in this study are marked as filled triangles, with previously reported bat CoVs as open triangles. Middle letters identify the viral host: H, human; C, civet; B, bat; Bo, bovine; M, murine; Ca, canine; F, feline.

TABLE 2 Comparison of full genomic lengths and ORF amino acid identities of SARS and SARS-like CoVs[a]

FL or ORF	LYRa11 length (aa)	Tor2		Rs3367		Rf1		Rp3	
		Length (aa)	% aa identity	Length (aa)	% aa identity	Length (aa)	% aa identity	Length (aa)	% aa identity
FL (in nt)	29,805	29,751		29,792		29,709		29,736	
1a	4,382	4,377	95.1	4,382	95.1	4,377	93.9	4,380	95.4
1b	2,628	2,641	98.9	2,628	99.0	2,628	98.6	2,628	98.9
S	1,259	1,255	89.6	1,256	89.9	1,241	79.0	1,241	81.1
3	274	274	91.3	274	91.6	274	81.5	274	89.1
4	NP	154	NA	114	NA	114	NA	NP	NA
E	76	76	98.7	76	98.7	76	94.8	76	98.7
M	221	221	97.7	221	95.2	221	95.5	221	95.9
7	63	63	95.3	63	91.7	63	89.1	63	87.5
8	122	122	94.3	122	94.1	122	91.1	122	93.5
9	44	44	91.1	44	90.5	44	93.3	44	91.9
10	NP	39	NA	NP	NA	122	NA	NP	NA
11	NP	84	NA	NP	NA	NP	NA	NP	NA
10b	121	NP	NA	121	81.1	NP	NA	121	79.5
N	422	422	97.9	422	97.8	421	95.5	421	97.9
13	98	98	96.0	98	93.7	97	82.7	97	86.7
14	70	70	94.4	70	92.6	70	84.5	70	91.5

[a] The accession numbers of Tor2, Rs3367, Rf1, and Rp3 are AY274119, KC881006, DQ412042, and DQ071615, respectively; FL, full genome sequence (nt); % aa identity shows amino acid sequence identity with LYRa11; NP, not present; NA, not available. The highest identities are shaded.

plicon were randomly picked for sequencing by the Sanger method in an ABI 3730 sequencer (Invitrogen). All strains in this study were named according to the following rules: the first two letters represent the sampling location, with the remaining letters identifying the host species and numbers referring to the sampling order.

Full genome sequencing. To obtain the full genome of LYRa11, 16 degenerate PCR primer pairs were designed using GeneFisher, based on human/civet SARS CoV and bat SARS-like CoV sequences available in GenBank, targeting almost the full length of the genome (sequences available upon request). For amplifying the terminal ends, 3′ and 5′ rapid amplification of cDNA ends (RACE) kits (TaKaRa) were employed. Viral cDNA was prepared as described above directly from positive samples and amplified using the Fast HiFidelity PCR kit (Tiangen). The amplicons were sequenced after blunt ligation into pZeroBack vector (Tiangen). Overlapping amplicons were assembled with SeqMan v.7.0 into full genomic sequences. Open reading frames (ORFs) of LYRa11 were determined by Vector NTI v.8, followed by comparison with those of other SARS CoVs and bat SARS-like CoVs.

Phylogenetic analysis of amplicons. All 440-bp-long amplicons were aligned with their closest phylogenetic neighbors in GenBank using ClustalW v.2.0. Representatives of different species in the genera *Alphacoronavirus* and *Betacoronavirus* as well as some unapproved species were included in the alignment. Phylogenetic and molecular evolutionary analyses were constructed by the maximum likelihood method using MEGA v.6 with the Tamura-Nei substitution model and a bootstrap value of 1,000 (33).

Morphological observation by electron microscopy. The positive swab was examined for viral particles of LYRa11 as per our previous description (34). Briefly, 100-μl swab suspensions were centrifuged at 120,000 × *g* for 3 h in an SW55Ti rotor (Beckman), and the resulting pellets were resuspended in 20 μl SM buffer (50 mM Tris, 10 mM $MgSO_4$, 0.1 M NaCl, pH 7.5) and directly negatively stained with 2% phosphotungstic acid for observation with a JEM-1200 EXII transmission electron microscope (JEOL).

