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Genomic Characterization of Severe Acute Respiratory Syndrome-Related Coronavirus in European Bats and Classification of Coronaviruses Based on Partial RNA-Dependent RNA Polymerase Gene Sequences▽†

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Bats may host emerging viruses, including coronaviruses (CoV). We conducted an evaluation of CoV in rhinolophid and vespertilionid bat species common in Europe. Rhinolophids carried severe acute respiratory syndrome (SARS)-related CoV at high frequencies and concentrations (26% of animals are positive; up to 2.4 × 10^8 copies per gram of feces), as well as two *Alphacoronavirus* clades, one novel and one related to the HKU2 clade. All three clades present in *Miniopterus* bats in China (HKU7, HKU8, and 1A related) were also present in European *Miniopterus* bats. An additional novel *Alphacoronavirus* clade (bat CoV [BtCoV]/BNM98-30) was detected in *Nyctalus leisleri*. A CoV grouping criterion was developed by comparing amino acid identities across an 816-bp fragment of the RNA-dependent RNA polymerases (RdRp) of all accepted mammalian CoV species (RdRp-based grouping units [RGU]). Criteria for defining separate RGU in mammalian CoV were a >4.8% amino acid distance for alphacoronaviruses and a >6.3% distance for betacoronaviruses. All the above-mentioned novel clades represented independent RGU. Strict associations between CoV RGU and host bat genera were confirmed for six independent RGU represented simultaneously in China and Europe. A SARS-related virus (BtCoV/BM48-31/Bulgaria/2008) from a *Rhinolophus blasii* (Rhi bla) bat was fully sequenced. It is predicted that proteins 3b and 6 were highly divergent from those proteins in all known SARS-related CoV. Open reading frame 8 (ORF8) was surprisingly absent. Surface expression of spike and staining with sera of SARS survivors suggested low antigenic overlap with SARS CoV. However, the receptor binding domain of SARS CoV showed higher similarity with that of BtCoV/BM48-31/Bulgaria/2008 than with that of any Chinese bat-borne CoV. Critical spike domains 472 and 487 were identical and similar, respectively. This study underlines the importance of assessments of the zoonotic potential of widely distributed bat-borne CoV.

Coronaviruses (CoV; order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*) are enveloped viruses with unusually large plus-strand RNA genomes of 26 to 32 kb (1–3). According to a current proposal to the International Committee of Taxonomy of Viruses (ICTV) (8), they are classified into 3 genera containing viruses pathogenic for mammals (*Alpha-* and *Betacoronavirus*) and, foremost, birds (*Gammacoronavirus*). Alpha-, beta-, and gammacoronaviruses are also referred to as CoV groups 1, 2, and 3 (4, 21, 34). In humans, there are four prototypic human CoV (hCoV) that cause endemic and epidemic respiratory disease, including the human alphacoronaviruses 229E and NL63 and the human betacoronaviruses OC43 and HKU1 (25, 27, 55, 56). The severe acute respiratory syndrome (SARS) CoV is an additional human betacoronavirus that circulated temporarily in the human population during 2002 to 2003, resulting in an epidemic of SARS, with a 10% case fatality rate (14, 17, 38, 49). Other important CoV include the transmissible gastroenteritis virus of swine (TGEV) and the feline infectious peritonitis virus (FIPV), which together define the species *Alphacoronavirus* 1 in the genus *Alphacoronavirus*. This genus currently contains seven more species that include the porcine epidemic diarrhea virus (PEDV) species, as well as hCoV 229E and NL63, each of which defines an independent *Alphacoronavirus* species. The genus *Betacoronavirus* includes the species *Betacoronavirus* 1, defined by the bovine CoV, hCoV OC43, and the species murine hepatitis

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virus (MHV), as well as five more species, including one founded by the SARS-related CoV. The genus *Gammacoronavirus* contains a species defined by the avian infectious bronchitis virus (IBV), one defined by a Munia bird CoV, and a single beluga whale CoV. The current proposal for the classification of independent CoV species demands an amino acid identity of less than 90% in all of seven defined functional domains in the seven nonstructural proteins (NSP), NSP3, -5, and -12 to -16, encoded in CoV open reading frame 1ab (ORF1ab).

Within the spectrum of potential zoonotic viruses, CoV may be regarded as particularly significant because of their potential to be transmitted via respiratory or fecal-oral routes, resulting in a proven ability to cause major epidemics after host transition. Studies undertaken in search of the natural host of SARS CoV identified related CoV in rhinolophid bats (genus *Rhinolophus*, family *Rhinolophidae*, order *Chiroptera*) in China (28, 31). Further investigations identified members of the majority of known mammalian CoV species in bats, as well as several additional species that exclusively occurred in bats (20, 28, 31, 40, 41, 53, 58). While no gammacoronaviruses have been found in bats, bats may be natural reservoirs at least of most mammalian CoV, i.e., alpha- and betacoronaviruses. Anecdotally, bat CoV (BtCoV) appear to be linked to specific bats, with closely related CoV sequences being associated with the same bat species (or genus) in considerably distant geographic regions and with considerably distant CoV cooccurring in the same habitat in different bat species (20, 60). Unfortunately, the currently proposed criteria for a CoV species classification are difficult to meet when only field samples are available. Because BtCoV have never been isolated in cell culture and bat feces contribute a strong background of substances that inhibit reverse transcription PCR (RT-PCR), it is rather difficult to sequence full BtCoV genomes and to analyze seven different genes from the CoV ORF1ab, as formally required, to assign CoV species (8). Consequently, it has been difficult in the past to determine the significance and genealogies of novel CoV from bats. According to the concept that occupation of novel hosts and subsequent restriction to these hosts might result in CoV speciation, even a tentative and provisional delineation of species in field-collected BtCoV could greatly facilitate the prediction of associations between virus and host.

Due to recent implications that bats are infected with a number of highly pathogenic viruses, including viruses of the genera *Lyssavirus* and *Henipavirus* as well as the family *Filoviridae* (3, 12, 24, 29, 36, 51, 62), it is highly important to learn more about the ecology of bat-borne viruses. In contrast to most of the aforementioned viruses, which are carried by tropical fruit bats (*Megachiroptera*), the predominant hosts of mammalian CoV are insect- and blood-eating *Microchiroptera*, which are not limited to tropical climates. One paramount question therefore is that of the geographic extent of CoV reservoirs.

Better knowledge of taxonomic associations between bats and CoV could enable predictions of CoV geographic ranges, since habitats of bats are well characterized in many regions. In China, members of the genus *Rhinolophus* have been specifically associated with SARS-related CoV. We investigated in this study whether SARS-related CoV might also occur in Europe in bats of the same genus.

Five different species of rhinolophid bats inhabit large parts of Europe, including Spain, France, and the United Kingdom, as well as most countries of southeastern Europe (Fig. 1). All five European species share a small area of overlapping occurrences (sympatry) on the Balkan Peninsula and in the eastern Mediterranean, including in Turkey, Bulgaria, and Greece (7). In this study, we examined European rhinolophids in their area of sympatry for their CoV genetic range, prevalence, and seasonality. A surrogate classification criterion for tentative CoV species based on sequences of RNA-dependent RNA polymerases (RdRp) was developed, and the entire genome of a SARS-related CoV from European rhinolophids was determined. The resulting taxonomic associations between bats and CoV were used to derive predictions of potential geographic ranges of novel CoV. Finally, the complete spike protein reading frames of human SARS CoV, SARS-related BtCoV/Rp3, and the European SARS-related CoV were expressed on the surfaces of BHK-21 cells and stained with a set of anti-SARS CoV sera in order to determine antigenic relatedness within the extended SARS-related CoV species.

MATERIALS AND METHODS

Sample collection and processing. No bats were killed for this study. For all capturing and sampling of bats, permission was obtained from the Bulgarian Ministry of Environment and Water. Geographic coordinates of all seven sampling sites in Bulgaria are given in Table 1. Sampling was performed in the spring and autumn of 2008 (months of April and September). Bats were identified on site by trained field biologists. Additionally, mitochondrial DNA in representative fecal samples was amplified and sequenced for species confirmation as described previously (57). Bats were caught with mist nets within 2 to 5 meters' distance from the entrances of caves and tunnels. Captured bats were freed from nets immediately and put into cotton bags for 2 to 15 min to allow them to calm down before examination. Species, sex, reproductive status, forearm length, and body mass were determined. While being kept in bags, bats produced fecal pellets that were collected with clean tweezers and spiked into RNAlater RNA stabilization solution (Qiagen, Hilden, Germany) for sample processing. Duplicate sampling of individual bats was prevented by marking the toes of captured bats with nail polish upon first capture. Approximately 100 mg of bat feces was suspended in 500 μl of RNAlater (Qiagen, Hilden, Germany) and homogenized by vortexing. Of the suspensions, 50 μl was suspended into 560 μl of buffer AVL from the Qiagen viral RNA minikit and processed further according to the instructions of the manufacturer. The elution volume was 50 μl.

Analysis of samples by RT-PCR. Reverse transcription-PCR (RT-PCR) covering the family *Coronaviridae* was done as previously described, yielding amplicons of 455 bp located in the highly conserved motifs in the RdRp that encode LMGWDYPKCD and MMILSDDAV (10). Following nucleotide sequencing of initial amplicons, nested or real-time RT-PCR assays were designed for the same amplicon region (oligonucleotide sequences are available on request). The above-described 455-bp amplicons were extended toward the 5′ end of the genome by using downstream primers within the fragment and upstream primers based on alignments of phylogenetically related CoV from GenBank. The final size of sequences used for phylogenetic analyses was 816 bp. 5′ primer sequences for the different CoV lineages were SP3080, 5′-CTTCTTCTTTGCTCAGGATGGCAATGCTGC-3′; SP3195, 5′-ATACTTTGATTGTTACGATGGTGGCTG-3′; SP3374, 5′-CTATAACTCAAATGAATCTTAAGTATGC-3′; GrISP1, 5′-TTCTTTGCACAGAAGGGTGATGC-3′; and GrISP2, 5′-CTTTGCACAAAAAGGTGATGCWGC-3′.

***In vitro*-transcribed RNA standards.** Photometrically quantified *in vitro* RNA transcripts of all CoV lineages described in this study were generated as described previously (13). Briefly, the 455-bp amplicon from the initial screening assay was TA cloned, and plasmids were purified and reamplified with vector-specific oligonucleotides and finally *in vitro* transcribed using a T7 promoter-based Megascript kit (Applied Biosystems, Darmstadt, Germany). All six described real-time RT-PCR assays showed comparable lower limits of detection in the single-copy range. The *in vitro*-transcribed RNAs were used as calibration standards for virus quantification in bat fecal samples, as described previously (40).

