Isolation of Bat Sarbecoviruses, Japan

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Surveillance of bat betacoronaviruses is crucial for understanding their spillover potential. We isolated bat sarbecoviruses from *Rhinolophus cornutus* bats in multiple locations in Japan. These viruses grew efficiently in cells expressing *R. cornutus* angiotensin converting enzyme-2, but not in cells expressing human angiotensin converting enzyme-2, suggesting a narrow host range.

Human betacoronaviruses are divided into 2 pathotypes: endemic viruses, such as human coronavirus OC43 (HCoV-OC43) and HCoV-HKU1, which cause mild respiratory symptoms (*1*), and highly pathogenic viruses comprising severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus, and SARS-CoV-2, which have caused outbreaks in the past 2 decades (*1*,*2*). Because all these highly pathogenic human betacoronaviruses are considered to have originated from bat-derived viruses (*2*–*6*), surveillance of bat betacoronaviruses is crucial for understanding and assessing the spillover potential of betacoronaviruses in humans.

Bats belonging to the genus *Rhinolophus* are considered natural reservoirs of sarbecoviruses because most have been detected in *Rhinolophus* bats in countries in Asia (*3*–*8*), as well as in countries in Europe and Africa (*9*,*10*). We previously identified a bat sarbecovirus, Rc-o319, from *Rhinolophus cornutus* bats in the Iwate Prefecture of Japan, which was shown to phylogenetically belong to the SARS-CoV-2 lineage (*7*).

Vesicular stomatitis virus–based pseudotyped virus having the Rc-o319 spike (S) protein was able to infect cells expressing *R. cornutus* angiotensin-converting enzyme 2 (RcACE2), but not those expressing human angiotensin-converting enzyme 2 (hACE2), suggesting that the Rc-o319 virus uses RcACE2 as its receptor (*7*). Sarbecoviruses detected in China and other countries in Asia were shown to vary genetically; however, the distribution and genetic variation of bat sarbecoviruses in Japan have not yet been determined.

Despite surveillance-based genetic detection of numerous bat sarbecoviruses, cultivable viruses have been rarely isolated to date, leading to the application of a pseudovirus system as described above to analyze their entry mechanisms into cells. Receptor selectivity assessed in this system does not necessarily correspond to functional receptor specificity of intact bat sarbecovirus (*11*), emphasizing the need for cultivable virus for assessment of its spillover potential of bat sarbecoviruses. We report detection, isolation, and genetic and biologic characterization of cultivable bat sarbecoviruses from several locations in Japan.

The Study

We collected fecal samples from bats belonging to the *R. cornutus* and *R. ferrumequinum* species in Niigata, Chiba, and Shizuoka Prefectures (Appendix Figure 1, panel A, https://wwwnc.cdc.gov/EID/article/28/12/22-0801-App1.pdf). Using real-time reverse transcription PCR, we successfully detected the envelope gene sequence of sarbecovirus in 1 or 2 *R. cornutus* bat samples in each prefecture (Table 1). In contrast, all *R. ferremuquinum* bat samples were negative. These data suggested that bat sarbecoviruses are distributed among *R. cornutus* bats at various locations in Japan.

In our previous study, we showed that a vesicular stomatitis virus–based pseudotyped virus possessing the S protein of Rc-o319 sarbecovirus from *R. cornutus* only infected RcACE2-expressing cells, but not hACE2-expressing or other *Rhinolophus* ACE2–expressing cells (*7*). Therefore, to isolate bat sarbecoviruses, we established RcACE2-stably expressing