S1 expression and antigenicity assay. To characterize the antigenic reactivity of S proteins of bat SARS-like CoVs with human SARS CoV antibody, S1 fragments of human SARS CoV BJ01 (AY278488) and bat SARS-like CoVs LYRa11 and Rp3 (DQ071615) were expressed as fusion proteins with enhanced green fluorescent protein (EGFP) in BHK-21 cells and subjected to Western blot analysis using human convalescent-phase serum from a SARS patient in 2003. Briefly, the S1 fragment of SARS CoV BJ01 (nt 3 to 2028 of the S gene) was amplified from pcDNA3.1-S. The corresponding S1 fragments of LYRa11 and Rp3 were amplified from the above-described cDNA and commercially synthesized (GenScript). Three S1 fragments were inserted into pEGFP-C1 (Clontech) between XhoI and BamH I restriction sites to construct three S1 expressing plasmids, pEGFP-BJ, pEGFP-LY, and pEGFP-Rp3. These three plasmids, along with pEGFP-C1 (as a control), were transiently expressed in BHK-21 cells using FuGENE HD transfection reagent (Promega). Total proteins were harvested 24 h posttransfection with M-PER mammalian protein extraction reagent (Thermo Scientific), and concentration was measured by the BCA protein assay kit (Tiandz). A total of 20 μg total protein was boiled in 2× protein loading buffer (Tiangen) for 10 min, separated on 10% SDS-PAGE, and transferred onto a nitrocellulose membrane (Millipore). The blocked membrane was then incubated with primary antibody mixture (SARS-convalescent human serum, rabbit anti-EGFP antibody [Beyotime], and 5% skimmed milk [vol/vol/vol = 1:1:1,000]) at 4°C overnight followed by a secondary antibody mixture (peroxidase-conjugated mouse anti-human antibody [ZSGB-Bio], IRDye 800CW goat anti-rabbit secondary antibody [LI-COR Biosciences], and 5% skimmed milk [vol/vol/vol = 3:5:15,000]) at room temperature for 2 h. The washed membrane was then scanned in an Odyssey infrared imaging system (LI-COR Biosciences) at 700-nm and 800-nm wavelengths to detect EGFP protein and then reacted with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and scanned using LAS-4000 Image Reader (Fujifilm) to detect S1 protein.

Recombination analysis. To detect possible recombination between SARS and SARS-like CoVs, the full-length genomic sequence of LYRa11 was aligned with selected human/civet SARS CoVs (Tor2, AY274119; BJ01, AY278488; SZ3, AY304486) and bat SARS-like CoVs (Rp3, DQ071615; Rf1, DQ412042; Rs672, FJ588686; Rm1, DQ412043; Rs3367, KC881006; B41, DQ084199; B24, DQ022305; Yunnan2011, JX993988; and HKU3, GQ153542) using ClustalW v.2.0. The aligned sequences were initially scanned for recombinational events using the Recombination Detection Program (RDP; version 4) with MaxChi and Chimaera methods using 0.6 and 0.05 fractions of variable sites per window, respectively (35, 36). The potential recombination events between LYRa11, Rs3367, Yunnan2011, and Rf1 suggested by RDP with strong *P* values ($<10^{-20}$) were investigated further by similarity plot and bootscan analyses using SimPlot v.3.5.1 (35–37). Maximum likelihood trees of four genomic re-

