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Detection and Characterization of Bat Sarbecovirus Phylogenetically Related to SARS-CoV-2, Japan

Appendix

Additional Methods

Sample Collection

We captured 4 *Rhinolophus cornutus* bats in a cave in Iwate prefecture, Japan, in 2013, with permission from the prefectural local government. Each bat was kept in a separate plastic bag. Fresh feces samples were collected, transferred into tubes containing sterilized saline, and frozen in dry ice. We released the bats after feces collection.

Reverse Transcription-PCR

RNA was extracted from the feces samples using an RNeasy PowerMicrobiome Kit (QIAGEN, https://www.qiagen.com). Next, we detected the partial RNA-dependent RNA polymerase (RdRp) gene of sarbecovirus in 2 samples by real-time reverse transcription-PCR by using RNA-direct SYBR Green Realtime PCR Master Mix (TOYOBO, https://www.toyobo-global.com) and a pair of primers (5′-CATATGCAGTAGTGGCATCA-3′ and 5′-GCTGTAACTTGTCACATCGT-3′) that were designed based on a previous report (*1*).

Next-Generation Sequencing

A cDNA library was prepared from RNA extracted from the feces sample by using the SMARTer Stranded RNA-Seq Kit (Takara-Bio, https://www.takarabio.com). The library

was sequenced by using a NovaSeq 6000 (Illumina, https://www.illumina.com) sequencer. The read sequences were mapped on RaTG13 (GenBank accession no. MN996532), and the Rc-o319 sequence was determined by using CLC genomic workbench version 8.0.1 (QIAGEN, https://www.qiagen.com) software. The sequence was deposited in GenBank (accession no. LC556375).

Phylogenetic Analysis

The nucleotide and amino acid (aa) sequences of sarbecoviruses were aligned by using ClustalW version 2.1(Clustal, https://www.clustal.org). Phylogenetic trees were constructed by performing maximum-likelihood analysis with MEGA version X (*2*), in combination with 500 bootstrap replicates.

Plasmids

We cloned the spike protein (S) genes of Rc-o319 and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; 2019-nCoV/Japan/AI/I-004/2020, GenBank accession no. LC521925), with a 19–aa deletion at the C-terminus (pCAGGS-o319-S-del19), into protein expression pCAGGS vectors (pCAGGS-SARS-CoV-2-del19). Earlier reports have suggested that this deletion leads to the efficient production of vesicular stomatitis virus (VSV) pseudotyped with the S gene of SARS-CoV (*3*). We used the SARS-CoV S gene expression plasmid pKS-SARS-St19 (*3*) and the vesicular stomatitis Indiana virus glycoprotein (G)-expression plasmid pCAGGS-VSV-G (*4*). We also constructed angiotensin-converting enzyme 2 (ACE2)-expression plasmids derived from humans (hACE2; GenBank accession no. NM_001371415), greater horseshoe bats (*R. ferrumequinum*) (Rf-ACE2; GenBank accession no. AB297479), and Chinese rufous horseshoe bats (*R. sinicus*) (Rs-ACE2; GenBank accession no. KC881004). The hACE2 cDNA was amplified from RNA isolated from Caco-2 cells by RT-PCR, and the Rf-ACE2 and Rs-ACE2 genes were

artificially synthesized and cloned into pCAGGS plasmids (pCAGGS-hACE2, pCAGGS-Rs-ACE2, or pCAGGS-Rf-ACE2, respectively).

Because fresh RNA samples from *R. cornutus* bats were not available, the ACE2 gene of *R. cornutus* (Rc-ACE2) was amplified from the exon regions of genomic DNA extracted from the kidney of a bat carcass (GenBank accession no. LC564973) and cloned into the pCAGGS vector that was designated as pCAGGS-Rc-ACE2. We also prepared a chimeric bat ACE2 (referred to as Rc/Rf chimera), which consisted of the S interaction domain of Rc-ACE2 (aa positions 15–116) and the remaining region from Rf-ACE2, to form the expression plasmid pCAGGS-Rc/Rf-ACE2.