Whole-genome sequencing. One bat fecal sample showing the highest virus concentration of all European SARS-related CoV samples was used for whole-

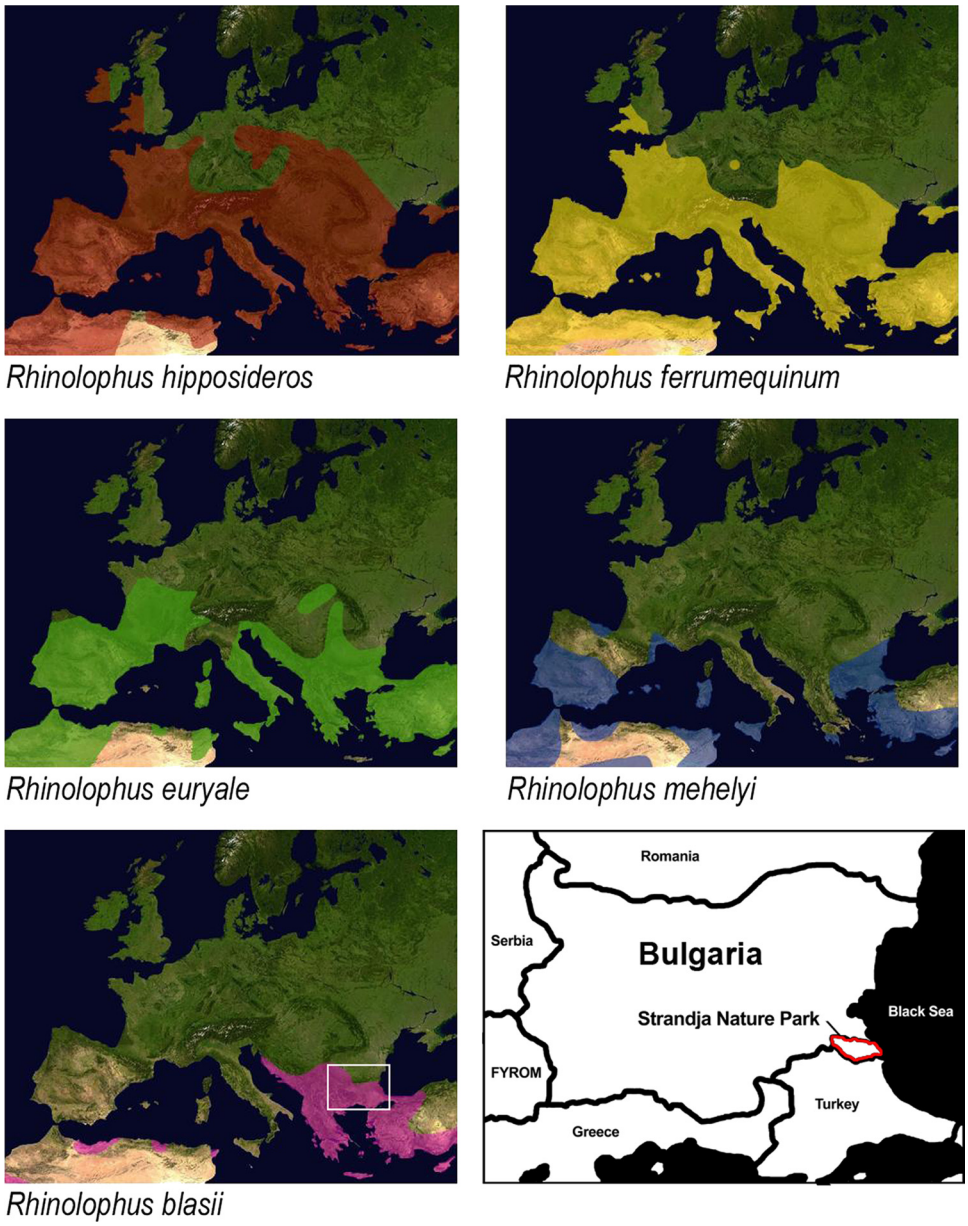

FIG. 1. Distribution of European rhinolophid bats. For each of the five rhinolophid bat species occurring in Europe, the area of distribution is depicted in separate colors (the underlying map of Europe was retrieved from http://visibleearth.nasa.gov). The map in the bottom left corner contains a white frame showing the position of the map in the bottom right corner, where the study region within Bulgaria is marked by a red outline. Plots are by A. Seebens and were adapted from reference 11a, with permission. FYROM, former Yugoslavian Republic of Macedonia.

genome sequencing. Based on related CoV genome sequences available in GenBank, sets of oligonucleotides that yielded small amplicons ranging from 150 to 300 bp at approximately every 5,000 bases along the SARS CoV genome were generated. Following nucleotide sequencing of amplicons, specific oligonucleotide primers were designed and long-range PCR was performed with the Expand high-fidelity (Roche, Penzberg, Germany) and Phusion DNA (New England Biolabs, Frankfurt, Germany) polymerase kits. Amplicons ranging from 4 to 6 kb were sequenced using primer walking. The 3′ end of the genome was determined using a 3′-RACE (rapid amplification of cDNA ends) strategy, based on gene-specific forward primers and inclusion of reverse primers by an oligo(dT)-driven reverse transcription step.

Phylogenetic analysis. Sanger sequencing of PCR products was done using dye terminator chemistry (Applied Biosystems, Darmstadt, Germany). Nucleic acid alignments were done based on amino acid codes by the BLOSUM algorithm in the MEGA 4 software package (52). A gap-free nucleotide alignment of 816 bp containing the novel viruses as well as CoV reference strains was generated (see Tables S1 and S2 in the supplemental material). Tree topologies were determined with MrBayes v3.1 (45). The analysis used a substitution model with GTR plus gamma plus an invariant site. Metropolis-coupled Markov chains (MCMC) of 6×10E6 iterations were sampled every 100 generations, resulting in 60,000 sampled trees. Two MCMC Monte Carlo (MCMCMC) analyses (1 cold chain and 3 heated chains each) were run in parallel, and results were compared and pooled. Convergence of chains was confirmed by the potential scale reduction factor statistic in MrBayes (19), as well as by visual inspection of each cold chain using TRACER (15).

Construction of expression plasmids. The construction of a protein gene of SARS CoV spike (CUHK-W1) in pCG1 was described previously (46). The spike gene of BtCoV/Rp3/*Rhinolophus pearsoni* [Rhi pea]/China/2004 was recloned from the plasmid pcDNA3.1-Rp3S by inserting the BamHI/XbaI-digested insert into the pCG1 vector (kindly provided by Roberto Cattaneo, Mayo Clinic Roch-

TABLE 1. Bat species tested for CoV and PCR assay positivity rates

Family	Species	No. of bats	No. of animals positive for indicated virus lineage (detection %)							Location (sampling season)[a]
			SARS related	HKU2 related	BB98-15	HKU7 related	HKU8 related	BtCoV1A related	BNM98-30	
Rhinolophidae	*Rhinolophus euryale*	243	78 (32.1)	8 (3.3)	25 (10.3)					BG1, BG2 (A), BG3, BG4, BG5 (A), BG7 (A)
	Rhinolophus blasii	82	13 (15.9)	3 (3.7)	7 (8.5)					BG1, BG2 (A), BG3, BG4, BG5 (A), BG7 (A)
	Rhinolophus ferrumequinum	45	6 (13.3)		8 (17.8)					BG1, BG2 (A), BG3 (A), BG4 (A), BG5, BG6 (A), BG7 (A)
	Rhinolophus mehelyi	13	4 (30.8)		3 (23.1)					BG1 (A), BG4
	Rhinolophus hipposideros	6								BG3 (A), BG5 (S)
Vespertilionidae	*Miniopterus schreibersii*	38				14 (36.8)	14 (36.8)	18 (47.4)		BG5 (A)
	Myotis bechsteinii	32								BG1, BG2 (A), BG3 (A), BG4, BG5 (S), BG7 (A)
	Barbastella barbastellus	12								BG2 (A), BG3 (A), BG6 (A)
	Myotis daubentonii	7								BG2 (A), BG3 (A), BG4 (A), BG7 (A), BG6 (A)
	Myotis emarginatus	5								BG3 (A), BG2 (A)
	Myotis myotis	3								BG2A)
	Nyctalus leisleri	3							1 (33.3)	BG7 (A)
	Pipistrellus pygmaeus	2								BG3 (A)
	Myotis alcathoe	2								BG2 (A), BG4 (S)
	Plecotus auritus	2								BG2 (A)
	Myotis nattereri	1								BG5 (S)
	Myotis mystacinus	1								BG4 (A)
	Myotis oxygnatus	1								BG5 (A)
	Myotis capaccini	1								BG5 (S)
Total		499	101 (20.2)	11 (2.2)	43 (8.6)	14 (2.8)	14 (2.8)	18 (3.6)	1 (0.2)	

[a] Samples were collected in both sampling seasons, spring (S) and autumn (A), unless a parenthetical season abbreviation follows the sampling site, in which case the samples were collected in that season. Sampling site coordinates (GPS map reference WGS84) were (country, type of habitat, geographic coordinates) as follows: BG1, Bulgaria, Elenas Cave, 42°9′6.0″N, 27°25′6.0″E; BG2, Bratanova Cave 42°0′21.0″N, 27°25′21.3″E; BG3, Bulgaria, Big Vupa, 42°9′32.0″N, 27°30′45.0″E; BG4, Bulgaria, Kaleto Cave 42°9′7.0″N, 27°21′28.0″E; BG5, Bulgaria, Roman Horse Cave, 42°5′30.7″N, 27°12′31.3″E; BG6, Malko Tarnovo Water Tunnel, 42°0′4.4″N, 27°31′21.1″E; and BG7, Bulgaria, Maharata Cave Area, 42°4′N, 27°46′E.

ester, Rochester, MN), to give pCG1-Rp3-S. The full-length S gene of European SARS-related BtCoV/BM48-31/*Rhinolophus blasii* [Rhi bla]/Bulgaria/2008 was cloned by PCR from cDNA prepared from original fecal material. Primers contained BamHI and XbaI restriction sites (forward, 5′-TTGGATCCATGAA ATTTTTGGCTTTTCTCTGTCTTCTTGGC-3′, reverse, 5′-TTTCTAGATTA TGTGTAATGTAACTTCACTCCAG-3′). The PCR product was purified using a PCR purification kit (Qiagen, Hilden, Germany) and digested overnight with BamHI and XbaI, and the digest was inserted into the vector pCG1 to yield pCG1-BtCoV/BM48-31-S. The open reading frame was deposited in GenBank (accession no. GU190215). For construction of a carboxy-terminal FLAG-tagged BtCoV/BM48-31/Rhi bla/Bulgaria/2008 spike protein, the reverse primer 5′-TT TCTAGATTACTTGTCGTCGTCGTCCTTGTAGTCTGTGTAATGTAACT TCACTCCAGTAAGCAC-3′ was used instead.

Immunofluorescence. BHK-21 cells grown on 12-mm-diameter coverslips were transfected with 1 μg of the respective plasmid DNA and 2 μl of Lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany), followed by incubation at 37°C for 24 h. The cells were fixed with 3% paraformaldehyde for 20 min at room temperature and permeabilized with 0.2% Triton X-100 –phosphate-buffered saline for 5 min. The spike proteins were detected with the antibodies indicated in the legend to Fig. 6 in phosphate-buffered saline containing 1% bovine serum albumin, followed by incubation with a fluorescein isothiocyanate-conjugated secondary antibody. Fluorescence was visualized with a Zeiss Axioplan 2 microscope.

Virus isolation attempts. Based on prior findings (18, 54), isolation of virus was attempted from feces suspended in RNAlater solution. Vero and CaCo2 cells were used, as well as primary cells from colon, lung, and kidney of *Myotis nattereri*, *Eidolon helvum*, and *Rousettus aegyptiacus* bats (unpublished data). No cytopathic effect was observed, and no virus growth was seen by RT-PCR, despite repeated trials (data not shown).

Statistical analyses. All analyses were performed with EpiInfo 3.5.1 (CDC, Atlanta, GA) and with SPSS 17 (SPSS, Munich, Germany).

Nucleotide sequence accession numbers. All sequences were submitted to GenBank under accession numbers GU190215 to GU190248 (see Table S2 in the supplemental material). GU190215 represents the full-genome sequence of the Bulgarian SARS-related bat coronavirus BtCoV/BM48-31/Rhi bla/Bulgaria/2008.

RESULTS

To obtain a comprehensive sample of European rhinolophid species, bats were studied in Strandja Park Sanctuary, Bulgaria, located in the area of sympatry of all five European rhinolophid species (Fig. 1) (2, 7). Bat feces were first tested by CoV broad-range RT-PCR (10) and sequenced. Detected viruses pertained to five major alphacoronavirus clades and one betacoronavirus. For each lineage, BLAST searches were done with default BLASTn settings to obtain alignments with closest phylogenetic neighbors. Redundant sequences were eliminated, and alignments were used to design lineage-specific RT-PCRs in order to increase detection sensitivity. Retesting increased the number of positive samples in the collection approximately 6-fold (202 RT-PCR products from 499 animals) (Table 1).

To obtain reliable resolution in phylogenetic analyses, we found earlier that it was insufficient especially for the betacoronaviruses to compare sequences of just the ~440-bp universal CoV RT-PCR fragment that is generally used in ecological investigations (10, 40, 59). Lineage-specific combinations of nested RT-PCR primers were therefore designed, and an 816-bp fragment of the 5′-terminal RdRp (NSP12) gene was successfully generated for representatives of all CoVs found in this study. Results of a Bayesian phylogenetic analysis based on 816-bp RdRp fragments are shown in Fig. 2.