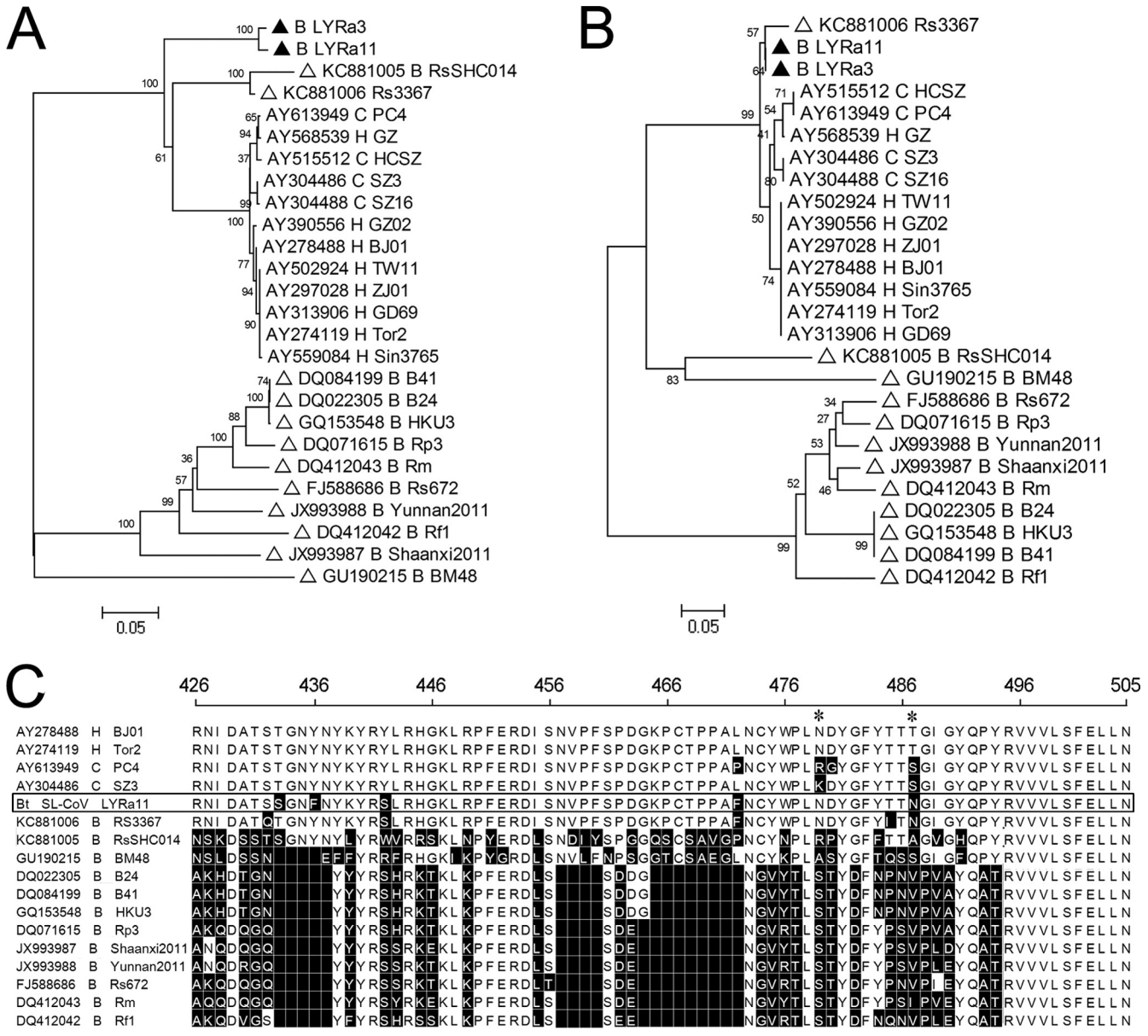

FIG 4 Characterization of S1 domains of SARS and SARS-like CoVs. (A) Phylogenetic analysis of entire S1 amino acid sequences based on the maximum likelihood method; (B) phylogenetic analysis of RBD amino acid sequences based on the maximum likelihood method; (C) sequence comparison of entire RBMs of SARS CoVs, LYRa11 (boxed), and other closely related bat SARS-like CoVs. The sequences of SARS-like CoVs in this study are marked as filled triangles, with other bat SARS-like CoVs as open triangles. Middle letters: H, human SARS CoV; C, civet SARS CoV; B, bat SARS-like CoV. Amino acid (aa) positions refer to SARS CoV Tor2 (AY274119). Critical residues that play key roles in receptor binding are indicated with asterisks.

gions generated by four breakpoints were constructed to illustrate the phylogenetic origin of parental regions. The breakpoint nucleotide locations are based on the LYRa11 genome.

Nucleotide sequence accession numbers. The raw data of Solexa sequencing have been deposited in Short Reads Archives (SRA) under accession number SRA100822. All amplicon sequences, the S gene of LYRa3, and the full genome of LYRa11 generated in this study have been deposited in GenBank under accession numbers KF569973 to KF569997. All accession numbers of sequences from GenBank used in this study are shown in the figures.

RESULTS

Viral metagenomic analysis. After Solexa sequencing and read annotations, a total of 730,668 useful reads with an average length of 141 nt were generated, and 32,335 of them (4.43%) were noted to viruses, including double-stranded DNA (dsDNA), dsRNA, and single-stranded RNA (ssRNA) viruses of mammalian, plant, insect, or bacterial origin (Fig. 2).

Alphacoronavirus in bats. Of 216 coronavirus-related sequences, 177 matched to the helicase gene of alphacoronavirus, with 70% nucleotide identities. Pan-CoV RT-PCR screening showed that 11% (13/120) of bats from Xiangyun, 7% (7/100) from Bingchuan, and 6% (2/34) from Jinghong were alphacoronavirus positive (Table 1). Although six amplicon clones of each sample were randomly chosen for sequencing, they showed almost 100% nucleotide identities, indicating that each sample carried only one CoV variant. All amplicons and their closest phylo-