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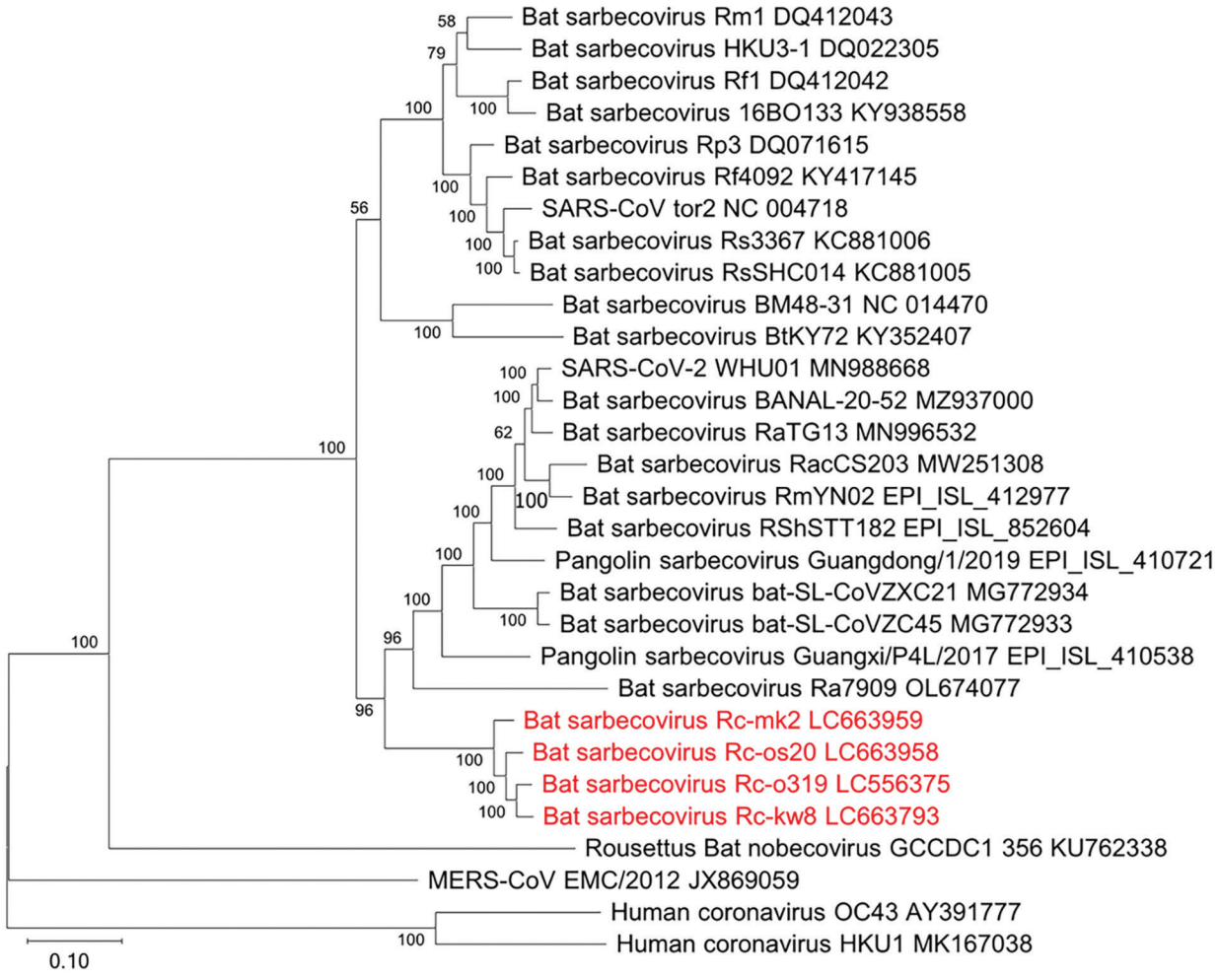

Figure 1. Phylogenetic tree of sarbecoviruses from bats in Japan, generated by using the full-genome nucleotide sequences with the maximum-likelihood analysis combined with 500 bootstrap replicates. Red indicates strains isolated in this study. Bootstrap values are shown above and to the left of the major nodes. GenBank accession numbers are indicated. Scale bars indicate nucleotide substitutions per site.

cells (Vero-RcACE2) based on Vero/TMPRSS2 cells. Using Vero-RcACE2 cells, we successfully isolated bat sarbecoviruses, which exhibited extensive cytopathic effect with syncytium formation (Appendix Figure 1, panel B) from real-time reverse transcription PCR–positive fecal samples from each prefecture. We designated the Niigata isolate as Rc-os20, the Chiba isolate as Rc-mk2, and the Shizuoka isolate as Rc-kw8. We further isolated the cultivable Rc-o319 strain by using Vero-RcACE2 cells.