All viruses with completed 816-bp RdRp gene sequences are listed along with their hosts in Table S2 in the supplemental material. In summary, three *Alphacoronavirus* clades occurred

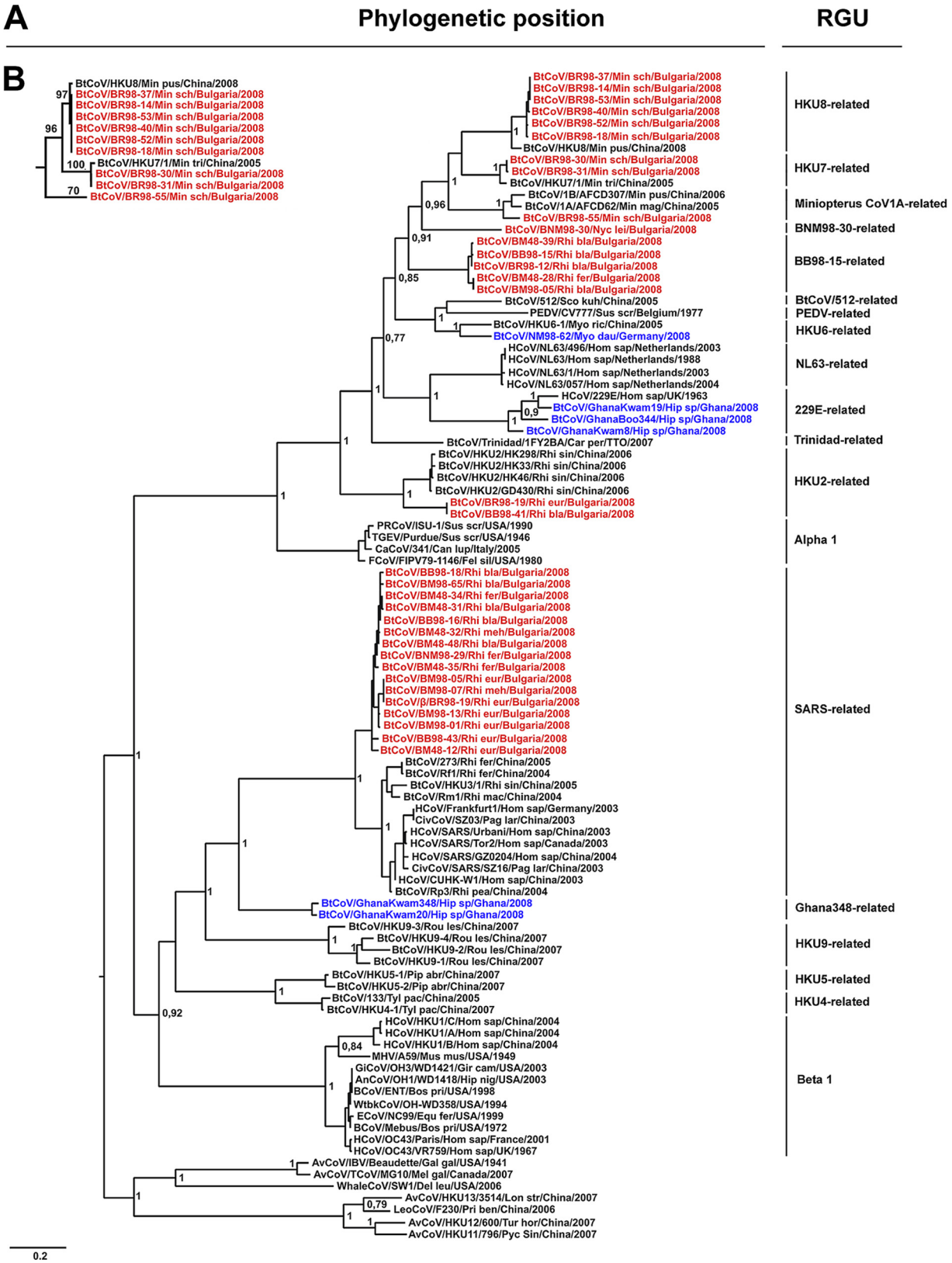

FIG. 2. RdRp-based phylogeny including novel bat coronaviruses. (A) Bayesian phylogeny of CoV on an 816-bp gap-free alignment of a fragment of the RNA-dependent RNA-polymerase (RdRp) gene corresponding to nucleotides 14781 to 15596 in SARS CoV strain Frankfurt 1 (GenBank accession no. AY291315). Analysis was done with MrBayes v3.1 (53). For clarity of presentation, only posterior probability values above 0.65 are shown and values at crown positions were removed. Novel European bat coronaviruses from this study are shown in red type. Additional European and African bat coronaviruses described previously by our group are shown in blue type. Taxa are named according to the following

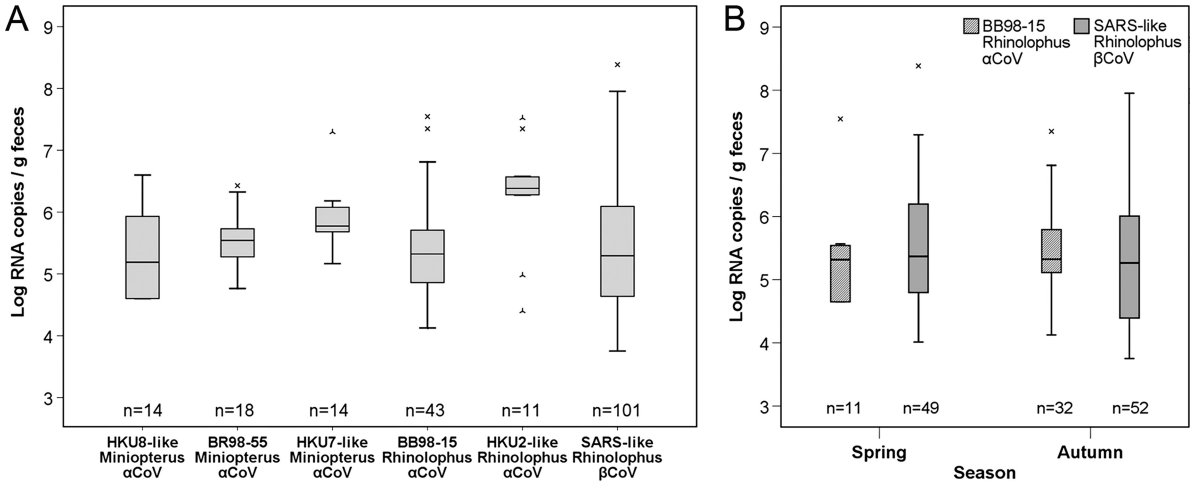

FIG. 3. Coronavirus shedding and seasonality. (A) Numbers of genome RNA copies per gram of feces of bats are shown for *Miniopterus* alphacoronaviruses and *Rhinolophus* alpha- and betacoronaviruses. (B) Virus concentrations of a *Rhinolophus* SARS-like betacoronavirus and a *Rhinolophus* alphacoronavirus are shown separately for each sampling season. Boxes show medians (horizontal lines) and interquartile ranges (box lengths). Whiskers represent extensions of the 25th or 75th percentiles by 1.5 times the interquartile range. Datum points beyond the whisker range are considered outliers and extreme values and are marked as x's and inverted y's, respectively.

exclusively in *Miniopterus* bats, one related to BtCoV/HKU7/1/*Miniopterus tristis* [Min tri]/China/2005, one related to BtCoV/HKU8/*Miniopterus pusillus* [Min pus]/China/2008, and one related to *Miniopterus* BtCoV-1A (Fig. 2A). Thirteen of these 38 *Miniopterus* bats were either doubly or triply infected. One unique alphacoronavirus (BtCoV/BNM98-30/*Nyctalus leisleri* [Nyc lei]/Bulgaria/2008) was detected in *Nyctalus leisleri*. Two *Alphacoronavirus* clades were detected in bats of the genus *Rhinolophus*, one related to BtCoV/HKU2/HK46/*Rhinolophus sinicus* [Rhi sin]/China/2006 and one unique lineage represented by BtCoV/BB98-15/Rhi bla/Bulgaria/2008.

SARS-related CoV, divided into two monophyletic clades, occurred exclusively in *Rhinolophus*, one of which preferentially occurs in Mediterranean horseshoe bats (*Rhinolophus euryale*), the other in *Rhinolophus blasii*, *Rhinolophus ferrumequinum*, and *Rhinolophus mehelyi* (Fig. 2A). Table 1 summarizes detection rates for all viruses. It should be mentioned that SARS-related CoV were detected at a significantly higher frequency than any alphacoronavirus (26% of 389 *Rhinolophus* bats, $P < 0.001$, $\chi^2 = 84.48$). Double infections occurred in 24 of 389 rhinolophids (6.2%), while two rhinolophids were triply infected (0.5%). All multiple infections included the SARS-related CoV.

In order to determine virus concentrations in fecal samples, specific real-time RT-PCRs with *in vitro*-transcribed RNA quantification standards were established for each of the above-described CoV clades. As shown in Fig. 3, there were no discernible differences in virus concentrations between clades as well as between sampling seasons (spring and autumn). Maximal concentrations were observed for the SARS-related CoV, reaching up to 2.4×10^8 copies per gram of feces.

Classification of CoV. Except for the SARS-related CoV described below, it was impossible to sequence larger genome portions necessary for species classification (8). To obtain a surrogate estimation of genetic taxonomical limits, distance matrices covering the 816-bp RdRp gene fragment were calculated for all available CoVs and summarized as shown in Fig. 4. In the nucleotide-based analysis, distances within and between potential taxonomic units were not clearly separated (Fig. 4A and B). At the amino acid level, however, distances always exceeded 32% between established CoV genera (Fig. 4C and D). Established *Alphacoronavirus* species were at least 5.9% distant from each other (Table 2). This also included BtCoV/HKU6, which has not been fully sequenced and is therefore not formally accepted as a CoV species (20, 60). *Betacoronavirus* species were always at least 6.3% distant from each other (Table 2).

In order to classify those CoV described here as well as in our recent studies on CoV in African and northern German

pattern: identification code/strain or isolate/typical host/country/collection year. Min pus, *Miniopterus pusillus*; Min sch, *Miniopterus schreibersii*; Min tri, *Miniopterus tristis*; Min mag, *Miniopterus magnater*; Nyc lei, *Nyctalus leisleri*; Sco kuh, *Scotophilus kuhlii*; Sus scr, *Sus scrofa*; Myo ric, *Myotis ricketti*; Myo dau, *Myotis daubentonii*; Hom sap, *Homo sapiens*; Hip sp, *Hipposideros* sp.; Car per, *Carollia perspicillata*; Rhi sin, *Rhinolophus sinicus*; Rhi eur, *Rhinolophus euryale*; Can lup, *Canis lupus familiaris*; Fel sil, *Felis silvestris*; Rhi bla, *Rhinolophus blasii*; Rhi fer, *Rhinolophus ferrumequinum*; Rhi meh, *Rhinolophus mehelyi*; Rhi mac, *Rhinolophus macrotis*; Pag lar, *Paguma larvata*; Rhi pea, *Rhinolophus pearsoni*; Rou les, *Rousettus leschenaulti*; Pip abr, *Pipistrellus abramus*; Tyl pac, *Tylonycteris pachypus*; Mus mus, *Mus musculus*; Gir cam, *Giraffa camelopardalis*; Hip nig, *Hippotragus niger*; Bos pri, *Bos primigenius*; Gal gal, *Gallus gallus*; Mel gal, *Meleagris gallopavo*; Del leu, *Delphinapterus leucas*; Lon str, *Lonchura striata*; Pri ben, *Prionailurus bengalensis*; Tur hor, *Turdus hortulorum*; Pyc sin, *Pycnonotus sinensis*. The right-hand column shows a classification of clades into RdRp-based grouping units (RGU). Hosts and GenBank accession numbers of all viruses are listed in Tables S1 and S2 in the supplemental material. (B) Distance-based phylogeny (neighbor-joining algorithm in MEGA) of the HKU7- and HKU8-related RGU confirming the monophyly of European and Chinese representatives in both clades.