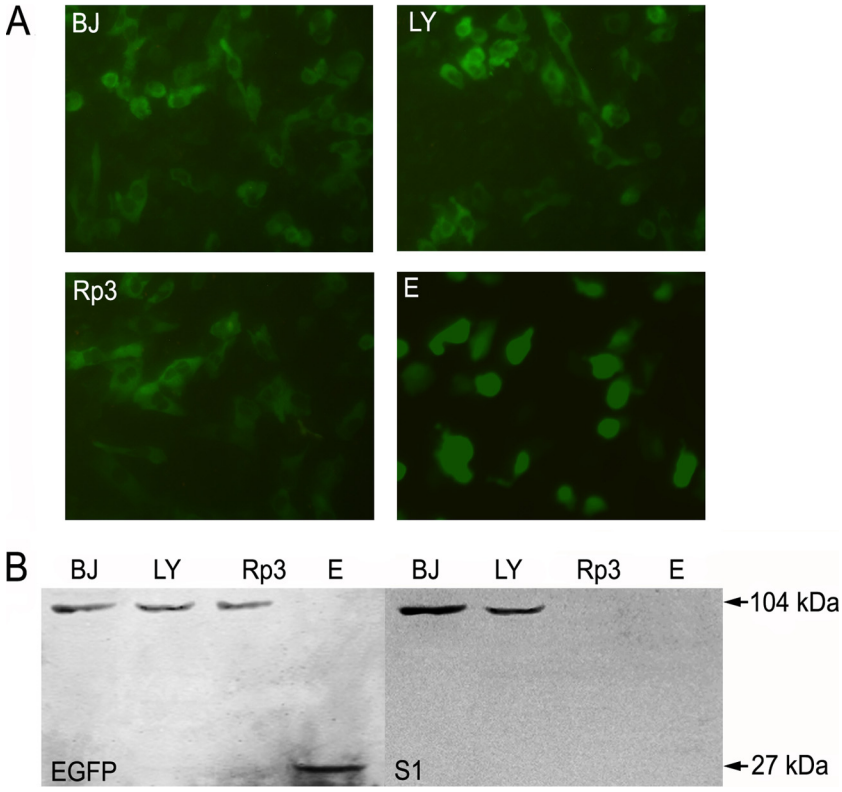

FIG 5 (A) Expression of EGFP-S1 fusion proteins in BHK-21 cells; (B) Western blot of expressed EGFP-S1 fusion proteins using rabbit anti-EGFP antibody (left) and SARS-convalescent human serum (right). The molecular masses are given on the right. BJ, LY, Rp3, and E, respectively, represent EGFP-S1 proteins of SARS CoV BJ01, bat SARS-like CoVs LYRa11 and Rp3, and EGFP control.

genetic neighbors from GenBank, along with representatives of 8 approved and several unclassified species in *Alphacoronavirus* (1), were aligned. As shown in Fig. 3, 22 amplicons grouped into five clades with 63 to 79% nucleotide identities between them and shared 80 to 91% identities with the viruses from Hong Kong, Guangdong, and Hainan in China, as well as from Spain (32, 38–40). Despite no individual carrying more than one clade, coinfection with different alphacoronaviruses did exist within a bat population in one location.

Betacoronavirus. The remaining 39 reads were annotated to ORF3 of SARS CoV with >91% nucleotide identities. Results of RT-PCR screening showed that 2/11 (18%) *Rhinolophus affinis* bats from Baoshan were positive for SARS-like CoVs, sharing 98.4% nucleotide identity in the RdRp gene with bat SARS-like CoV Rp3 which was detected in *Rhinolophus pearsonii* in Guangxi (12). These two amplicons shared 100% nucleotide identity (Fig. 3).

Full genomic sequence comparison. The complete genome of bat SARS-like CoV LYRa11 (KF569996) and the entire S gene of LYRa3 (KF569997) were obtained by sequencing several overlapping amplicons. The nucleotide identity of their complete S genes was 99%. The full genome of LYRa11 contained 29,805 nt, slightly larger than that of SARS CoVs and other bat SARS-like CoVs. It had 40.7% G+C content and the same 13 ORFs as strain Rp3 (Table 2). The full genome of LYRa11 shared ~91% nucleotide identity with those of SARS CoVs and the most recently reported SARS-like CoV Rs3367 (29), slightly higher than the highest identity with other bat SARS-like CoVs published previously (89%). LYRa11 ORFs were compared with human SARS CoV (Tor2) and three bat SARS-like CoVs (Rs3367, Rf1, and Rp3) (7, 12, 29). Table 2 shows that LYRa11 is more closely related to Tor2 and Rs3367 than to Rf1 and Rp3. In particular, its S gene shares >89% amino acid identity with Tor2 and Rs3367, significantly higher than ~80% amino acid identity with Rf1 and Rp3. However, ORF4 is absent from LYRa11, while it is present in Tor2 and Rs3367.

