1 Identification of a novel lineage bat SARS-related coronaviruses that use bat

2 ACE2 receptor

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14 Author contributions

15 Z-L.S and P.Z. conceived the study. X-L.Y organized sampling. B.H., H-R.S, B.L.

16 and Y.Z. performed viral genome sequencing and bioinformatics analysis. H.G., W.Z.,

17 A.L. and R.G. performed protein expression and RBD-ACE2 binding assays. H.G.

18 and H-F.L. performed pseudovirus work. H.G, H.B, P.Z and Z-L.S wrote the paper

19 with input from all authors.

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21

22 **Abstract**

23 Severe respiratory disease coronavirus-2 (SARS-CoV-2) causes the most devastating
 24 disease, COVID-19, of the recent century. One of the unsolved scientific questions
 25 around SARS-CoV-2 is the animal origin of this virus. Bats and pangolins are
 26 recognized as the most probable reservoir hosts that harbor the highly similar SARS-
 27 CoV-2 related viruses (SARSr-CoV-2). Here, we report the identification of a novel
 28 lineage of SARSr-CoVs, including RaTG15 and seven other viruses, from bats at the
 29 same location where we found RaTG13 in 2015. Although RaTG15 and the related
 30 viruses share 97.2% amino acid sequence identities to SARS-CoV-2 in the conserved
 31 ORF1b region, but only show less than 77.6% to all known SARSr-CoVs in genome
 32 level, thus forms a distinct lineage in the *Sarbecovirus* phylogenetic tree. We then
 33 found that RaTG15 receptor binding domain (RBD) can bind to and use *Rhinolophus*
 34 *affinis* bat ACE2 (RaACE2) but not human ACE2 as entry receptor, although which
 35 contains a short deletion and has different key residues responsible for ACE2 binding.
 36 In addition, we show that none of the known viruses in bat SARSr-CoV-2 lineage or
 37 the novel lineage discovered so far use human ACE2 efficiently compared to SARSr-
 38 CoV-2 from pangolin or some of the SARSr-CoV-1 lineage viruses. Collectively, we
 39 suggest more systematic and longitudinal work in bats to prevent future spillover
 40 events caused by SARSr-CoVs or to better understand the origin of SARS-CoV-2.

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42

43 **Keywords:** SARS-related coronavirus, Novel lineage, Bat, Reservoir host, ACE2,

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45

46 **Introduction**

47 SARS-CoV-2, a novel coronavirus that causes COVID-19 which was first identified
48 in late 2019 [1], took just a few months to sweep the globe. As the largest pandemic
49 in the past century in human history, it not only results in serious impact on human
50 health but also leads to stagnation in economics, travel, education and many other
51 societal functions globally.

52

53 The natural origin of SARS-CoV-2 is one of the unanswered scientific questions
54 about the COVID-19 pandemic. It is generally believed that SARS-CoV-2 is
55 transmitted from an animal reservoir host to human society through an or multiple
56 intermediate hosts [2]. The discovery of SARS-CoV-2 related viruses (SARSr-CoV-
57 2), RaTG13 and Pangolin-CoV from horseshoe bats and pangolin respectively, shed
58 light on the importance of these two groups as animal reservoirs of SARSr-CoV-2
59 viruses [1,3,4]. However, among the six critical residues of the receptor-binding
60 domain (RBD) in spike to interact with human ACE2 receptor, RaTG13 only shares
61 one with SARS-CoV-2 [5]. The RBD of RaTG13 has a lower binding affinity and
62 usage efficiency with human ACE2 though sharing 96% genome sequence identity to
63 SARS-CoV-2 [6-8]. One of the viruses derived from Malayan pangolin (*Manis*
64 *javanica*), Pangolin-CoV-GD, possesses six identical critical residues of RBD with
65 SARS-CoV-2 and displays a similar binding affinity to human ACE2 compared with
66 SARS-CoV-2, although it shares lower sequence identity to SARS-CoV-2 in genome
67 compared with RaTG13 [4,7,8]. Another SARSr-CoV-2 detected from bat
68 (*Rhinolophus malayanus*), RmYN02, contains a similar insertion at the S1/S2
69 cleavage site in the spike of SARS-CoV-2, but it has some deletions in the RBD and
70 fails to bind with human ACE2 [9]. Besides, more SARSr-CoV-2 viral genome

71 sequences from bats have been reported from Eastern China, Japan, and Southeast
72 Asian countries subsequently [10-13]. However, the progenitor virus that shares
73 >99% identical to SARS-CoV-2 was still undetermined.