We determined the full-genome sequence of all isolates by using next-generation sequencing and deposited the sequences in GenBank (accession nos. LC663958, LC663959, and LC663793). We found that sequence homologies were high (range 94.8%–96.8%) among all isolates from Japan (Table 2), However, Rc-mk2 and Rc-os20 lacked the entire open reading frame 8 coding region.

We also performed similarity plot analysis of entire genome sequence by using each isolate as a query,

Table 1. Detection of sarbecoviruses in *Rhinolophus* bats by RT-PCR, Japan*

Location	Bat species	No. samples	No. positive RT-PCR samples
Niigata	*R. cornutus*	26	2
	R. ferrumequinum	1	0
Chiba	*R. cornutus*	11	1
	R. ferrumequinum	16	0
Shizuoka	*R. cornutus*	21	2
	R. ferrumequinum	13	0

*RT-PCR was performed by using sarbecovirus consensus primers targeting the envelope gene. RT-PCR, reverse transcription PCR.

which indicated that similarities among isolates were high throughout the entire genome sequence, except for coding regions of the N-terminal domain (NTD) and receptor-binding domain (RBD) of the S gene, although NTDs of Rc-o319 and Rc-kw8 were conserved (Appendix Figure 2). No clear recombination among the isolates were observed as analyzed by RDP5 software (*12*). Phylogenetic analysis showed that the isolates from Japan formed a single genetic cluster and positioned in a clade containing SARS-CoV-2–related sarbecoviruses, which might be designated the Japanese clade of bat sarbecoviruses (Figure 1).

We aligned the receptor-binding motif of the S protein of isolates from Japan with that of other sarbecoviruses (Appendix Figure 3, panel A). We observed that all isolates had a 9-aa deletion in this motif, as previously observed in Rc-o319, and had relatively conserved residues with Rc-o319. In addition, phylogenetic tree analysis of RBD showed that strains from Japan were included in the clade of viruses that use ACE2 orthologs as a strain receptor (Appendix Figure 3, panel B). Therefore, we assumed that these new strains from Japan use RcACE2 as a receptor.

To test this hypothesis, we compared the replication of isolates from Japan with that of a control SARS-CoV-2 (B.1.1.7, Alpha variant) in Vero-RcACE2, Vero-hACE2, Vero-ACE2KO, and Vero/TMPRSS2 cells. Whereas the 4 bat isolates replicated well in Vero-RcACE2 only, they did not replicate in Vero/TMPRSS2, Vero-hACE2, or Vero-ACE2KO cells, suggesting their RcACE2-dependent infectivity. In contrast, we observed that SARS-CoV-2 replicated efficiently in Vero/TMPRSS2, Vero-RcACE2, and Vero-hACE2 cells, but not in Vero-ACE2KO cells (Figure 2), suggesting multiple ACE2-dependent infectivity, including that of *R. cornutus* bats. These data suggested that at isolates from Japan use only bRcACE2 as a receptor, showing narrow host specificity.

Table 2. Full-genome nucleotide identity for sarbecovirus isolates from bats, Japan

Isolate	Rc-o319	Rc-os20	Rc-kw8	Rc-mk2
Rc-o319	–	95.6%	96.8%	94.8%
Rc-os20	–	–	95.4%	95.4%
Rc-kw8	–	–	–	95.1%
SARS-CoV-2	81.5%	80.7%	81.4%	80.7%

Conclusions

We isolated bat sarbecoviruses from *R. cornutus* bats in several locations in Japan that were phylogenetically positioned in the same cluster of the SARS-CoV-2–related viruses. These isolates used only bat ACE2 as a receptor and did not replicate in hACE2-expressing cells, forming a unique type, and suggesting a low potential for human infection.

To our knowledge, this type of bat sarbecoviruses has not been previously isolated (*13*) because African green monkey Vero cells having highly similar ACE2 to hACE2 were used for viral isolation attempts in the previous studies (*4*,*5*). Cultivable bat sarbecoviruses provide a useful and powerful tool to determine their characteristics, such as receptor specificity and pathogenicity in animals, leading to elucidation of spillover potential.