Genetic and antigenic characterization of the S1 domain. The S gene encodes a spike protein which is a type I transmembrane, class I fusion protein and composed mainly of distinct N-terminal (S1) and conserved C-terminal (S2) domains. The S1 domain contains the receptor binding domain (RBD), which mediates receptor binding of the virus to host cells and determines the host spectrum (2). Comparative analysis showed that the S1 amino acid sequence of LYRa11 shared high identity (83.3 to 84.0%) with those of human/civet viruses and Rs3367 but low identity (62.4 to 66.6%) with those of other bat SARS-like CoVs (Fig. 4A). Bat SARS-like CoV strain BM48, identified in *Rhinolophus blasii* from Bulgaria, was significantly distinct (15), sharing 63.6 to 65.0% identity with other bat SARS-like CoVs (Fig. 4A). An RBD amino acid sequence comparison of LYRa11 with human/civet viruses and bat SARS-like CoVs showed that LYRa11 shares 92.5 to 94.6% identity with human/civet SARS CoVs and 95.1% with Rs3367. In contrast, other bat SARS-like CoVs, including BM48, share 58.7 to 61.3% amino acid identities with human/civet viruses (Fig. 4B). Further alignment of amino acid sequences of the entire receptor binding motif (RBM), a core part of the RBD, showed a close

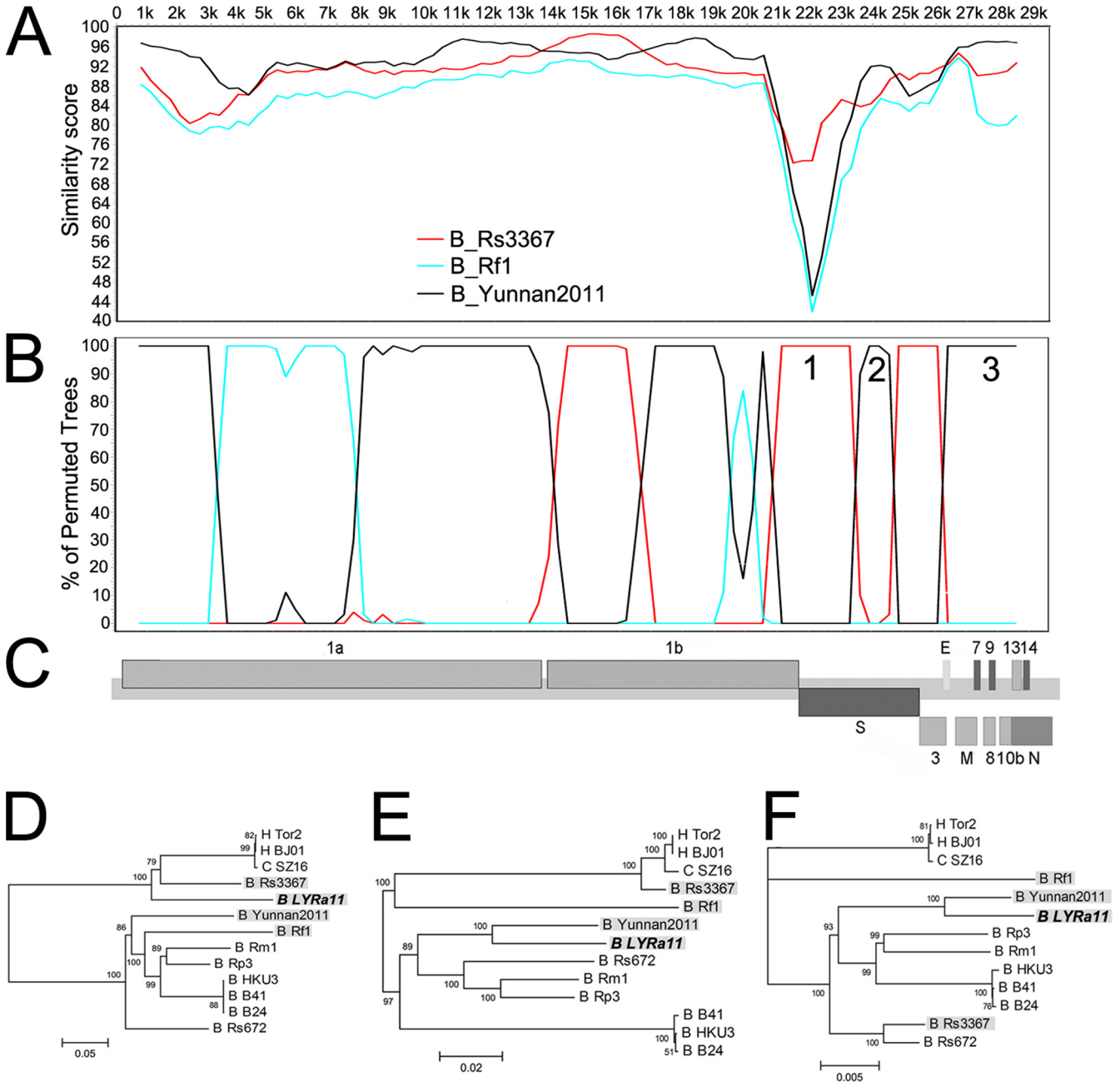

FIG 6 Recombination analysis of LYRa11 and other SARS-like CoVs. Similarity plots (A) and bootscan analyses (B) were conducted with LYRa11 as the query and bat SARS-like CoVs, including Rs3367, Yunnan2011, and Rf1, as potential parental sequences. (C) A gene map of LYRa11 is used to position breakpoints. Four breakpoints at nt 20968, 23443, 24643, and 26143 in the LYRa11 genome were detected, generating three recombinant fragments, 1, 2, and 3. Phylogenetic trees were constructed based on the three fragments (D to F, corresponding to fragments 1 to 3) by the maximum likelihood method. LYRa11 (bold italic), Rs3367, Yunnan2011, and Rf1 used in SimPlot are shaded. Leading capitals: H, human SARS CoV; C, civet SARS CoV; B, bat SARS-like CoV.