Western Blot Analysis

HEK293T cells were transfected with pCAGGS-RcACE2, pCAGGS-hACE2, pCAGGS-RfACE2, pCAGGS-RsACE2, pCAGGS-Rc/RfACE2, or empty pCAGGS vectors. One day after transfection, the cells were lysed with SDS sample buffer and subjected to western blotting by using rabbit anti-ACE2 antibody (Abcam, https://www.abcam.com).

Production of VSV-Pseudotyped Virus

VSVΔG*-GFP, which expresses a GFP reporter gene instead of the viral G gene, was used to produce VSV pseudotyped with S genes from sarbecoviruses, as described in earlier reports (*3*,*5*). HEK293T cells seeded on 6-well plates were transfected with 2 μg of either pCAGGS-o319-S-del19, pCAGGS-SARS-CoV-2-del19, pKS-SARS-St19, or control pCAGGS-VSV-G. At 24 h post-transfection, the cells were infected with VSVΔG*-GFP and incubated for 24 h. The culture fluid was collected, centrifuged, filtered through a 0.45-μm filter to remove cells and cell debris, and stored at −80°C until use. The pseudotyped viruses with Rc-o319 S were designated as VSV-Rc-o319, for SARS-CoV S were designated VSV-SARS, for SARS-CoV-2 S were designated VSV-SARS-2, and for VSV-G were designated VSV-VSV-G. The viruses pseudotyped with sarbecovirus S proteins were incubated with the

anti-VSV-G neutralizing antibody I1 (*6*) for 30 min at 21°C to eliminate the remaining VSVΔG*-GFP.

Cell Entry Assay

HEK293T cells seeded on 24-well plates were transfected with 0.5 μg of either pCAGGS-Rc-ACE2, pCAGGS-Rf-ACE2, pCAGGS-Rs-ACE2, pCAGGS-Rc/Rf-ACE2, pCAGGS-hACE2, or control empty pCAGGS plasmids, and incubated for 24 h. Each pseudotyped virus (200 μL) was used to inoculate each ACE2-expression cell culture. After incubation at 37°C for 1 h, the cells were washed once with Opti-MEM (Thermo Fisher Scientific, https://www.thermofisher.com) and incubated with Opti-MEM at 37°C for 20 h. The number of GFP-positive cells within 1 microscopic field (3.1 mm^2) was counted under an Axio Vert.A1 fluorescent microscope (Carl Zeiss, https://www.zeiss.com). The virus titers were determined as the number of GFP-positive cells per well in a 24-well plate (1.9 cm^2), calculated for 5 microscopic fields. The virus titers are expressed in terms of the average values with standard deviations from 3 independent experiments.

Cell Fusion Assay

HEK293T cells were cotransfected with 1 of the S-expression plasmids (pCAGGS-o319-S-del19, pCAGGS-SARS-CoV-2-del19, or pKS-SARS-St19) and 1 of the ACE2-expression plasmids (pCAGGS-Rc-ACE2, pCAGGS-Rf-ACE2, pCAGGS-Rs-ACE2, or pCAGGS-hACE2), with or without the TMPRSS2-expression plasmid, and a fluorescent reporter Venus-expression plasmid (*7*) for the convenient visualization of fused cells under a fluorescence microscope. After transfection, the cells were incubated at 37°C for 24 h. The fused cells were observed under an Axio Vert.A1 fluorescence microscope (Carl Zeiss). As a control, we confirmed that no appreciable syncytium was observed in HEK293T cells transfected with each S-expression plasmid, which indicates that endogeneous hACE2 in these cells did not affect the results of the cell fusion assay.