Rhinolophus spp. bats are relatively short-distance migrants (*14*) and lack frequent cross-contact between bat groups, explaining why most genome sequences were highly conserved among strains from Japan. Exceptions were the RBD-coding and NTD-coding regions of the S gene, which show high variation caused by immune pressure (*15*), suggesting

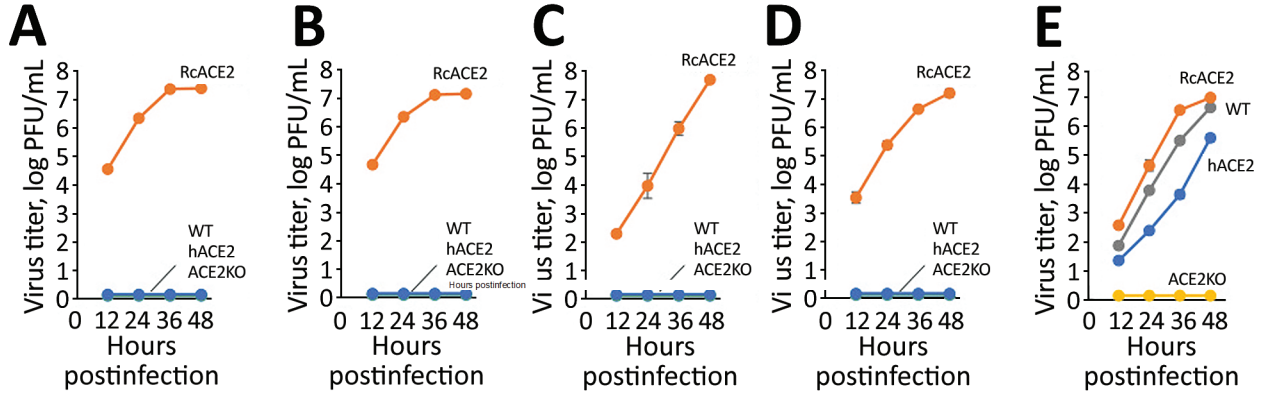

Figure 2. Growth kinetics of sarbecovirus isolates from bats in Japan. *Rhinolophus cornutus* bat isolates Rc-o319 (A), Rc-os20 (B), Rc-mk2 (C), and Rc-kw8 (D) or SARS-CoV-2 (B.1.1.7) (E) were inoculated into Vero/TMPRSS2 (WT), Vero-RcACE2 (RcACE2), Vero-hACE2 (hACE2), or Vero-ACE2KO (ACE2KO) cells at a multiplicity of infection of 0.01. The culture supernatants were collected at the indicated time points, and viral titers were determined by using a plaque assay. Data are reported as the mean titer with standard deviations from 3 independent experiments. ACE2, angiotensin converting enzyme 2; hACE2, human ACE2; RcACE2, *R. cornutus* ACE2; WT, wild-type

that they diverged relatively recently from the undefined ancestral virus. Because sarbecoviruses might mutate and infect humans by intermediate hosts in wildlife or livestock, epidemiologic studies of sarbecoviruses in wildlife, including bats, need to be conducted on a long-term basis for risk assessment of their zoonotic potential.

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Appendix

Materials and Methods

Cells and Virus

Vero/TMPRSS2 cells (*1*,*2*) were kindly provided by Dr. Makoto Takeda (National Institute of Infectious Diseases, Tokyo, Japan) and maintained in Dulbecco modified Eagles medium (DMEM; Nacalai Tesque, https://www.nacalai.co.jp) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cells were cultured in 5% CO_2 at 37°C. SARS-CoV-2 (B.1.1.7, α variant, UT-HP127–1Nf/Human/2021/Tokyo) was propagated in Vero/TMPRSS2 cells, and aliquots were stored at −80°C.

Sample Collection

We collected 88 fresh fecal samples, 58 from *Rhinolophus cornutus* and 30 from *Rhinolophus ferrumequinum* bats living in caves, abandoned mines, or abandoned tunnels in Niigata, Chiba, and Shizuoka prefectures in Japan (Table 1). When bats were densely packed during the daytime roost, plastic sheets were placed under the roost for 1–2 h. Fresh feces that dropped onto the sheets were collected. When bats were sporadically placed during the daytime roost, we captured bats after obtaining permission from the prefectural

local governments (no. 962 for Chiba and no. 311 for Shizuoka) and kept each bat in a separate nonwoven fabric bag. Feces excreted by the bat in the bag were collected and bats were released. Fecal samples were transferred into tubes containing phosphate-buffered saline supplemented with 200 U/mL penicillin, 200 μg/mL streptomycin, and 0.25 μg/mL amphotericin B, and immediately frozen in dry ice.