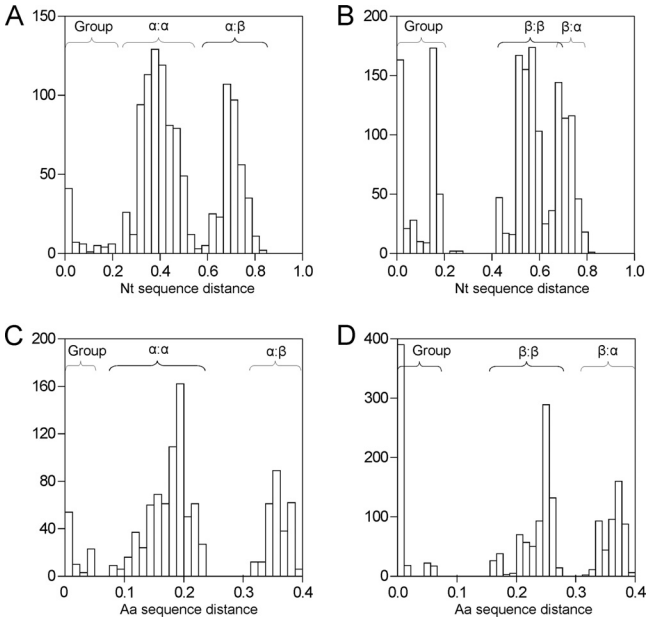

FIG. 4. Distribution of pairwise divergence scores between coronaviruses. Jukes-Cantor corrected nucleotide sequence distances among members of the genus *Alphacoronavirus* (A) and *Betacoronavirus* (B); uncorrected amino acid percentage distances among alphacoronaviruses (C) and betacoronaviruses (D). The *y* axis indicates the number of pairwise sequence comparisons. Braces indicate pairwise distances within groups, within a genus, and between genera. Pairwise distances between coronaviruses were calculated with MEGA 1.0 (62). Seven betacoronaviruses from different groups were used for comparison with all alphacoronaviruses. Similarly, nine alphacoronaviruses were used for comparison with all betacoronaviruses. The distribution of pairwise distances was plotted with SYSTAT 11.

bats (20, 40), amino acid distances of the novel viruses from related reference species and phylogenetic neighbors were calculated as listed in Table 3. In a first approach, the abovementioned amino acid distance criteria were used to classify viruses into RdRp grouping units (RGU) according to distances between established species (Table 2). However, it became clear that the two prototype viruses defining the HKU8 species, i.e., BtCoV/HKU8/Min pus/China/2008 and BtCoV/HKU7/1/Min tri/China/2005 (6), were each represented by closely related European viruses that in turn were separated from each other by the same distance as that between BtCoV/HKU8 and BtCoV/HKU7. This independent relationship was strongly supported by Bayesian-likelihood-based phylogeny (Fig. 2A) and by distance-based phylogeny using the neighbor-joining method (Fig. 2B). Because of the paraphyly of both the European and Chinese representatives of the HKU8 species, the separation criterion for RGU within the genus *Alphacoronavirus* was reduced to a 4.8% amino acid distance. This corresponded to the separation limit between BtCoV/HKU8 and BtCoV/HKU7, yielding two independent RGU within the formally defined BtCoV/HKU8 species. The following criteria for defining separate RGU in mammalian CoV were thus adopted: a >4.8% amino acid distance in the analyzed 816-bp fragment for alphacoronaviruses and a >6.3% amino acid distance for betacoronaviruses.

Using these criteria, two novel RGU were defined in the genus *Alphacoronavirus*, represented by BtCoV/BB98-15/Rhi bla/Bulgaria/2008 and BtCoV/BNM98-30/Nyc lei/Bulgaria/2008 (refer to the right-hand column in Fig. 2A for RGU designations). Another novel RGU was defined for a virus in direct phylogenetic relationship to SARS CoV represented by BtCoV/GhanaKwam20/*Hipposideros* sp. [Hip spe]/Ghana/2008, which was described previously to occur in Ghanaian *Hipposideros* bats by our group (40).

Six more viruses were determined to be extensions of six existing RGU, as shown in Fig. 2A (related to HKU8, HKU7, *Miniopterus* BtCoV1A, HKU6, HKU2, and SARS). Of note, within all six RGU, the respective Chinese and European members were carried by corresponding bat genera (Fig. 2A and Table S2 in the supplemental material). To test the hypothesis that associations between CoV RGU and their hosting bat genus are valid across wide geographies, *Nyctalus leisleri* bats were sampled in Munster, Germany, 2,000 km from Strandja Park. A virus of 97.7% nucleotide and 100% amino acid identity with the Bulgarian *Nyctalus* virus BtCoV/BNM98-30/Nyc lei/Bulgaria/2008 was retrieved in one of eight bats. No other CoV were found.

Full genome analysis and distinctive features of European SARS-related CoV. Virus isolation was attempted without success from Vero cells and CaCo2 cells, as well as from several primary bat cell cultures (data not shown). However, a comprehensive design of nested RT-PCR primer sets allowed for a determination of the entire sequence of BtCoV/BM48-31/Rhi bla/Bulgaria/2008 (here referred to as BM48-31), except the 5′-most 72 nucleotides (nt), directly from fecal material of a single Blasius's horseshoe bat (*Rhinolophus blasii*). As shown in Fig. S1 in the supplemental material, the phylogenetic position of BM48-31 in relation to prototype alpha- and betacoronaviruses was preserved along all analyzed genome portions, including those for NSP1, NSP3, NSP5 (3C-like protease), and NSP13 (helicase), as well as the structural S, E, M, and N proteins. No further attempts were therefore made to identify evidence of recombination. Pairwise amino acid identities with fully sequenced SARS-related CoV are listed by protein domain in Table 4. According to criteria of the ICTV proposal (8), BM48-31 was a member of the SARS-related CoV species, confirming RGU classification. Three proteins showed the most significant deviations from SARS CoV.

Low conservation of open reading frame 3b. The SARS CoV ORF3b, which was shown to counteract type I interferon (IFN) induction in human cells (26), was not conserved in BM48-31, which comprises only 115 instead of 154 amino acids (aa). ORF3b in Chinese SARS-related BtCoV/Rf1/*Rhinolophus ferrumequinum* [Rhi fer]/China/2004 and BtCoV/273/Rhi fer/China/2005 also comprised only 114 aa, while the respective domains in all other known SARS-like bat viruses, including the most SARS-related virus, BtCoV/Rp3/Rhi pea/China/2004, were even shorter. In the 115-aa ORF3b of BM48-31, identity with SARS CoV was 44.3% and similarity was 57.4%. In contrast, the 114-aa protein putatively encoded by BtCoV/Rf1/Rhi fer/China/2004 was 91.2% identical and 93.0% similar to that of SARS CoV, respectively.

Loss of open reading frame 8. The ORF8 domain that acquired a 29-nt deletion during host transition of the SARS CoV was missing entirely from the BM48-31 genome, unlike with all other SARS-related CoV (5, 23, 28, 37, 43). In order to

TABLE 2. Percent amino acid identities within and between established mammalian CoV species

Species	% amino acid identity to alphacoronavirus:											% amino acid identity to betacoronavirus:				
	HKU8	HKU7	1A	512	PEDV	HKU6	NL63	229E	Trinidad	HKU2	Alpha1	SARS	HKU9	HKU5	HKU4	Beta1
HKU8	100															
HKU7[a]	95.2	100														
BtCoV1A[b]	93.7–94.1	92.3	98.9–100													
BtCoV512	82.0	81.6	82.4	100												
PEDV	84.2	82.7	83.8–84.6	88.6	100											
HKU6[c]	85.7	84.9	85.3–85.7	91.5	90.8	100										
NL63[d]	83.8	81.2–81.6	81.6–82.0	79.8	82.4	82.0–82.4	99.6–100									
229E	82.7	82.7	81.6	77.6	79.4	81.2	81.2–81.6	100								
Trinidad	84.9	84.6	83.5	83.1	85.3	84.6	83.8	80.5	100							
HKU2[e]	81.2	81.6	80.9	77.6	79.4	79.8	77.2	82.0	79.4	100						
Alpha1[f]	80.5–80.9	80.9–81.2	79.8–80.1	77.2–77.6	78.7	79.4–79.8	77.9	77.6–77.9	77.9–78.3	80.9–81.6	98.9–100					
SARS[g]	62.1–63.2	61.4–62.5	61.4–62.5	67.3–68.0	64.7–65.8	65.8–66.9	62.5–63.6	62.5–63.2	64.0–64.7	64.0–65.1	64.7–66.5	98.5–100				
HKU9[h]	64.3–65.1	63.2–64.0	63.6–64.7	65.1–65.4	65.4–66.2	65.4–66.2	62.5–63.6	62.1–62.5	64.0–64.7	64.0–65.1	65.4–66.9	78.7–80.1	97.8–100			
HKU5[i]	65.8	64.3	64.7–65.1	66.9	65.4	66.2	62.5	62.9	63.6–64.0	64.3	67.3–67.6	75.0–76.5	74.3–75.4	99.6–100		
HKU4[j]	64.3	62.9	63.6–64.0	66.9	65.1	65.8	62.5	63.2	64.3	63.2	65.4–66.2	77.2–78.3	74.6–75.4	93.7	100	
Beta1[k]	63.2–64.7	62.1–63.2	64.7–66.2	64.0–66.2	64.0–66.2	64.3–66.5	60.7–61.8	60.3–61.8	61.8–62.5	62.5–64.7	64.7–65.8	73.5–76.5	72.4–76.1	74.3–75.4	73.9–75.4	93.4–100

[a] HKU7 is not a formally accepted species because no full genome is available.
[b] The species BtCoV1A comprises *Miniopterus* CoV 1A and 1B.
[c] The species HKU6 is not a formally accepted species because no full genome is available; we assume that it is a species because it overlaps (in those parts where it is sequenced) the species definitions of both its neighbors, PEDV and BtCoV/512, which in turn are formally classified species. This suggests that a 10% threshold is slightly too conservative to separate CoV species.
[d] The species NL63 comprises types Amsterdam 1, 057, and 496, as well as Rotterdam.
[e] The species HKU2 comprises types HK/46, HK/33, HK/298, and GD/430.
[f] The species *Alphacoronavirus* 1 comprises feline CoV, canine CoV, transmissible gastroenteritis virus of swine (TGEV) strain Purdue, and porcine respiratory CoV (PRCV).
[g] The species SARS comprises SARS-related viruses from humans and civets, as well as bat viruses Rp3, Rm1, Rf1, 273, and HKU3-1.
[h] The species HKU9 comprises HKU9 types 1 to 4.
[i] The species HKU5 comprises HKU5 types 1 and 2.
[j] The species HKU4 comprises HKU4-1 and BtCoV/133.
[k] The species *Betacoronavirus* 1 comprises human CoV OC43 and HKU1, as well as mouse hepatitis virus (MHV) and viruses related to bovine CoV (giraffe, waterbuck, equine, and antelope CoV).