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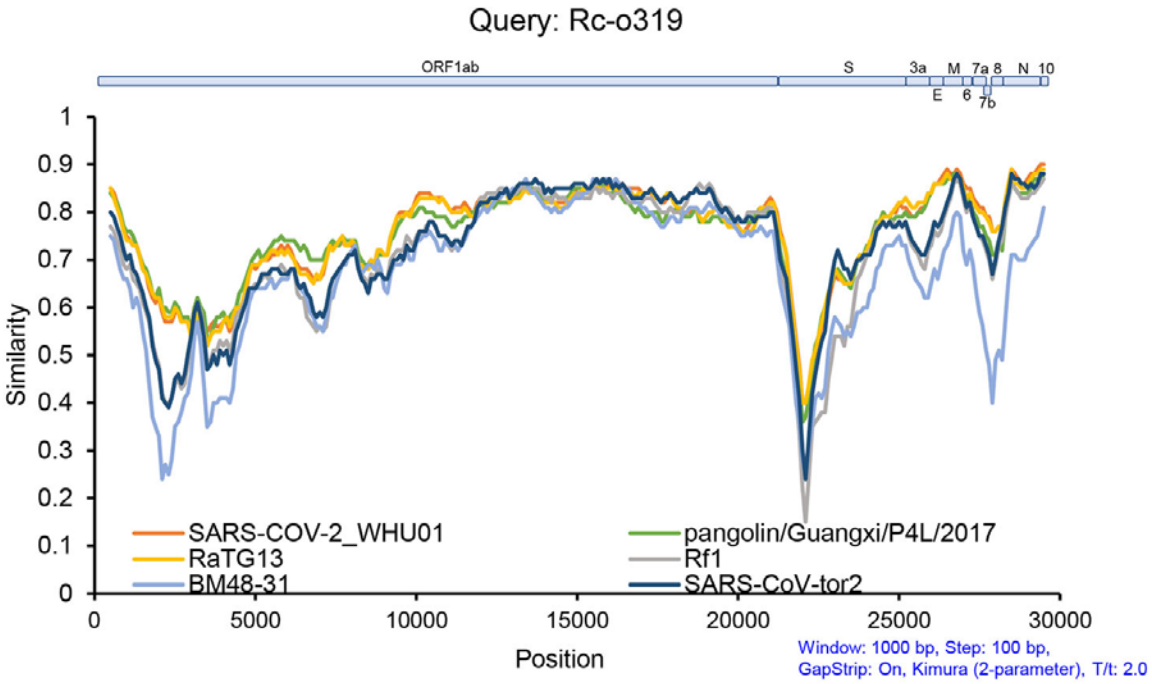
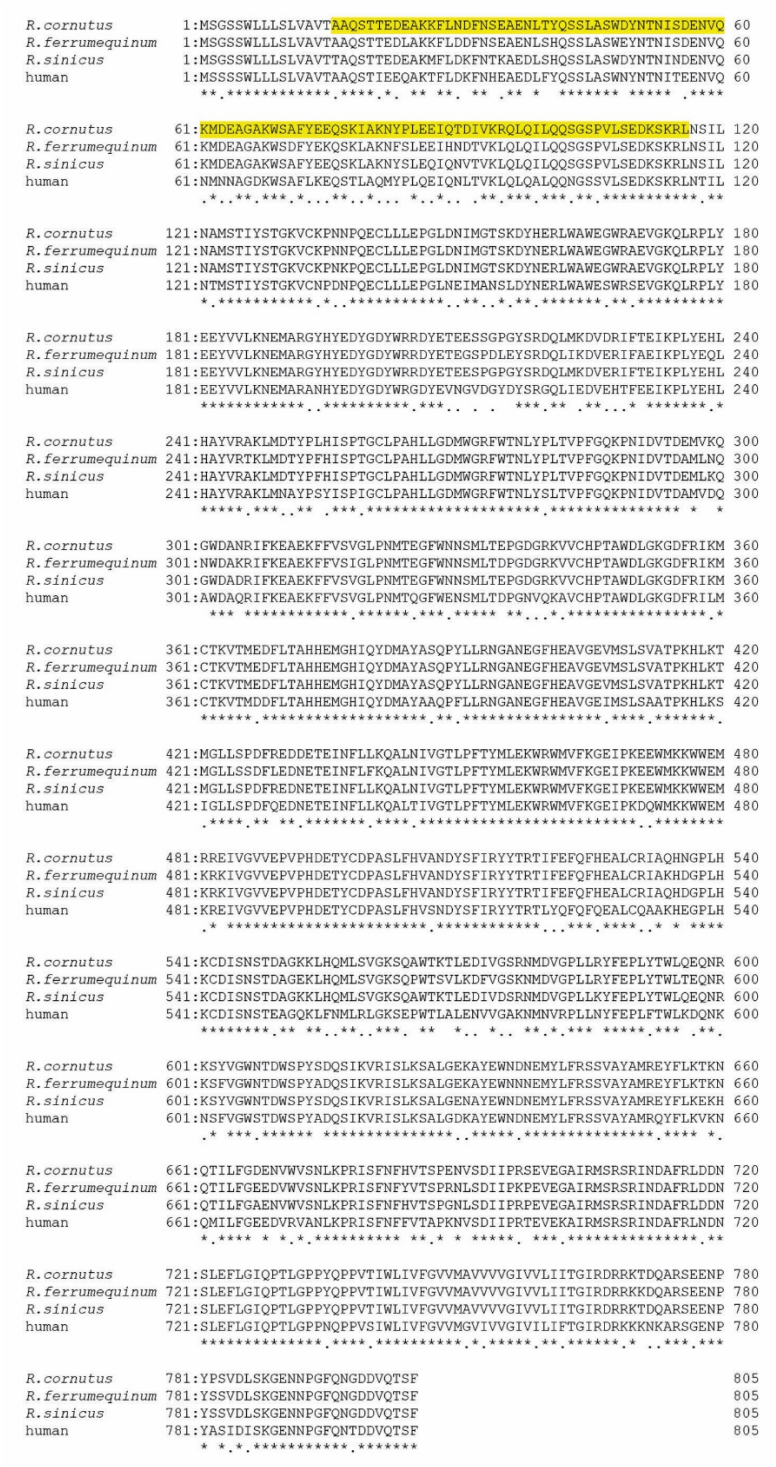
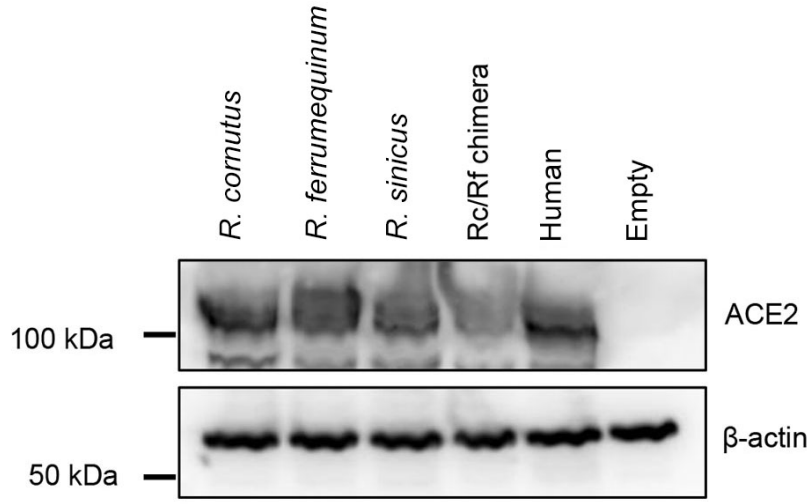