Reverse Transcription PCR

RNA was extracted from the fecal samples by using the RNeasy PowerMicrobiome Kit (QIAGEN, https://www.qiagen.com), and the partial envelope gene of sarbecovirus was detected in RNA samples by real-time reverse transcription-PCR (rRT-PCR) using the RNA-direct SYBR Green Realtime PCR Master Mix (Toyobo, https://www.toyobo-global.com) and a pair of primers (5′-TCGGAAGAGACAGGTACGTT-3′ and 5′-TCGAAGCGCAGTAAGGATGG-3′) that were designed to target a highly conserved region of the sarbecovirus envelope gene.

Establishment of ACE2 Stably Expressing Cells

We constructed a plasmid, pCAGGS-blast, by inserting the XhoI-EcoRV fragment of pMXs-IRES-Bsd (*3*), which contains the encephalomyocarditis virus internal ribosomal entry site and blasticidin-resistant gene, into the *Xho*I and *Stu*I sites of the pCAGGS-MCS vector. Open reading frame sequences of RcACE2 or hACE2 were PCR-amplified from pCAGGS-RcACE2- or pCAGGS-hACE2-expressing plasmids (*4*) and cloned into *Eco*RI- and *Xho*I-digested pCAGGS-blast plasmids by using NEBuilder (New England Biolabs, https://www.neb.com). Vero/TMPRSS2 cells were transfected with pCAGGS-blast-

RcACE2 or pCAGGS-blast-hACE2 plasmids by using the PEI MAX Transfection Reagent (Polysciences, https://www.polysciences.com).

Transfected cells were treated with 10 μg/mL blasticidin S (Kaken Pharmaceutical, https://www.kaken.co.jp) 1 d posttransfection, and blasticidin S-resistant cells were selected and cloned. Highly susceptible cell clones for the pseudotyped viral infection were selected by screening using GFP-expressing VSV-pseudotyped virus possessing the S protein of Rc-o319 or SARS-CoV-2 (*4*), generating RcACE2- or hACE2-stably expressing Vero/TMPRSS2 cells (namely Vero-RcACE2 or Vero-hACE2, respectively).

Establishment of ACE2-Knockout Cells

We generated ACE2-knockout Vero/TMPRSS2 cells (Vero-ACE2KO) by knocking out the corresponding genes using the CRISPR/Cas9 system. The target sequence for the ACE2 gene (5′-TGCTGCTCAGTCCACCATTG-3′) was designed by using CRISPR direct (https://crispr.dbcls.jp) and cloned into plentiCRISPR plasmids (*5*). Addgene plasmid #52961, a gift from Dr. Feng Zhang) using NEBuilder (New England Biolabs). Vero/TMPRSS2 cells were transfected with an ACE2-targeting plasmid by using PEI MAX (Polysciences). At 24-h posttransfection, the cell supernatant was replaced with medium containing 10 μg/mL puromycin. Drug-resistant clones were randomly selected, and their genomic DNA was sequenced. Cells having insertions or deletions (in/dels) in the targeted gene were chosen for further analysis.

Isolation of Bat Sarbecoviruses

Fecal samples positive for the partial envelope gene of sarbecovirus were homogenized in TissueLyser II (QIAGEN) by using 0.1-mm glass beads (Tomy Seiko, https://bio-tomys-co-jp) in phosphate-buffered saline containing 200 U/mL penicillin, 200 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. The supernatants were collected after centrifugation at 5000 × g for 5 min at 4°C and diluted 100-fold in cell maintenance medium (DMEM supplemented with 1% fetal bovine serum, 200 U/mL penicillin, 200 μg/mL streptomycin, and 0.25 μg/mL amphotericin B). Diluents were inoculated into 6-well plates containing Vero-RcACE2 cells, and plates were incubated for 60 min at 37°C after removing the inoculum. Wells were then washed once with cell maintenance medium, another 2 mL of cell maintenance medium was added to each well, and incubated at 37°C. Supernatants from cells that exhibited cytopathic effects were collected at 3–4 d postinoculation and were passed through a 0.22-μm filter. The successful isolation of viruses was confirmed by rRT-PCR, and isolates were propagated in Vero -RcACE2 cells, and aliquots were stored at −80°C.