74
75 Bats also carry SARSr-CoV with all the genetic building blocks of SARS-CoV-1,
76 which jumped to human in 2002 [14]. Therefore, investigation of bat SARSr-CoVs is
77 not only important for tracing the origin and immediate progenitor viruses of SARS-
78 CoV-2, but also critical for public health measures to prevent future outbreaks caused
79 by this species of viruses. Here, we report the genome characterization and viral
80 receptor analysis of a novel lineage of SARSr-CoVs in Tongguan town, Mojiang
81 county, Yunnan province in China in 2015, the same location where we found bat
82 RaTG13 in 2013 [1].

83

84 **Methods**

85 ***Bat Sampling and Coronavirus Detection***

86 Sampling of bat was conducted in Mojiang county, Yunnan province at May 2015.
87 Bats were released after anal swabs sampling. Samples were aliquoted and stored at -
88 80 °C until use. RNA was extracted using the High Pure Viral RNA Kit (Roche,
89 Basel, Switzerland). Partial RdRp was amplified using the SuperScript III OneStep
90 RT-PCR and Platinum Taq Enzyme kit (Invitrogen, Carlsbad, CA, USA) by family-
91 specific degenerate seminested PCR. The PCR products were gel purified and
92 sequenced with an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City,
93 CA). The sequences were blasted against the GenBank database.

94

95 ***Genome Sequencing***

96 For SARSr-CoV positive RNA extractions, next-generation sequencing (NGS) was
 97 performed using BGI MGISEQ 2000. NGS reads were first processed by Cutadapt
 98 (v.1.18) to eliminate the possible contamination. Then the clean reads were assembled
 99 into genomes using Geneious (v11.0.3) and MEGAHIT (v1.2.9). PCR and Sanger
 100 sequencing were used to fill the genome gaps. To amplify the terminal ends, a
 101 SMARTer RACE 5`/3`kit (Takara) was used. Bat species identification was based on
 102 the partial sequence of cytochrome c oxidase subunit I (COI) gene.

103

104 ***Phylogenetic Analysis***

105 Routine sequence management and analysis were carried out using DNASTar.
 106 Sequence alignments were created by ClustalW implemented in MEGA6 with default
 107 parameters. Maximum Likelihood and Neighbour-joining phylogenetic trees were
 108 generated using the Jukes-Cantor model with 1000 bootstrap replicates in the MEGA6
 109 software package. Similarity plot analysis of the full-length genome sequences was
 110 conducted by Simplot 3.5.1. The genome ID used in the analysis are MN996528 for
 111 SARS-CoV-2, AY278488 for SARS-CoV-1, MN996532 for the bat SARSr-CoV
 112 RaTG13, MG772933 for ZC45, MW251308 for RacCS203, LC556375 for Rc-o319,
 113 KF367457 for WIV1, DQ022305 for HKU3-1, MT121216 for pangolin-CoV-GD
 114 strain, MT072864.1 for pangolin-CoV-GX strain, EPI_ISL_412977 for bat SARSr-
 115 CoV RmYN02, EPI_ISL_852604 for RshSTT182. The National Genomics Data
 116 Center of China ID for the eight novel lineage SARSr-CoVs are:
 117 GWHBAUM01000000- GWHBAUT01000000.

118

119 ***Expression Constructs, Protein Expression, and Purification***

120 Codon-optimized RBD genes from the following viruses were used (see above
121 genome accession number): SARS-CoV-2 (spike aa 330-583), SARS-CoV-1 (spike aa
122 317–569), RaTG13 (spike aa 330-583), pangolin-CoV-GD (spike aa326-579),
123 pangolin-CoV-GD (spike aa 330-583), RaTG15 (spike aa 317-566). They were
124 synthesized (Sangon Biotech, Shanghai, China) and placed into the expression vector
125 with an N-terminal signal peptide and an S-tag as described previously [15]. The
126 ectodomains of human ACE2 (aa 19–615, accession number: AB046569) and
127 *R.affinis* ACE2 (aa 19–615, accession number: MT394204) were amplified and
128 cloned into the same expression vector as above.

129
130 The RBD and ACE2 proteins used for the BLI binding assay were produced in HEK
131 293T/17 cells. Cells were transiently transfected with expression plasmids using
132 Lipofectamine 3000 (Life Technologies), washed twice with D-Hanks solution 6 h
133 post-transfection, and followed with culturing in fresh 293T FreeStyle expression
134 medium (Life Technologies) at 37°C in a humidified 5% CO₂ incubator. The
135 supernatant were harvested 48 h post-transfection and centrifuged at 4000 × *g* for 10
136 min at 4°C. Clarified supernatant were purified by S-tag agarose beads and eluted
137 with 3 M MgCl₂. The purified protein was finally buffered with PBS and quantified
138 using Qubit 2 Fluorometer (Thermo Fisher Scientific), and stored at -80 °C until use.