TABLE 3. Amino acid identities in RGU fragments of novel and recently described BtCoV with selected CoV species

CoV clade	Amino acid identity across 272 amino acids within the 816-bp fragment of reference strain[m]:											
	HKU8	HKU7	BtCoV 1[k]	BtCoV/ 512	PEDV	HKU6	NL63	229E	BtCoV Trinidad	HKU2	SARS[l]	HKU9
EU-HKU8-like[a]	**99.6**	95.6	94.5–94.1	82.0	84.2	85.7	83.8	82.7	85.3	81.2	62.1–63.2	64.3–65.1
EU-HKU7-like[b]	95.6	**99.6**	92.3	81.6	82.7	84.9	81.2–81.6	82.7	84.9	81.6	61.4–62.5	63.2–64.0
EU-MinCoV1-like[c]	92.3	90.1	**96.0–96.7**	81.2	83.8	84.6	81.2	80.5	82.7	79.4	61.8–62.9	63.6–64.7
BNM98-30-like[d]	89.7	87.1	88.2–89.0	84.6	84.6	87.5	85.3–85.7	81.2	85.7	79.4	62.9–63.6	64.3–65.1
BB98-15-like[e]	88.2–88.6	86.0–86.4	87.5–88.2	85.3–85.7	89.0–89.3	86.8–87.1	85.3–85.7	80.9–81.2	82.4–86.0	82.0–82.4	63.6–74.7	65.8–66.5
EU-HKU6-like[f]	84.6	83.1	84.2–84.6	90.8	92.3	**96.0**	83.1	80.9	84.9	79.4	65.8–66.9	66.9–67.6
EU-HKU2-like[g]	81.2	81.2	81.5	77.9	79.8	80.1	77.2	82.0	84.6	**98.2**	64.0–65.1	64.3–65.4
GH-229E-like[h]	72.7–84.2	82.7–84.2	82.0–83.1	76.8–77.9	78.7–79.8	81.6–82.4	79.8–83.1	**95.2–97.1**	78.7–80.9	82.4–83.1	61.1–64.3	61.4–62.5
EU-SARS-like[i]	62.1–62.5	61.4–61.8	61.4–61.8	66.9–67.3	64.7–65.1	66.2–66.5	62.5–62.9	62.5–62.9	63.6–64.0	64.0–64.3	**98.5–99.6**	77.9–79.0
Ghana348-like[j]	66.2	65.1	66.2–66.5	65.8	65.1	65.8	62.9–63.6	64.7	65.4	66.2	82.7–84.2	82.7–83.8

[a] BtCoV/BR98-53/Min sch/Bulgaria/2008; BtCoV/BR98-14/Min sch/Bulgaria/2008; BtCoV/BR98-37/Min sch/Bulgaria/2008; BtCoV/BR98-40/Min sch/Bulgaria/2008; BtCoV/BR98-52/Min sch/Bulgaria/2008; BtCoV/BR98-18/Min sch/Bulgaria/2008.

[b] BtCoV/BR98-30/Min sch/Bulgaria/2008; BtCoV/BR98-31/Min sch/Bulgaria/2008.

[c] BtCoVBtCoV/BR98-55/Min sch/Bulgaria/2008.

[d] BtCoV/BNM98-30/Nyc lei/Bulgaria/2008.

[e] BtCoV/BB98-15/Rhi bla/Bulgaria/2008; BtCoV/BM48-39/Rhi bla/Bulgaria/2008; BtCoV/BR98-12/Rhi bla/Bulgaria/2008; BtCoV/BM98-05/Rhi bla/Bulgaria/2008; BtCoV/BM48-28/Rhi fer/Bulgaria/2008.

[f] BtCoV/NM98-62/Myo dau/Germany/2008.

[g] BtCoV/BB98-41/Rhi bla/Bulgaria/2008; BtCoV/BR98-19/Rhi eur/Bulgaria/2008.

[h] BtCoV/GhanaKwam8/Hip sp/Ghana/2008; BtCoV/GhanaKwam19/Hip sp/Ghana/2008; BtCoV/GhanaBuo344/Hip sp/Ghana/2008.

[i] BtCoV/BB98-16/Rhi bla/Bulgaria/2008; BtCoV/BB98-18/Rhi bla/Bulgaria/2008; BtCoV/BM98-65/Rhi bla/Bulgaria/2008; BtCoV/BM48-34/Rhi fer/Bulgaria/2008; BtCoV/BM48-48/Rhi blaBulgaria/2008; BtCoV/BM48-32/Rhi meh/Bulgaria/2008; BtCoV/BM48-31/Rhi bla/Bulgaria/2008; BtCoV/BNM98-29/Rhi fer/Bulgaria/2008; BtCoV/BM48-35/Rhi fer/Bulgaria/2008; BtCoV/BR98-19/Rhi eur/Bulgaria/2008; BtCoV/BM98-07/Rhi meh/Bulgaria/2008; BtCoV/BM98-13/Rhi eur/Bulgaria/2008; BtCoV/BM98-05/Rhi eur/Bulgaria/2008; BtCoV/BM98-01/Rhi eur/Bulgaria/2008; BtCoV/BB98-43/Rhi eur/Bulgaria/2008; BtCoV/BM48-12/Rhi eur/Bulgaria/2008.

[j] BtCoV/GhanaKwam348/Hip sp/Ghana/2008; BtCoV/GhanaKwam20/Hip sp/Ghana/2008.

[k] BtCoV/1A/Min mag/China/2005; BtCoV/1B/Min pus/China/2006.

[l] CoV clade SARS-like comprised: BtCoV/HKU3/1/Rhi sin/China/2005; BtCoV/Rf1/Rhi fer/China/2004; BtCoV/Rm1/Rhi mac/China/2004; BtCoV/273/Rhi fer/China2005; HCoV/SARS/Tor2/Hom sap/Canada/2003; HCoV/SARS/Urbani/Hom sap/China/2003; HCoV/CUHK-W1/Hom sap/China/2003; HCoV/Frankfurt1/Hom sap/Germany/2003; HCoV/SARS/GZ0204/Hom sap/China/2004; CivCoV/SZ03/Pag lar/China/2003; CivCoV/SARS/SZ16/Pag lar/China/2003; BtCoV/Rp3/Rhi pea/China2004.

[m] Amino acid identity was calculated with MEGA 4 using the pairwise deletion option. The alignment comprised 816 bp located in the viral RNA-dependent RNA polymerase (RdRp) gene. Amino acid distance overlaps between novel BtCoV (columns) and CoV species (rows) are printed in boldface.

determine how recently the ORF8 region had been lost in the European SARS-related viruses, representatives of both European SARS-related CoV sublineages from all possible hosts (*Rhinolophus ferrumequinum*, *R. blasii*, and *R. mehelyi*, *R. euryale*) were amplified with primers flanking the deleted region (compared to sequences of Chinese SARS-related viruses). The deletion was present in all viruses, as shown in Fig. S2 in the supplemental material, suggesting a loss of the ORF8 region already in an ancestor common to all European SARS-related viruses. Nucleotide sequencing further confirmed almost 100% conservation in the amplified genomic region.

Spike protein. The spike protein has been shown to mediate virus entry and to contain binding domains of virus-neutralizing antibodies that provide relevant protection against infection in a mouse model (9). BtCoV/Rp3/Rhi pea/China/2004, which is most closely related to the human SARS CoV, was 78.0% identical and 86.8% similar to SARS CoV in the whole Spike protein. For comparison, the Spike protein of BM48-31 identified in this study was slightly more distant from that of SARS CoV, at 74.7% identity and 83.8% similarity. Because the binding of the SARS CoV spike protein to its human receptor, ACE-2, is determined by aa 319 to 518 (22, 30), this region was analyzed as shown in Fig. 5. Whereas the region homologous to the SARS CoV receptor binding domain (RBD) showed two deletions in all Chinese SARS-related BtCoV, BM48-31 had deletions in only one region. With the receptor binding motif of SARS CoV (aa 424 to 495), the corresponding sequence in BM48-31 showed 67% similarity (BLOSUM62 similarity matrix). For comparison, similarities in this region between SARS CoV and the closest Chinese BtCoV, BtCoV/HKU3/1/Rhi sin/China/2005 and BtCoV/Rp3/Rhi pea/China/2004, were only 36 and 33%, respectively. The two most critical domains for interaction of SARS CoV spike with ACE-2, aa 472 and 487, were identical (aa 472) or highly similar (aa 487), as were their contexts, in BM48-31 (22, 42). Phylogenetic analysis confirmed that the RBDs of SARS CoV and related civet CoV were closer in relation to corresponding sequences in BM48-31 than to those in Chinese SARS-related CoV from bats (Fig. 5C).

For a functional estimate of antigenic relatedness within the SARS-like group of CoV, the entire spike protein reading frames of SARS CoV, BtCoV/Rp3/Rhi pea/China/2004, and BM48-31, followed by C-terminal FLAG tags, were cloned into the expression vector pCG1 downstream of a cytomegalovirus promoter and transfected into BHK-21 cells. Transfected cells were confirmed to express spike proteins on their surfaces by anti-FLAG immunofluorescence assay (IFA) staining (Fig. 6B). Cells were then stained with a polyclonal rabbit serum raised against a recombinant SARS CoV spike protein or, alternatively, with sera of two different human SARS patients, one from China and one from Germany. As shown in Fig. 6A, the rabbit serum showed reactivity with SARS CoV and BtCoV/Rp3/Rhi pea/China/2004, while both human sera reacted only with the SARS CoV spike protein. The BM48-31 spike protein was recognized by none of the sera, including the rabbit serum.

TABLE 4. Amino acid identity and similarity of European SARS-like BtCoV BM48-31 nonstructural proteins with established SARS-like viruses

Protein[a]	Size (aa)[b]	Identity or similarity	% identity or similarity[c] with:							
			SARS CoV Tor2	Civet SARS CoV SZ16	Bat SARS CoV Rp3	Bat SARS CoV Rf1	Bat SARS CoV Rm1	Bat SARS CoV HKU3	Bat SARS CoV 273	Bat SARS CoV 279
Nsp1	179	Identity	77.7	77.7	77.7	74.3	74.3	77.1	74.3	75.4
		Similarity	89.9	89.9	89.4	87.2	88.3	89.9	87.2	88.8
Nsp2	639	Identity	70.7	70.9	70.3	72.3	70.6	71.7	72	70.7
		Similarity	85	85	84.5	84.5	84.8	83.7	84.1	85
Nsp3	1,912	Identity	75.6	75.6	75.9	74.3	74.4	75.7	74.5	74.6
		Similarity	86.5	86.4	87.1	86	86	86.6	86.2	86.3
Nsp4	501	Identity	84.4	85	84.2	85.4	85.4	85.6	85.4	85.6
		Similarity	92.6	93.2	92.4	93.4	93	92.8	93.4	93
Nsp5	306	Identity	94.4	94.4	94.1	94.1	93.8	94.4	94.1	94.1
		Similarity	97.7	97.7	97.4	97.7	97.4	97.7	97.7	97.7
Nsp6	290	Identity	90.3	90.3	89.7	90.3	90.3	90.3	90.3	90.3
		Similarity	96.9	96.9	96.9	95.9	96.9	96.6	95.9	96.9
Nsp7	83	Identity	94	94	95.2	95.2	95.2	95.2	95.2	95.2
		Similarity	100	100	100	100	100	100	100	100
Nsp8	198	Identity	98	98	97	97.5	98.5	97	97.5	98.5
		Similarity	99	99	99	98.5	99.5	98.5	98.5	99.5
Nsp9	113	Identity	99.1	99.1	98.2	99.1	98.2	98.2	99.1	98.2
		Similarity	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1
Nsp10	139	Identity	97.1	97.1	97.1	96.4	95	97.1	96.4	94.2
		Similarity	97.8	97.8	97.8	97.8	96.4	97.8	97.8	95.7
Nsp12	932	Identity	97.7	97.7	97.7	97.2	97.9	97.4	97.2	97.9
		Similarity	98.9	98.9	98.6	98.6	98.7	98.9	98.6	98.7
Nsp13	601	Identity	97.7	97.7	97.7	97.5	97.3	97.2	97.5	97.7
		Similarity	99.3	99.3	99.3	99.2	99	99.3	99.2	99.2
Nsp14	527	Identity	95.6	95.6	95.6	94.5	94.5	94.9	95.8	94.9
		Similarity	98.3	98.3	98.5	97.5	97.7	98.5	98.7	97.9
Nsp15	346	Identity	91.9	91.9	91.6	91	90.2	91.9	91.9	91.6
		Similarity	96.5	96.5	96	95.1	95.1	96.5	96.5	96
Nsp16	298	Identity	91.6	92	92	92	92	92.6	92	91.6
		Similarity	96.7	96.7	96.7	97	96.3	97.3	97	96.3
Spike	1,259	Identity	74.7	74.6	74.5	74.5	73.5	74.4	73.5	74.5
		Similarity	83.8	83.7	84.5	84.6	83.6	84.2	83.6	84.6
3a	271	Identity	69.2	69.6	69.6	69.6	66.3	67.4	66.3	69.6
		Similarity	82.6	83.3	83.3	83	81.9	82.2	81.9	83
E	76	Identity	92.2	92.2	92.2	90.9	90.9	92.2	90.9	90.9
		Similarity	94.8	94.8	94.8	93.5	93.5	94.8	93.5	93.5
M	227	Identity	88.3	88.3	88.3	88.3	87.8	88.3	87.8	88.3
		Similarity	92.2	92.2	92.2	91.7	91.7	92.2	91.7	91.7
6	62	Identity	50	50	53.1	53.1	48.4	53.1	48.4	53.1
		Similarity	78.1	78.1	78.1	78.1	76.6	78.1	76.6	78.1
7a	118	Identity	57	56.9	57.7	57.7	56.1	56.1	56.1	57.7
		Similarity	72.4	72.4	74.8	75.6	74.8	75.6	74.8	75.6
7b	40	Identity	64.4	64.4	64.4	64.4	64.4	60	64.4	64.4
		Similarity	68.9	68.9	68.9	68.9	68.9	68.9	68.9	68.9
N	417	Identity	87.7	87.5	87.2	87.5	86.1	86.8	86.8	87
		Similarity	92.4	91.7	92.9	92.4	91.7	92.7	92.4	92.2

[a] Nsp11 is not included because it is not uniformly mapped in the SARS CoV genome. NSP3b is not included because of major differences in the predicted reading frame sizes between strains (refer to the text). ORF8 is absent from the BtCoV/BM48-31 genome.