Next-Generation Sequencing

A cDNA library was prepared from RNA extracted from bat sarbecoviral isolates by using the TruSeq Stranded Total RNA LT Sample Prep Kit Gold (Illumina, https://www.illumina.com) for Rc-mk2 and Rc-kw8 strains or the MGIEasy RNA Directional Library Prep kit (MGI, https://en.mgi-tech.com) for the Rc-os20 strain. Libraries of Rc-mk2 and Rc-kw8 strains were sequenced by using a Novaseq 6000 Sequencer (Illumina), and those of Rc-os20 were sequenced by using a DNBSEQ-G400RS

sequencer (MGI). Read sequences were mapped to the Rc-o319 genome sequence (GenBank accession no. LC556375), and sarbecoviral sequences were determined by using CLC Genomic Workbench Version 8.0.1 software (QIAGEN). Sequences of Rc-os20, Rc-mk2, and Rc-kw8 have been deposited in GenBank (accession nos. LC663958, LC663959, and LC663793, respectively).

Phylogenetic Analysis

The nucleotide sequences of sarbecoviruses were aligned by using ClustalW version 2.1 (Clustal, https://www.clustal.org). Phylogenetic trees were then constructed by performing a maximum-likelihood analysis by using EGA version X (*6*) in combination with 500 bootstrap replicates.

Evaluation of Viral Growth in Cells

Cells were inoculated with viruses at a multiplicity of infection of 0.01 and incubated for 1 h for viral adsorption. After removing the inocula, cells were incubated in cell maintenance medium, and the supernatants were collected at 12-h intervals. Viral titers were measured by using a plaque assay, in which cells inoculated with diluted viruses were overlaid and incubated with DMEM containing 1% agarose and 1% fetal calf serum for 2 d, followed by staining with crystal violet before counting plaques.