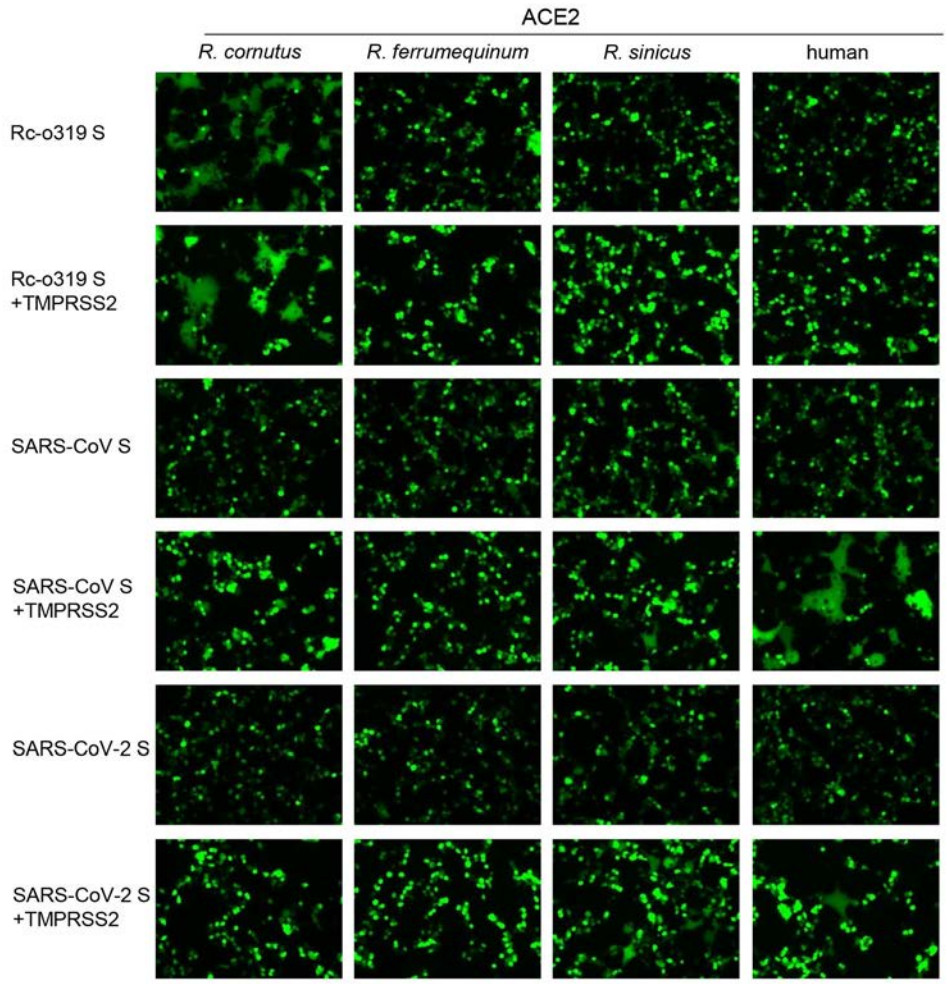
Appendix Figure 1. Similarity plot analysis of sarbecovirus Rc-o319 sequenced from little Japanese horseshoe bats (*Rhinolophus cornutus*) and genetically related to human SARS-CoV-2, Japan. Full-length genome sequence of Rc-o319 was used as query. Representative sequences from sarbecoviruses were used as references. ORF1ab, open reading frame 1ab; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.