genetic relationship of LYRa11 to SARS CoVs and Rs3367 but a much less close relationship with other bat SARS-like viruses (Fig. 4C). European bat SARS-like CoV BM48 has a 4-residue deletion (aa 433 to 436) and differs considerably in amino acid composition from the RBM of human/civet and other bat viruses, while previously reported bat viruses have 17- or 18-residue deletions (aa 433 to 437, 457 to 468, and 472). In contrast, LYRa11 and Rs3367 have no deletion and have almost completely the same sequence as SARS CoVs. Of the 2 critical residues in RBM that play key roles in receptor recognition and enhancement of receptor binding (24, 41, 42), only 1 mutation, T487N, was observed in LYRa11 and Rs3367 compared with SARS CoVs (Fig. 4C).

Based on the results described above, to further characterize antigenic reactivity of LYRa11 with SARS CoV-specific antibody in comparison to that of SARS CoV BJ01 and the representative bat SARS-like CoV Rp3, S1 proteins of these three viruses were successfully expressed in BHK-21 cells (Fig. 5A) and then subjected to Western blot analysis (Fig. 5B). In Western blotting,

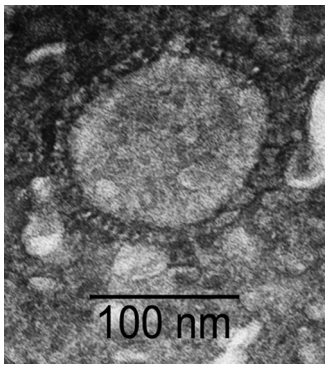

FIG 7 CoV-like particle considered to be LYRa11.

anti-EGFP antibody detected three EGFP-S1 proteins (104 kDa) as well as the EGFP control (27 kDa), indicating correct expression and effective transfer of the proteins to the membrane, while SARS-convalescent human serum reacted specifically with EGFP-S1 proteins of BJ01 and LYRa11, but not with those of Rp3 and the EGFP control. These results indicate that LYRa11 is antigenically more closely related to SARS CoV than the representative bat SARS-like CoV Rp3.

Recombination analysis. Due to its unique mechanism of RNA replication, the CoV genome has high-frequency RNA recombination between different strains (43). The potential recombination events between LYRa11 and the other 12 human/civet and bat SARS-like CoVs were initially predicted using the RDP program. Results showed that several fragments of LYRa11 were potential recombinants from Rs3367 and Yunnan2011 when LYRa11 was set as a query, and four breakpoints were detected in the LYRa11 genome, generating three recombinational fragments (Fig. 6B). Detailed analysis of LYRa11, Rs3367, Yunnan2011, and Rf1 using similarity plot and bootscan analysis of SimPlot supported the above-given prediction and generated three recombinant fragments covering nt 20968 to 23443 (fragment 1, including partial nsp16 and the entire S1 domain), nt 23444 to 24643 (fragment 2, partial S2 domain), and nt 26143 to the end (fragment 3, including the entire ORF E, M, 7, 8, 9, 10b, N) (Fig. 6A to C). Phylogenetic analyses based on these parental regions suggested that fragment 1 of LYRa11 was recombinant from lineages that had ultimately evolved into Rs3367 (Fig. 6D), while fragments 2 and 3 of LYRa11 were recombinants from lineages of Yunnan2011 (Fig. 6E and F).

Morphological observation. Pellets of ultracentrifuged rectal material were resuspended in SM buffer and examined by transmission electron microscopy (TEM). Three spherical enveloped viruslike particles of about 130 nm in diameter were observed, each in a separate field of vision. Surface spikes were apparent, but not with the typical coronavirus morphology (Fig. 7). To justify considering these as coronaviruses, therefore, the sample was subjected to RT-PCR for detection of CoV, respirovirus, morbillivirus, henipavirus, avulavirus, rubulavirus, and pneumovirus in *Paramyxoviridae* and influenza virus A in *Orthomyxoviridae* using published methods (44, 45). Results showed that the sample was positive only for coronavirus.