[b] Number of amino acid residues in the predicted protein in BtCoV/BM48-31.

[c] Identity of listed reference strains with BtCoV/BM48-31, based on gap-free pairwise amino acid alignments using the BLOSUM62 substitution matrix. Accession numbers of comparison strains were as follows: human SARS CoV TOR2, NC_004718; civet SARS CoV SZ16, AY572038; bat SARS CoV Rp3, DQ071615; bat SARS CoV Rm1, DQ412043; bat SARS CoV Rf1, DQ412042; bat SARS CoV HKU3-1, DQ022305; bat SARS CoV 273, DQ648856; and bat SARS CoV 279, DQ648857. Nsp11 has not been clearly mapped on the SARS-like CoV genome and is not included in this comparison.

DISCUSSION

CoV are predominantly associated with *Microchiroptera*, which are not limited to tropical climates. Their geographic range might therefore be considerably wider than that of other bat-borne viruses. In this study, we have demonstrated that SARS-related CoV exist in Europe. In addition to identifying these betacoronaviruses, we have identified novel alphacoronavirus lineages in rhinolophid and vespertilionid bats that might in part represent novel CoV species. To enable a taxonomic assignment of BtCoV from field data with reasonable effort, we have developed a surrogate criterion for defining tentative coronavirus species based on distance matrices involving an 816-bp fragment from the RdRp (RdRp grouping units [RGU]). The 816-bp fragment can easily be analyzed in samples showing evidence of CoV in generic screening assays (9, 41) and is sufficient, as demonstrated in this study, to re-

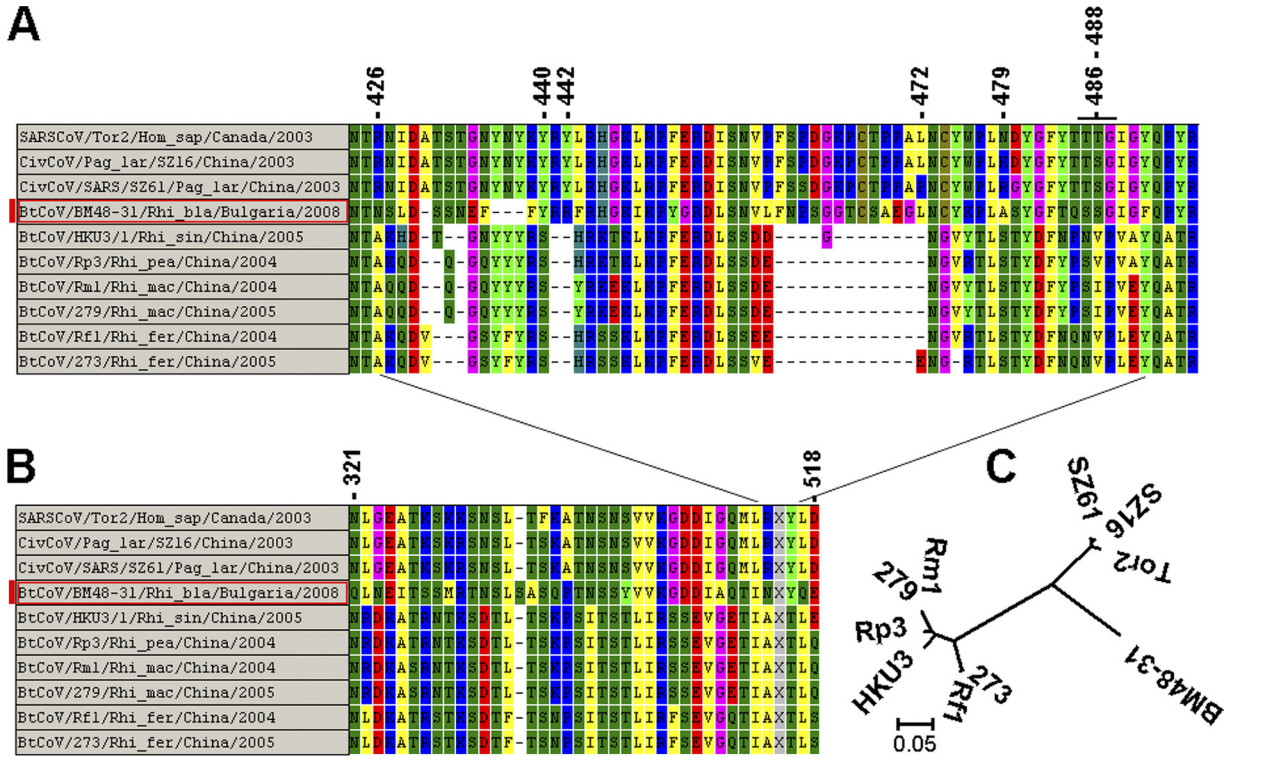

FIG. 5. Comparison of the receptor-binding domains of SARS CoV spike proteins with homologous sequences in civet and bat CoV. SARS CoV spike protein residues 319 to 518 were aligned with homologous regions of closely related civet and bat CoV. The European SARS-related CoV BtCoV/BM48-31/Rhi bla/Bulgaria/2008 is highlighted in red. The alignment was done in MEGA and corrected manually, with preference for parsimonious nucleotide exchanges rather than gap extensions. (A) Sequences homologous to the receptor binding motif (RBM) of SARS CoV. Those amino acids interacting directly with the human ACE-2 molecule are identified by numbers. (B) Alignment of only those amino acids upstream and downstream of the SARS CoV RBM that vary across strains. The alignment in panel A corresponds to the column marked with X's. (C) Amino acid-based neighbor-joining phylogeny, using the p distance model in an alignment of sequences homologous to the SARS CoV RBD, aa 319 to 512.

solve all currently agreed upon CoV species. It is acknowledged that the CoV species classification as defined recently in a joint proposal to the ICTV has to be met before a CoV species can be formally declared. However, for most field-sampled CoV, this task is impossible to complete. Even if only surrogate criteria are available, it will be practically useful in ecological investigations to assign preliminary connections between specific taxonomic units of bats and specific CoV in order to assess potential geographic ranges of CoV reservoirs. With six different CoV RGU as examples, our study revealed that European and Chinese representatives were consistently associated with the same bat host genera. Moreover, the Ghana348-related RGU that constitutes the closest phylogenetic neighbor of the SARS-related RGU was detected in bats of the genus *Hipposideros*, which in turn is the closest phylogenetic neighbor of the genus *Rhinolophus* (40). We can thus conclude that associations exist between CoV RGU and bat genera. If we assume that RGU are predictive of species, this suggests that CoV speciation may have frequently been caused by extensions of host ranges of ancestral CoV into novel bat genera. On the other hand, because host associations rarely extend to bat species, it appears that CoV may experience limited genetic barriers toward host switching within bat genera.

Virus-host associations can be used in a prospective manner to predict geographic distributions of reservoir-borne CoV. For example, we have identified in this study a novel alphacoronavirus RGU in *Nyctalus leisleri.* The concept of taxonomic association suggests that similar viruses might occur in other regions of the world harboring *Nyctalus* bats, including most of northern Europe and the boreal regions of the Asian continent. Indeed, such a virus could be confirmed in a specimen of the same genus from Germany, 2,000 km away. Similarly, there was one *Rhinolophus*-associated CoV that occurred as an independent RGU in Bulgaria but that has never been detected in China (represented by BtCoV/BB98-15). We may predict that this virus can also be found in *Rhinolophus* bats elsewhere in Europe and Asia.

The availability of a full genome sequence of the Bulgarian SARS-related CoV allows for an unequivocal classification of the virus as an extension of the recognized SARS CoV species. The existence of SARS-related CoV in a considerably larger geographic area than previously assumed demands a comprehensive approach to characterizing the relevance of this virus as a potential zoonotic agent. The complete genome sequence enables the comparison of several features that have been functionally characterized for SARS CoV, in order to derive hypotheses for future experimental work. For example, the important papain-like protease domain of NSP3 that has been shown to interact with IRF3 phosphorylation and nuclear translocation and thereby to prevent induction of type I inter-

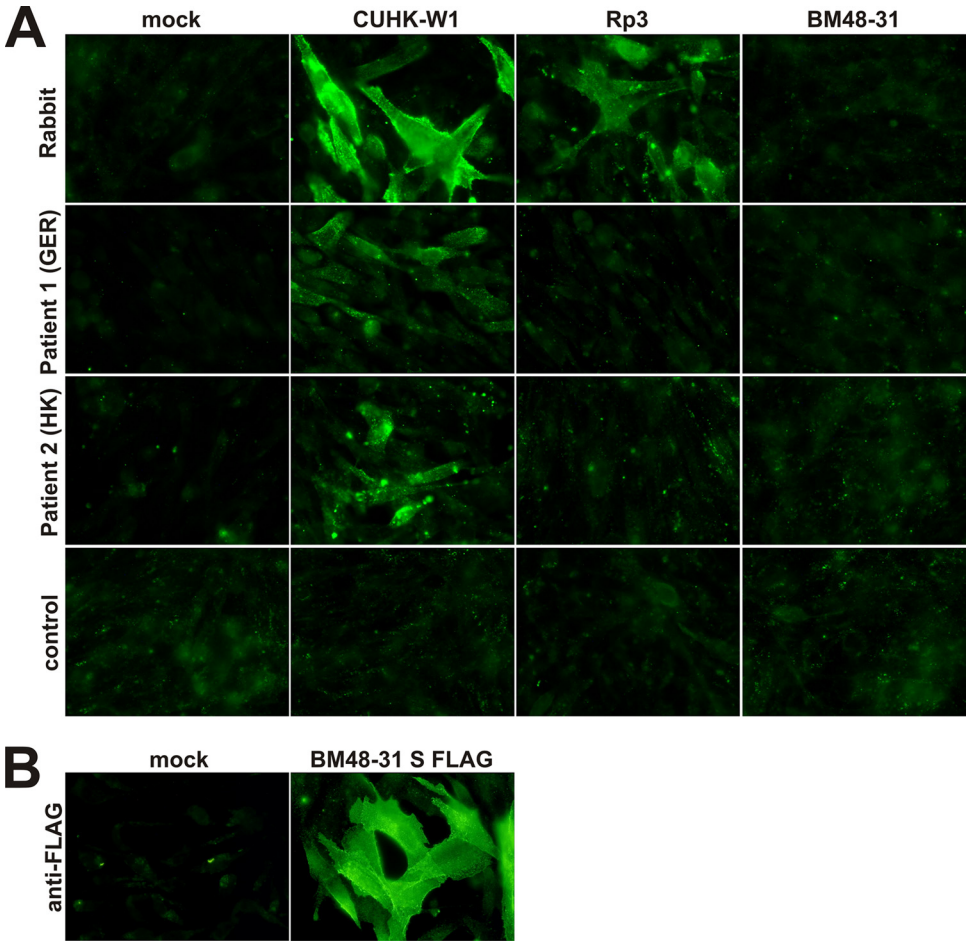

FIG. 6. Immunofluorescence staining of SARS CoV and SARS-related spike proteins. (A) The SARS CoV spike protein and SARS-related spike proteins from bats (strains BtCoV/Rp3/Rhi pea/China/2004 and BtCoV/BM48-31/Rhi bla/Bulgaria/2008) were expressed in BHK-21 cells. Cells were fixed and permeabilized, and immunofluorescence was performed by using a polyclonal rabbit serum raised against SARS CoV spike protein (CUHK-W1 strain) (top panels) or human patient sera (middle and bottom panels) (patient 1 from Germany [GER] and patient 2 from Hong Kong [HK]). After incubation with IgG-specific fluorescein isothiocyanate (FITC)-labeled secondary antibodies, fluorescence was visualized by fluorescence microscopy. (B) For detection of FLAG-tagged spike protein of BtCoV/BM48-31/Rhi bla/Bulgaria/2008, a monoclonal anti-FLAG antibody was used, followed by incubation with a secondary FITC-conjugated antibody.