Appendix Figure 2. Alignment of ACE2 amino acid sequence from *Rhinolophus cornutus*, *R. ferrumequinum*, *R. sinicus,* and humans. Deduced amino acid sequence of *R. cornutus* ACE2 RNA was aligned with those of *R. ferrumequinum, R. sinicus,* and human ACE2. Highlighted yellow region was considered the spike protein binding region of *R. cornutus* ACE2. ACE2, angiotensin-converting enzyme 2.



Appendix Figure 3. Western blot analysis showing expression of angiotensin-converting enzyme 2 in transfected cells from bats and humans. HEK293T cells that transiently expressed *Rhinolophus cornutus*, *R. ferrumequinum*, *R. sinicus*, Rc/Rf chimera (spike protein [S] interaction domain from *R. cornutus* and the remaining from *R. ferrumequinum*), or human ACE2. ACE2, angiotensin-converting enzyme 2; HEK293T cells, human embryonic kidney 293T cells.



Appendix Figure 4. Results of fusion assay in which HEK293T cells were cotransfected with S-expression plasmids and ACE2 from bat and human sarbecoviruses. S-expression plasmids of Rc-o319, SARS-CoV, or SARS-CoV-2 and expression plasmids of Rc-ACE2, Rf-ACE2, Rs-ACE2, or hACE2, and fluorescent reporter Venus-expression plasmid with and without TMPRSS2-expression plasmid were incubated for 24 h to assess syncytium formation. At 24 h post-transfection, the cells were observed under a fluorescence microscope. ACE2, angiotensin-converting enzyme 2; hACE2, human ACE2; HEK293T cells, human embryonic kidney 293T cells; Rc-o319, sarbecovirus identified in this study; Rc-ACE2, *Rhinolophus cornutus* ACE2; Rf-ACE2, *R. ferrumequinum* ACE2; Rs-ACE2, *R. sinicus* ACE2; S, spike glycoprotein; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TMPRSS2, transmembrane serine protease 2.