DISCUSSION

Following identification of the first bat CoV in 2005 (11, 12), further CoVs have been discovered in different bat species within China (summarized in Table 3 and Fig. 1). To date, CoVs have been found in 20 bat species within 4 families from 13 provinces and Hong Kong (11–14, 16, 20, 29, 38, 40). Among these bat species, 10 were in the family *Vespertilionidae*, 8 in *Rhinolophidae*, with one in each of *Molossidae* and *Pteropodidae*, suggesting that *Vespertilionidae* and *Rhinolophidae* comprise the main hosts of CoVs. Within the above-named families, the genera *Miniopterus* and *Myotis* were found to harbor only alphacoronaviruses, while bats from the genera *Pipistrellus*, *Tylonycteris*, and *Rhinolophus* harbored both alpha- and betacoronaviruses. Table 3 also shows that alphacoronaviruses have a wider host range and show greater genetic diversity in bats than betacoronaviruses. In addition to China, countries reporting bat alphacoronaviruses include Japan (46), the United States (47), Spain (32), Germany (48), and Ghana (21). Studies have shown that natural infection of various bats with various alphacoronaviruses is globally distributed, and bats are susceptible hosts of alphacoronaviruses. In addition, bats can also harbor diverse betacoronaviruses. According to the 9th Report of ICTV, since the first betacoronaviruses, i.e., SARS-like CoVs, were identified in bats, there have been 4 bat betacoronavirus species identified within the *Betacoronavirus* genus (1). More recently, some viruses related to Middle East respiratory syndrome (MERS) CoV have been discovered in different bat species in South Africa, Ghana, and Saudi Arabia (49–51). It is apparent that more betacoronaviruses will be identified in bat populations, although not as abundantly as alphacoronaviruses. All of the above indicate that alpha- and betacoronaviruses have different circulation and transmission dynamics in bat populations. Among the carriers of betacoronaviruses, which are most associated with emerging human infectious diseases, *Rhinolophus* spp. have been the main hosts found to harbor SARS-like CoVs in China and therefore have been considered to be the natural hosts of SARS CoVs (11, 12, 29). With the increasing number of SARS-like CoVs identified in bats since 2005, the host range of SARS-like CoVs has extended from *Rhinolophus* spp. to *Chaerephon* spp. in China and *Hipposideros* and *Chaerephon* spp. in Africa (13–21). Most SARS-like CoVs from non-*Rhinolophus* spp. show far greater genetic distance to SARS CoVs than those from *Rhinolophus* spp. This is especially true for viruses from Africa, which share less than 83% full genomic identities with SARS CoVs (17, 19, 21), suggesting that the circulation of SARS-like CoVs is restricted mainly to *Rhinolophus* spp. but with wide geo-locations.

Our attempt to amplify the full S gene of SARS-like CoVs from positive samples was successful, but amplification of the full S gene of alphacoronaviruses failed, possibly due to high sequence diversity as well as the limited sample amount. Instead, a 440-bp highly conserved region of the RdRp gene was amplified to construct the phylogenetic tree in the present study. This region is useful to analyze the diversity although cannot accurately determine the evolutionary status of CoVs (20). Using this region, 5 clades of alphacoronavirus were identified from 4 of 5 bat species in 3 of the 4 sampled locations, while betacoronavirus was from only one species in a single location (Table 1, Fig. 1), indicating that bats in Yunnan have an abundant diversity of CoVs. In the present study, SARS-like CoV was detected only in 2 of 14 bats in Baoshan. This sample size was too small to permit detection of alphacoronaviruses, but betacoronaviruses were not found in 254 bats from the other three locations, which supports the conclusion that there is a restricted distribution of betacoronaviruses in the bat population. Taken all together, these data show that circulation and

TABLE 3 Bat CoV distribution in China

Location	CoV type[a]																							
	Vespertilionidae												*Rhinolophidae*											
	Miniopterus pusillus	*Miniopterus schreibersii*	*Miniopterus magnater*	*Pipistrellus abramus*	*Pipistrellus pipistrellus*	*Myotis ricketti*	*Myotis daubentonii*	*Myotis davidii*	*Tylonycteris pachypus*	*Scotophilus kuhlii*	*Rhinolophus sinicus*	*Rhinolophus ferrumequinum*	*Rhinolophus pearsonii*	*Rhinolophus macrotis*	*Rhinolophus affinis*	*Rhinolophus hipposideros*	*Rhinolophus pusillus*	*Hipposideros pomona*	*Molossidae Chaerephon plicata*	*Pteropodidae Rousettus leschenaulti*
Hong Kong	α		α	α		α			α		α/β(S)									
Yunnan						α	α	α			β(S)	α			α/β(S)	α			β(S)	
Guangdong		α				α			β									α		α
Guangxi		α				α							β(S)				β(S)			β(S)
Fujian		α			β					α										
Hainan											β(S)									
Guizhou						α														
Jiangxi						α														
Anhui		α				α						β								
Hubei												β(S)		β(S)	β(S)					
Sichuan				β								β								
Henan				β								β								
Shandong											β(S)	β	α							
Shaanxi																	β(S)			

[a] α, alphacoronavirus; β, betacoronavirus. S in parentheses means only positive for SARS-like CoV.

transmission dynamics of alpha- and betacoronaviruses in bats are different.