feron fell significantly below the general level of identity observed in the nonstructural proteins (>94%), at only 84.3 to 86.5% (11). The Bulgarian virus might show a discernible difference from SARS CoV in its ability to interact with the human interferon system. Reverse genetics experiments are under way to investigate this important implication (39). Another interesting domain whose differential functionality should be studied in comparison with that of SARS CoV is the ADRP domain in NSP3, with a level of identity to prototypic SARS-like CoVs of only 64.4 to 69.1%. This domain is conserved across the alphavirus-like superfamily but is of yet-unclear function in CoV replication (16, 35, 61). Within the genome portion containing structural and accessory genes, ORF3b has been identified as an important interferon antagonist (26). While among the structural proteins E, M, and N the level of identity is generally around 90%, a low degree of conservation in ORF3b among the now-extended range of SARS-related CoV speaks against a generally important role of the encoded protein for virus replication. This difference might identify one of the distinctive features causing the high pathogenicity of the epidemic 2002–2003 SARS CoV as opposed to the obviously mild courses following postepidemic zoonotic transitions of closely related viruses in 2003-2004 (33, 50).

A strikingly low identity with prototypic members of the SARS CoV species existed in protein 6 (48.4 to 53.1%), which has been determined to interfere with type I interferon-mediated signaling via inhibition of nuclear translocation of STAT1 (26, 63). Again, it would be warranted to study differences that may exist between both viruses' proteins 6 in their ability to ablate interferon (IFN) signaling. Yet another interesting observation is that identities between ORF7b proteins were clearly higher than those between ORF7a proteins, suggesting that ORF7b, whose translation in cell culture is probably mediated by ribosomal leaky scanning, is differentially selected and, thus, probably expressed *in vivo* in bats. Interestingly, the entire region encoded by subgenomic RNA 8 was not present in BM48-31. This includes ORF8, whose 29-nucleotide deletion in human SARS CoV as opposed to in SARS-like bat and civet viruses has been hypothesized to represent a major func-

tional hallmark in the transition of SARS CoV to humans (5, 23, 28, 43). The absence of ORF8 in both sublineages of European SARS-related CoV suggests that protein 8 may not be generally essential for the maintenance of SARS-related CoV in bats. Confirmation in a BtCoV animal model is warranted, yet such models are not available so far.

Finally, the CoV spike protein mediates entry of virus into host cells and acts as a functionally relevant virus neutralization domain targeted by virus-neutralizing antibodies. Interestingly, neither human sera nor a high-titer rabbit serum raised against a recombinant trimeric SARS CoV spike protein bound the BM48-31 spike protein, while at least some expectable degree of cross-reactivity was observed among SARS CoV and a close relative from Chinese bats, BtCoV/Rp3/Rhi pea/China/2004 (28, 31, 43). Even in the absence of virus neutralization tests, we may therefore draw the preliminary conclusion that it would be of little value to use existing SARS CoV vaccine candidates for an immunization of the population in case of an epidemic caused by the European SARS-related virus.

Another highly relevant experimental task is to determine whether the spike protein of BM48-31 might mediate entry into human or domestic animal cells. It has been shown previously that members of the Chinese BtCoV lineage in the SARS-related species cannot utilize the human SARS CoV entry receptor, ACE-2 (44). However, the RBD of BM48-31 is related considerably more closely to the SARS CoV RBD than that of any other BtCoV characterized so far. The most critical domains for receptor binding in SARS CoV, i.e., aa 472 and aa 487, as well as their contexts, are present and highly similar (22, 32, 42). To reflect adaptive processes in nature, it would therefore be highly interesting to simulate the behavior of the BM48-31 spike protein (or RBD) in the context of a replicating SARS CoV. It has already been demonstrated that recombinant CoV can be pseudotyped with BtCoV spike proteins and rescued with replicative activity (1, 47, 48). It should therefore be possible to study adaptive mechanisms of the BM48-31 RBD during transition to novel hosts by passaging of recombinant viruses in cells of other mammals. However, as a first prerequisite, such experiments would require susceptible cell cultures from bats, either from the genus *Rhinolophus* or ultimately from the exact species yielding BM48-31, *R. blasii.* Such cells are not available to us so far.

In summary, this study shows approaches to utilizing data from field investigations for ecological and experimental assessments of the zoonotic potential of bat-borne CoV. Bats are a major current focus of zoonotic risk assessment, due to their large population sizes, their ability to fly, and their relatedness with other mammals. Coronaviruses provide important models for studying ecological and molecular mechanisms of virus transition from bats to other mammals, including humans.

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REFERENCES

1. **Becker, M. M., R. L. Graham, E. F. Donaldson, B. Rockx, A. C. Sims, T. Sheahan, R. J. Pickles, D. Corti, R. E. Johnston, R. S. Baric, and M. R. Denison.** 2008. Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. Proc. Natl. Acad. Sci. U. S. A. **105:**19944–19949.
2. **Benda, P., T. Ivanova, I. Horáček, V. Hanák, J. Červený, J. Gaisler, A. Gueorguieva, B. Petrov, and V. Vohralík.** 2003. Bats (Mammalia: Chiroptera) of the eastern Mediterranean. Part 3. Review of bat distribution in Bulgaria. Acta Soc. Zool. Bohem. **67:**245–357.
3. **Calisher, C. H., J. E. Childs, H. E. Field, K. V. Holmes, and T. Schountz.** 2006. Bats: important reservoir hosts of emerging viruses. Clin. Microbiol. Rev. **19:**531–545.
4. **Cavanagh, D.** 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. **142:**629–633.
5. **Chinese Sars Molecular Epidemiology Consortium.** 2004. Molecular evolution of the SARS coronavirus during the course of the SARS epidemic in China. Science **303:**1666–1669.
6. **Chu, D. K., J. S. Peiris, H. Chen, Y. Guan, and L. L. Poon.** 2008. Genomic characterizations of bat coronaviruses (1A, 1B and HKU8) and evidence for co-infections in Miniopterus bats. J. Gen. Virol. **89:**1282–1287.
7. **Csorba, G., P. Ujhelyi, and N. Thomas.** 2003. Horseshoe bats of the world (Chiroptera: Rhinolophidae). Alana Ecology Ltd., Shropshire, United Kingdom.
8. **de Groot, R. J., J. Ziebuhr, L. L. Poon, P. C. Woo, P. J. Talbot, P. J. M. Rottier, K. V. Holmes, R. Baric, S. Perlman, L. Enjuanes, and A. E. Gorbalenya.** 2008. Taxonomic proposal to the ICTV Executive Committee. Revision of the family Coronaviridae. International Committee on Taxonomy of Viruses. http://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/vertebrate-2008/1230/download.aspx.
9. **Deming, D., T. Sheahan, M. Heise, B. Yount, N. Davis, A. Sims, M. Suthar, J. Harkema, A. Whitmore, R. Pickles, A. West, E. Donaldson, K. Curtis, R. Johnston, and R. Baric.** 2006. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS Med. **3:**e525.
10. **de Souza Luna, L. K., V. Heiser, N. Regamey, M. Panning, J. F. Drexler, S. Mulangu, L. Poon, S. Baumgarte, B. J. Haijema, L. Kaiser, and C. Drosten.** 2007. Generic detection of coronaviruses and differentiation at the prototype strain level by reverse transcription-PCR and nonfluorescent low-density microarray. J. Clin. Microbiol. **45:**1049–1052.
11. **Devaraj, S. G., N. Wang, Z. Chen, Z. Chen, M. Tseng, N. Barretto, R. Lin, C. J. Peters, C. T. Tseng, S. C. Baker, and K. Li.** 2007. Regulation of IRF-3-dependent innate immunity by the papain-like protease domain of the severe acute respiratory syndrome coronavirus. J. Biol. Chem. **282:**32208–32221.

11a. **Dietz, C., D. Nill, and O. von Helversen.** 2009. Handbook of the bats of Europe and Northwest Africa, A & C Black Publishers, Ltd., London, United Kingdom.

12. **Dobson, A. P.** 2005. Virology. What links bats to emerging infectious diseases? Science **310:**628–629.
13. **Drexler, J. F., B. Kupfer, N. Petersen, R. M. Grotto, S. M. Rodrigues, K. Grywna, M. Panning, A. Annan, G. F. Silva, J. Douglas, E. S. Koay, H. Smuts, E. M. Netto, P. Simmonds, M. I. Pardini, W. K. Roth, and C. Drosten.** 2009. A novel diagnostic target in the hepatitis C virus genome. PLoS Med. **6:**e31.
14. **Drosten, C., S. Gunther, W. Preiser, S. van der Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
15. **Drummond, A. J., and A. Rambaut.** 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. **7:**214.
16. **Egloff, M. P., H. Malet, A. Putics, M. Heinonen, H. Dutartre, A. Frangeul, A. Gruez, V. Campanacci, C. Cambillau, J. Ziebuhr, T. Ahola, and B. Canard.** 2006. Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains. J. Virol. **80:**8493–8502.
17. **Eickmann, M., S. Becker, H. D. Klenk, H. W. Doerr, K. Stadler, S. Censini, S. Guidotti, V. Masignani, M. Scarselli, M. Mora, C. Donati, J. H. Han, H. C. Song, S. Abrignani, A. Covacci, and R. Rappuoli.** 2003. Phylogeny of the SARS coronavirus. Science **302:**1504–1505.
18. **Forster, J. L., V. B. Harkin, D. A. Graham, and S. J. McCullough.** 2008. The