139

140 ***Bio-layer Interferometry Binding Assays***

141 Binding assays between RBDs and ACE2 proteins were performed using the Octet
142 RED system (ForteBio, Menlo Park, CA, USA) in 96-well microplates at 30°C with
143 shaking at 1000 rpm as described previously [15]. Briefly, the RBD was biotinylated
144 using EZ-Link NHS-LC-LC-Biotin (Thermo Fisher Scientific, Waltham, MA, USA).

145 The Streptavidin Biosensors were activated for 200s prior to coupling with 50 µg/mL
 146 biotinylated RBD proteins for 600s. A baseline were collected in the kinetic buffer (1
 147 M NaCl, 0.1% BSA, 0.02% Tween-20; pH 6.5) for 200s before immersing the sensors
 148 in a 1:2 serial diluted ACE2 proteins for 900s and then dissociation in the same
 149 kinetic buffer for another 900s. Data analysis from the ForteBio Octet RED
 150 instrument includes reference subtraction. Inter-step correction and Y-alignment were
 151 used to minimize tip-dependent variability. Curve fitting were performed in a 1:1
 152 model using the Data Analysis Software v7.1 (ForteBio, Menlo Park, CA, USA). The
 153 mean K_{on} , K_{off} values were determined with a global fit applied to all data. The
 154 coefficient of determination (R^2) for these interactions was close to 1.0.

155

156 ***Pseudovirus Entry Assays***

157 Pseudotyped VSV- Δ G particles were generated as previously described with minor
 158 adjustments [16]. Briefly, HEK 293T/17 cells were seeded at 6-well-plate and
 159 transfected with plasmids contain codon-optimized SARSr-CoV-2 spike at a 70%
 160 confluency using Lipofectamine 3000. At 6 h post-transfection, the medium was
 161 replaced with fresh DMEM+10%FBS medium. At 24 h after transfection, cells were
 162 incubated with VSV-G-pseudotyped VSV Δ G/Fluc at 37°C for 1 h. Cells were
 163 subsequently washed five times and supplied with fresh DMEM + 10% FBS medium
 164 + anti-VSV-G antibody (Kerafast). Cell-free supernatants were harvested at 24 h after
 165 transduction, then centrifuged at $4000 \times g$ for 10 min at 4°C. The virus particles were
 166 used for infection directly.

167

168 The 48-well-plate was treated with Poly-L-lysine solution (Sigma) before seeded
 169 HEK293T/17 cells. Cells were transiently transfected with equal amounts of human
 170 ACE2, *R. affinis* ACE2 or empty vector plasmids at 70% confluency. At 24 h post-
 171 transfection, the cells were incubated with same amounts of S-pseudotyped virions for
 172 1 h at 37°C, then washed twice with PBS solution, and supplemented with DMEM
 173 containing 10% FBS. Luciferase activity was determined using a GloMax
 174 luminometer (Promega Biotech Co. Ltd., Beijing, China) 48 h after infection.
 175 Infection experiments were performed independently in triplicate with three technical
 176 replications each time.

177

178 ***Quantification of Pseudotyped Virus Particles using RT-PCR***

179 Viral RNA of all VSV-spike pseudovirus particles were extracted from 200ul
 180 supernatant using the High Pure Viral RNA Kit (Roche, Cat. No. 11858882001)
 181 following the supplier's manual. Quantification of pseudovirus by real-time PCR was
 182 performed using HiScript® II One Step qRT-PCR SYBR Green Kit (Vazyme, Cat.
 183 No. Q221-01). The gene of VSV P protein were amplified and synthesized *in vitro*
 184 using mMESSAGE mMACHINE® Kit (Life technologies, Cat. No. AM1344) to
 185 serve as a standard. Viral copy numbers were calculated according to the standard
 186 curve. Primers used for transcription *in vitro* were: VSV (P protein)-F1:
 187 GTTCGTGAGTATCTCAAGTCCT, VSV (P protein)-R2-T7:
 188 TAATACGACTCACTATAGGGAGAGCCTTGATTGTCTTCAATTTCTGG,
 189 primers used for real-time PCR were described as previously [17].