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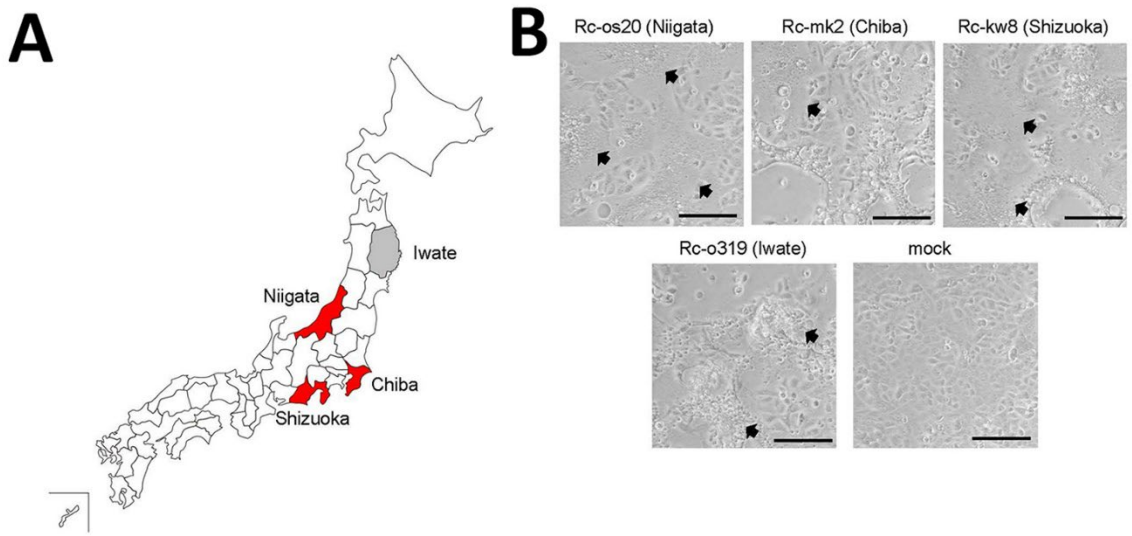
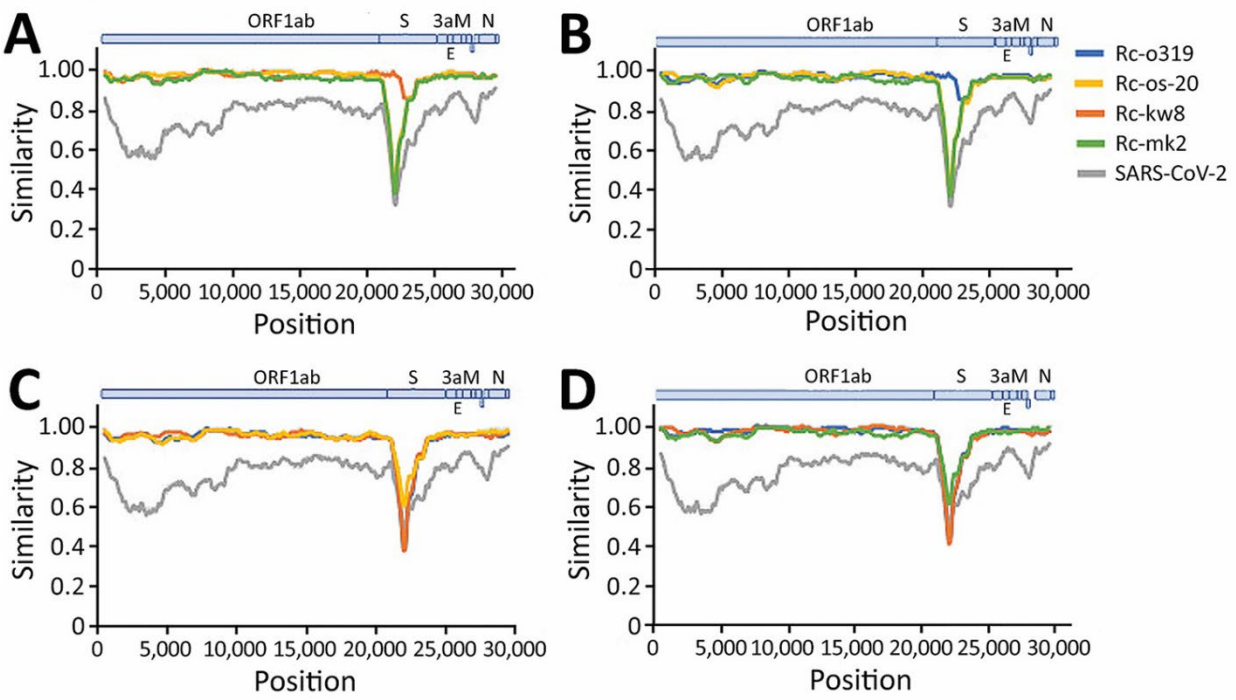
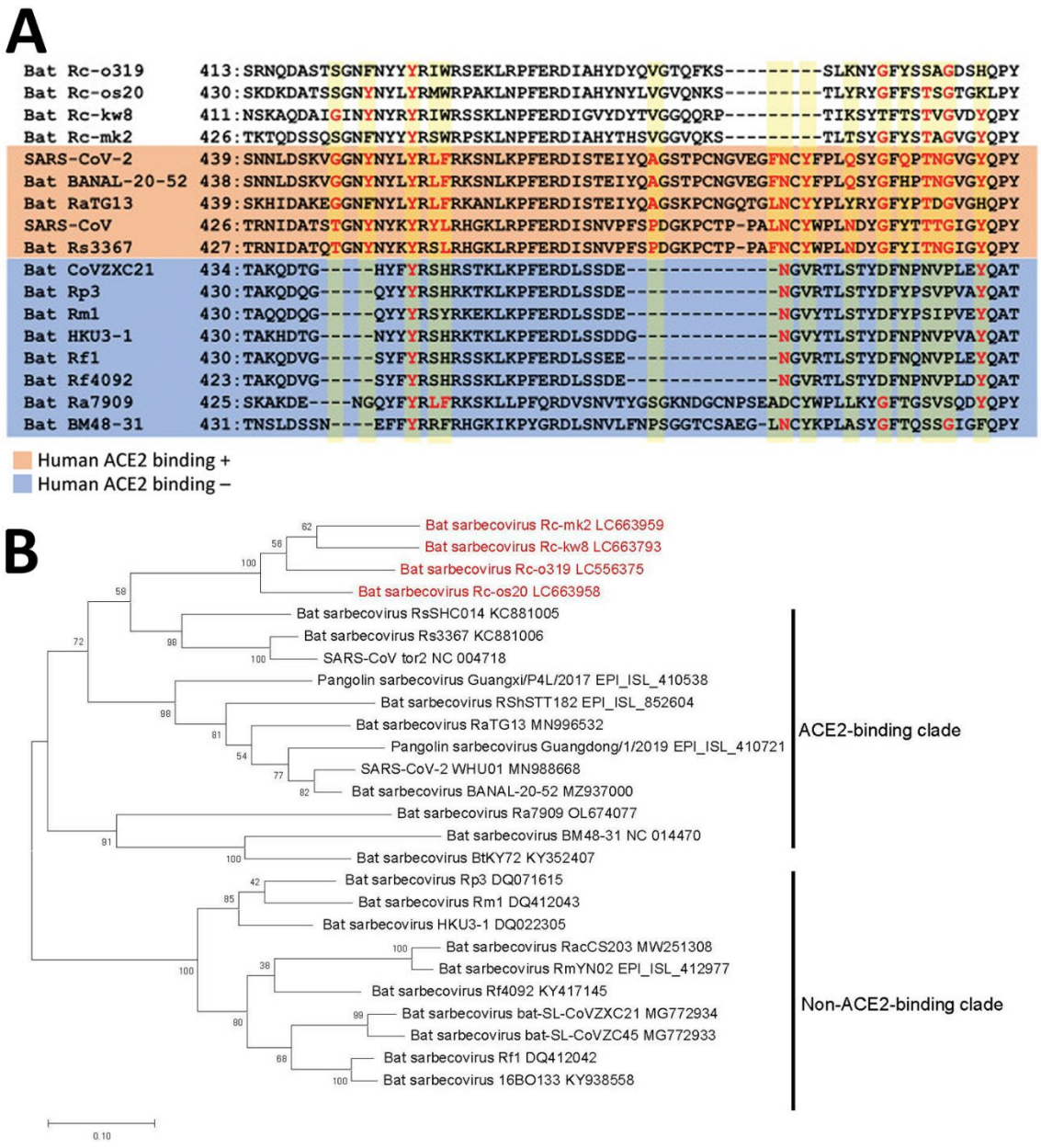