effect of sample type, temperature and RNAlater on the stability of avian influenza virus RNA. J. Virol. Methods **149:**190–194.
19. **Gelman, A., and D. B. Rubin.** 1996. Markov chain Monte Carlo methods in biostatistics. Stat. Methods Med. Res. **5:**339–355.
20. **Gloza-Rausch, F., A. Ipsen, A. Seebens, M. Gottsche, M. Panning, J. F. Drexler, N. Petersen, A. Annan, K. Grywna, M. Muller, S. Pfefferle, and C. Drosten.** 2008. Detection and prevalence patterns of group I coronaviruses in bats, northern Germany. Emerg. Infect. Dis. **14:**626–631.
21. **Gonzalez, J. M., P. Gomez-Puertas, D. Cavanagh, A. E. Gorbalenya, and L. Enjuanes.** 2003. A comparative sequence analysis to revise the current taxonomy of the family Coronaviridae. Arch. Virol. **148:**2207–2235.
22. **Graham, R. L., and R. S. Baric.** 2010. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. J. Virol. **84:**3134–3146.
23. **Guan, Y., B. J. Zheng, Y. Q. He, X. L. Liu, Z. X. Zhuang, C. L. Cheung, S. W. Luo, P. H. Li, L. J. Zhang, Y. J. Guan, K. M. Butt, K. L. Wong, K. W. Chan, W. Lim, K. F. Shortridge, K. Y. Yuen, J. S. Peiris, and L. L. Poon.** 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science **302:**276–278.
24. **Halpin, K., P. L. Young, H. E. Field, and J. S. Mackenzie.** 2000. Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. J. Gen. Virol. **81:**1927–1932.
25. **Isaacs, D., D. Flowers, J. R. Clarke, H. B. Valman, and M. R. MacNaughton.** 1983. Epidemiology of coronavirus respiratory infections. Arch. Dis. Child. **58:**500–503.
26. **Kopecky-Bromberg, S. A., L. Martinez-Sobrido, M. Frieman, R. A. Baric, and P. Palese.** 2007. Severe acute respiratory syndrome coronavirus open reading frame (ORF) 3b, ORF 6, and nucleocapsid proteins function as interferon antagonists. J. Virol. **81:**548–557.
27. **Larson, H. E., S. E. Reed, and D. A. Tyrrell.** 1980. Isolation of rhinoviruses and coronaviruses from 38 colds in adults. J. Med. Virol. **5:**221–229.
28. **Lau, S. K., P. C. Woo, K. S. Li, Y. Huang, H. W. Tsoi, B. H. Wong, S. S. Wong, S. Y. Leung, K. H. Chan, and K. Y. Yuen.** 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc. Natl. Acad. Sci. U. S. A. **102:**14040–14045.
29. **Leroy, E. M., B. Kumulungui, X. Pourrut, P. Rouquet, A. Hassanin, P. Yaba, A. Delicat, J. T. Paweska, J. P. Gonzalez, and R. Swanepoel.** 2005. Fruit bats as reservoirs of Ebola virus. Nature **438:**575–576.
30. **Li, F., W. Li, M. Farzan, and S. C. Harrison.** 2005. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science **309:**1864–1868.
31. **Li, W., Z. Shi, M. Yu, W. Ren, C. Smith, J. H. Epstein, H. Wang, G. Crameri, Z. Hu, H. Zhang, J. Zhang, J. McEachern, H. Field, P. Daszak, B. T. Eaton, S. Zhang, and L. F. Wang.** 2005. Bats are natural reservoirs of SARS-like coronaviruses. Science **310:**676–679.
32. **Li, W., S. K. Wong, F. Li, J. H. Kuhn, I. C. Huang, H. Choe, and M. Farzan.** 2006. Animal origins of the severe acute respiratory syndrome coronavirus: insight from ACE2–S-protein interactions. J. Virol. **80:**4211–4219.
33. **Liang, G., Q. Chen, J. Xu, Y. Liu, W. Lim, J. S. Peiris, L. J. Anderson, L. Ruan, H. Li, B. Kan, B. Di, P. Cheng, K. H. Chan, D. D. Erdman, S. Gu, X. Yan, W. Liang, D. Zhou, L. Haynes, S. Duan, X. Zhang, H. Zheng, Y. Gao, S. Tong, D. Li, L. Fang, P. Qin, and W. Xu.** 2004. Laboratory diagnosis of four recent sporadic cases of community-acquired SARS, Guangdong Province, China. Emerg. Infect. Dis. **10:**1774–1781.
34. **Masters, P. S.** 2006. The molecular biology of coronaviruses. Adv. Virus Res. **66:**193–292.
35. **Nargi-Aizenman, J. L., C. M. Simbulan-Rosenthal, T. A. Kelly, M. E. Smulson, and D. E. Griffin.** 2002. Rapid activation of poly(ADP-ribose) polymerase contributes to Sindbis virus and staurosporine-induced apoptotic cell death. Virology **293:**164–171.
36. **Nel, L. H., and W. Markotter.** 2007. Lyssaviruses. Crit. Rev. Microbiol. **33:**301–324.
37. **Oostra, M., C. A. de Haan, and P. J. Rottier.** 2007. The 29-nucleotide deletion present in human but not in animal severe acute respiratory syndrome coronaviruses disrupts the functional expression of open reading frame 8. J. Virol. **81:**13876–13888.
38. **Peiris, J. S., K. Y. Yuen, A. D. Osterhaus, and K. Stohr.** 2003. The severe acute respiratory syndrome. N. Engl. J. Med. **349:**2431–2441.
39. **Pfefferle, S., V. Krahling, V. Ditt, K. Grywna, E. Muhlberger, and C. Drosten.** 2009. Reverse genetic characterization of the natural genomic deletion in SARS-coronavirus strain Frankfurt-1 open reading frame 7b reveals an attenuating function of the 7b protein in-vitro and in-vivo. Virol. J. **6:**131.
40. **Pfefferle, S., S. Oppong, J. F. Drexler, F. Gloza-Rausch, A. Ipsen, A. Seebens, M. A. Muller, A. Annan, P. Vallo, Y. Adu-Sarkodie, T. F. Kruppa, and C. Drosten.** 2009. Distant relatives of severe acute respiratory syndrome coronavirus and close relatives of human coronavirus 229E in bats, Ghana. Emerg. Infect. Dis. **15:**1377–1384.
41. **Poon, L. L., D. K. Chu, K. H. Chan, O. K. Wong, T. M. Ellis, Y. H. Leung, S. K. Lau, P. C. Woo, K. Y. Suen, K. Y. Yuen, Y. Guan, and J. S. Peiris.** 2005. Identification of a novel coronavirus in bats. J. Virol. **79:**2001–2009.
42. **Qu, X. X., P. Hao, X. J. Song, S. M. Jiang, Y. X. Liu, P. G. Wang, X. Rao, H. D. Song, S. Y. Wang, Y. Zuo, A. H. Zheng, M. Luo, H. L. Wang, F. Deng, H. Z. Wang, Z. H. Hu, M. X. Ding, G. P. Zhao, and H. K. Deng.** 2005. Identification of two critical amino acid residues of the severe acute respiratory syndrome coronavirus spike protein for its variation in zoonotic tropism transition via a double substitution strategy. J. Biol. Chem. **280:**29588–29595.
43. **Ren, W., W. Li, M. Yu, P. Hao, Y. Zhang, P. Zhou, S. Zhang, G. Zhao, Y. Zhong, S. Wang, L. F. Wang, and Z. Shi.** 2006. Full-length genome sequences of two SARS-like coronaviruses in horseshoe bats and genetic variation analysis. J. Gen. Virol. **87:**3355–3359.
44. **Ren, W., X. Qu, W. Li, Z. Han, M. Yu, P. Zhou, S. Y. Zhang, L. F. Wang, H. Deng, and Z. Shi.** 2008. Difference in receptor usage between severe acute respiratory syndrome (SARS) coronavirus and SARS-like coronavirus of bat origin. J. Virol. **82:**1899–1907.
45. **Ronquist, F., and J. P. Huelsenbeck.** 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:**1572–1574.
46. **Schwegmann-Wessels, C., X. Ren, and G. Herrler.** 2006. Intracellular transport of the S proteins of coronaviruses. Adv. Exp. Med. Biol. **581:**271–275.
47. **Sheahan, T., B. Rockx, E. Donaldson, D. Corti, and R. Baric.** 2008. Pathways of cross-species transmission of synthetically reconstructed zoonotic severe acute respiratory syndrome coronavirus. J. Virol. **82:**8721–8732.
48. **Sheahan, T., B. Rockx, E. Donaldson, A. Sims, R. Pickles, D. Corti, and R. Baric.** 2008. Mechanisms of zoonotic severe acute respiratory syndrome coronavirus host range expansion in human airway epithelium. J. Virol. **82:**2274–2285.
49. **Snijder, E. J., P. J. Bredenbeek, J. C. Dobbe, V. Thiel, J. Ziebuhr, L. L. Poon, Y. Guan, M. Rozanov, W. J. Spaan, and A. E. Gorbalenya.** 2003. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J. Mol. Biol. **331:**991–1004.
50. **Song, H. D., C. C. Tu, G. W. Zhang, S. Y. Wang, K. Zheng, L. C. Lei, Q. X. Chen, Y. W. Gao, H. Q. Zhou, H. Xiang, H. J. Zheng, S. W. Chern, F. Cheng, C. M. Pan, H. Xuan, S. J. Chen, H. M. Luo, D. H. Zhou, Y. F. Liu, J. F. He, P. Z. Qin, L. H. Li, Y. Q. Ren, W. J. Liang, Y. D. Yu, L. Anderson, M. Wang, R. H. Xu, X. W. Wu, H. Y. Zheng, J. D. Chen, G. Liang, Y. Gao, M. Liao, L. Fang, L. Y. Jiang, H. Li, F. Chen, B. Di, L. J. He, J. Y. Lin, S. Tong, X. Kong, L. Du, P. Hao, H. Tang, A. Bernini, X. J. Yu, O. Spiga, Z. M. Guo, H. Y. Pan, W. Z. He, J. C. Manuguerra, A. Fontanet, A. Danchin, N. Niccolai, Y. X. Li, C. I. Wu, and G. P. Zhao.** 2005. Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. Proc. Natl. Acad. Sci. U. S. A. **102:**2430–2435.
51. **Swanepoel, R., S. B. Smit, P. E. Rollin, P. Formenty, P. A. Leman, A. Kemp, F. J. Burt, A. A. Grobbelaar, J. Croft, D. G. Bausch, H. Zeller, H. Leirs, L. E. Braack, M. L. Libande, S. Zaki, S. T. Nichol, T. G. Ksiazek, and J. T. Paweska.** 2007. Studies of reservoir hosts for Marburg virus. Emerg. Infect. Dis. **13:**1847–1851.
52. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
53. **Tang, X. C., J. X. Zhang, S. Y. Zhang, P. Wang, X. H. Fan, L. F. Li, G. Li, B. Q. Dong, W. Liu, C. L. Cheung, K. M. Xu, W. J. Song, D. Vijaykrishna, L. L. Poon, J. S. Peiris, G. J. Smith, H. Chen, and Y. Guan.** 2006. Prevalence and genetic diversity of coronaviruses in bats from China. J. Virol. **80:**7481–7490.
54. **Uhlenhaut, C., and M. Kracht.** 2005. Viral infectivity is maintained by an RNA protection buffer. J. Virol. Methods **128:**189–191.
55. **Vabret, A., J. Dina, S. Gouarin, J. Petitjean, V. Tripey, J. Brouard, and F. Freymuth.** 2008. Human (non-severe acute respiratory syndrome) coronavirus infections in hospitalised children in France. J. Paediatr. Child Health **44:**176–181.
56. **Vabret, A., T. Mourez, S. Gouarin, J. Petitjean, and F. Freymuth.** 2003. An outbreak of coronavirus OC43 respiratory infection in Normandy, France. Clin. Infect. Dis. **36:**985–989.
57. **Vallo, P., A. GuillénServent, P. Benda, D. B. Pires, and P. Koubek.** 2008. Variation of mitochondrial DNA reveals high cryptic diversity in Hipposideros caffer complex. Acta Chiropt. **10:**193–206.
58. **Vijaykrishna, D., G. J. Smith, J. X. Zhang, J. S. Peiris, H. Chen, and Y. Guan.** 2007. Evolutionary insights into the ecology of coronaviruses. J. Virol. **81:**4012–4020.
59. **Woo, P. C., S. K. Lau, C. M. Chu, K. H. Chan, H. W. Tsoi, Y. Huang, B. H. Wong, R. W. Poon, J. J. Cai, W. K. Luk, L. L. Poon, S. S. Wong, Y. Guan, J. S. Peiris, and K. Y. Yuen.** 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J. Virol. **79:**884–895.
60. **Woo, P. C., S. K. Lau, K. S. Li, R. W. Poon, B. H. Wong, H. W. Tsoi, B. C. Yip, Y. Huang, K. H. Chan, and K. Y. Yuen.** 2006. Molecular diversity of coronaviruses in bats. Virology **351:**180–187.
61. **Xu, Y., L. Cong, C. Chen, L. Wei, Q. Zhao, X. Xu, Y. Ma, M. Bartlam, and Z. Rao.** 2009. Crystal structures of two coronavirus ADP-ribose-1′-monophosphatases and their complexes with ADP-ribose: a systematic structural analysis of the viral ADRP domain. J. Virol. **83:**1083–1092.
62. **Yob, J. M., H. Field, A. M. Rashdi, C. Morrissy, B. van der Heide, P. Rota, A. bin Adzhar, J. White, P. Daniels, A. Jamaluddin, and T. Ksiazek.** 2001. Nipah virus infection in bats (order Chiroptera) in peninsular Malaysia. Emerg. Infect. Dis. **7:**439–441.
63. **Zhou, H., D. Ferraro, J. Zhao, S. Hussain, J. Shao, J. Trujillo, J. Netland, T. Gallagher, and S. Perlman.** 2010. The N terminal region of severe acute respiratory syndrome coronavirus protein 6 induces membrane rearrangement and enhances virus replication. J. Virol. **84:**3542–3551.