The gene encoding spike protein S is the highly variable region within the CoV genome. The S protein consists mainly of S1 and S2 domains, the former containing RBM (aa 426 to 518) within RBD (aa 319 to 518). RBM, which determines the host tropism of CoV by binding cell receptor ACE2, is the most variable region (2, 24, 52). The RBM of SARS CoVs is a unique element which initiates viral infection by specifically binding to the ACE2 receptor of human and civet cells. In this process, two critical amino acid residues on RBM (479N and 487T) determine the efficiency of receptor binding since substitution of both abolishes viral binding to human ACE2, thereby abrogating the viral infection (41, 42). Substitution of either residue alone, however, has no significant impact on human ACE2 binding (24). Of significance is the fact that the S1 domain of bat SARS-like CoVs reported before 2013 has a very low nucleotide similarity to that of SARS CoVs (Fig. 4A and B), and there are several key deletions and mutations in their RBM (Fig. 4C) which distinguish them from SARS CoVs and make them incapable of infecting humans and civets via binding to ACE2 (11, 12, 24–27). In contrast, the LYRa11 in our study and Rs3367 reported recently (29) have high sequence identity with the S1 domain of SARS CoVs, showing almost exactly the same RBM sequence, with a single amino acid substitution among the two key sites determining host tropism (Fig. 4A to C). This makes Rs3367 able to use human ACE2 for potentially direct human infection and to be crossly neutralized by convalescent-phase sera of SARS patients (29). This property is probably shared by LYRa11 since its S1 domain, in addition to having very high sequence identify with Rs3367, is efficiently recognized by SARS-convalescent human serum (Fig. 5B). The clear serological and RBM sequence evidences show that LYRa11 is antigenically very close to SARS CoV. All results given above strongly suggest that LYRa11 and Rs3367 have the potential to directly infect civets and humans and, as gap-filling viruses between previously reported bat SARS-like and human SARS CoVs, might be deemed progenitors of SARS CoVs. In consideration of the 91% full genomic identity with Rs3367, lack of ORF4, and its isolation site being >350 km from Kunming, where Rs3367 was identified (Fig. 1B), the two viruses are distinct. It is reasonable to speculate that more LYRa11- or Rs3367-like viruses will be isolated from bats in the future.

Due to their unique mechanism of viral RNA replication, CoVs are prone to recombination during double infections (43). Previous studies have suggested that SARS CoVs were likely recombinants originating from strains Rp3 and Rf1 (13, 35), while Rs3367 recombined from lineages that had evolved into human/civet SARS CoV and bat SARS-like CoV Rs672 (29). Our analysis of the recombination events among LYRa11 and other SARS or SARS-like CoVs using RBD and SimPlot and the results suggest that LYRa11 is a recombinant descending from lineages that had ultimately evolved into Rs3367 and Yunnan2011, both of which were detected in Yunnan Province (16, 29). On this basis, it appears that SARS-like CoVs have been circulating in Yunnan bats for a long time, with obvious genetic recombination during virus transmission between bat species.

Our attempts to isolate infectious virus from the bat rectal samples failed, and only a few CoV-like particles were observed directly from rectal samples after ultracentrifugation. Reasons for believing these to be coronaviruses have been provided in Results, although the uncharacteristic morphology of the surface projections remains to be explained. Only a few petal-shaped spikes were observed on the surface of the virions (Fig. 7). Spikes, however, are comprised mainly of S1 and S2 domains, which, respectively, form the globular portion and the stalk (2). Studies have shown that S1 is not strongly associated with S2 and is easily detached from the virion during excessive freeze-thawing or ultracentrifugation (53–55); hence, the observation of only a few intact spikes in our preparation might be ascribed to damage or loss of S1.

In conclusion, Yunnan is a region with diverse alpha- and betacoronaviruses. Due to the ease of recombination between different strains, more diverse bat CoVs are likely to be identified in the future in this region, with important public health implications. The identification of bat SARS-like CoVs unable to infect human and civet before 2013 prompted speculation about the existence of SARS-like CoVs able to directly infect human and civets via wild animals. This speculation has ended with the identification of LYRa11 and Rs3367, which are gap-filling viruses and likely have the ability to directly infect humans. The discovery of LYRa11, together with Rs3367, has provided an important clue to the origin of SARS CoV from bat SARS-like CoVs and presents the strongest evidence so far that bats are the natural hosts of SARS CoVs.

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