Appendix Figure 1. A) Sampling locations in Japan. Prefectures, where bats were captured, are indicated in red. Prefectures, where bat sarbecoviruses were detected in our previous study, are indicated in gray. B) Images of bat sarbecovirus-inoculated cells. Vero-RcACE2 cells were inoculated with fecal samples from *Rhinolophus cornutus* bats from several prefectures of Japan. After 1–2 d of inoculation, cytopathic effects with extensive syncytium formation (arrowheads) were observed. Scale bar indicates 200 μm.



Appendix Figure 2. Genetic analysis of 4 bat sarbecovirus isolates, Japan. Similarity plot analysis of isolates was performed by using the full-length genome sequence of A) Rc-o319, B) Rc-os20, C) Rc-kw8, or D) Rc-mk2 for comparison. SARS-CoV-2 virus was used as a reference. E, envelope; M, matrix; N, nucleocapsid; ORF, open reading frame; S, spike; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.



Appendix Figure 3. A) Alignment of the receptor-binding motif sequence of spike proteins of bat sarbecovirus isolates. Amino acid positions of receptor-binding motifs contacting human angiotensin converting enzyme-2 (ACE2) identified in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are indicated in yellow, residues identical to SARS-CoV or SARS-

CoV-2 are indicated in red, strains that are capable of binding to human ACE2 are indicated in orange, and strains that are incapable of binding to human ACE2 are indicated in blue. B) Phylogenetic tree of bat sarbecoviruses was generated using the receptor binding domain (RBD) nucleotide sequences with the maximum-likelihood analysis combined with 500 bootstrap replicates. Red text indicates the isolates in this study. Bootstrap values are shown above and to the left of the major nodes. Scale bars indicate nucleotide substitutions per site.