190

191 **Results**

192 **Identification of a novel lineage of SARSr-CoVs**

193 In tracing the origin of SARS-CoV-2 from bats, we identified RaTG13, which shares
194 96.2% genome identity to SARS-CoV-2 and is so far the closest genome [1].
195 Following the investigation, we identified eight SARSr-CoV sequences that share
196 93.5% sequence identity to SARS-CoV-2 in the 402-nt partial RdRp gene from bat
197 samples collected at the same place in 2015. Seven samples were from *Rhinolophus*
198 *stheno*, and the other one was from *Rhinolophus affinis* (Table S1). We thus
199 performed next-generation sequencing (NGS) for further analysis of these CoVs.
200 Whole genome sequences were obtained from all eight individual samples. The eight
201 SARSr-CoV genomes are almost identical, sharing more than 99.7% sequence
202 identity among each other. One strain designated RaTG15 was used as the
203 representative in the subsequent analysis.
204
205 In the seven conserved replicase domains used for coronavirus species classification,
206 RaTG15 is 95.3% or 92.5% identical to SARS-CoV-2 and SARS-CoV-1 respectively,
207 suggesting that it remains a member of the *SARSr-CoV* species in the *Sarbecovirus*
208 subgenus within *Betacoronavirus* genus, *Coronaviridae* family. Further, RaTG15 is
209 genetically close to SARS-CoV-2 in open reading frame 1b (ORF1b). In the complete
210 ORF1b region, RaTG15 showed 84.6~89.0% nucleotide identities and 95.6~97.3%
211 amino acid sequence identities to bat SARSr-CoV-2 from wildlife in China and
212 Southeast Asia, which includes bat CoVs RaTG13 and RmYN02 from Yunnan, Rc-
213 o319 from Japan, RshSTT182 from Cambodia, RacCS203 from Thailand, as well as
214 two different strains of pangolin-CoVs (Table S2). It is also conceivable that RaTG15
215 clusters with SARSr-CoV-2 in the phylogeny using full-length RdRp gene (Figure
216 S1A).
217

218 In contrast, similarity plot analysis reveals that beyond ORF1b, RaTG15 is
 219 remarkably distinct from both SARSr-CoV-2 and SARSr-CoV-1 in majority of the
 220 genome (Figure 1A). It exhibits less than 80% nucleotide identities in ORF1a, M and
 221 N genes and lower than 70% identities in S, ORF3, 6 and 7a/7b to all other SARSr-
 222 CoVs (Table S2). Overall, the full genome of the SARSr-CoV RaTG15 show 74.4%
 223 sequence identity to SARS-CoV-1 and 77.6% to sequence identity to SARS-CoV-2.
 224 Notably, RaTG15 show higher sequence identity to SARS-CoV-1 than to SARS-
 225 CoV-2 in the spike, E, M, N and ORF6 proteins. It also has almost equivalent
 226 homology to any other known SARSr-CoVs from bat or pangolin CoVs (Table S2).
 227 This mosaic profile suggests that this novel lineage viruses may be a results of
 228 recombination of different SARSr-CoVs.

229
 230 The result of phylogenetic analysis is in accordance with similarity plot. SARSr-CoVs
 231 mainly consists of two sub-lineages, the SARSr-CoV-1 and SARSr-CoV-2 (Figure
 232 1B). The latter one includes SARS-CoV-2 from pangolins and different *Rhinolophus*
 233 bats species recently reported in a wide range of areas in Asia. In the full-length
 234 genome tree and S gene tree, RaTG15 and the related viruses are distant from both of
 235 the two existing sub-lineages, and forms a well-supported novel lineage with the
 236 *sarbecoviruses* (Figure 1B and Figure S1B).

237

238 **In silico analysis of receptor binding domain (RBDs) of SARSr-CoVs**

239 We further examined the spike protein sequence of RaTG15 in comparison with other
 240 SARSr-CoV-2. The receptor-binding domain (RBD) of the RaTG15 spike is highly
 241 divergent from other *sarbecoviruses*, with 72.6% amino acid sequence identity to
 242 SARS-CoV-2 and 68.6%-73.3% identities to related bat and pangolin CoVs. Unlike

243 RmYN02 and RacCS203, the RaTG15 RBD does not contain the deletion
 244 corresponding to aa 473-486 (deletion 2) of the SARS-CoV-2 spike which determines
 245 ACE2 usage based on previous reports [18]. However, aligned with SARS-CoV-2 and
 246 RaTG13, a short deletion is noted at the position corresponding to aa 444-447
 247 (deletion 1). The location of this deletion is similar to the one in the spike of
 248 RshSTT182, a SARS-CoV-2-related CoV identified in *Rhinolophus shameli* from
 249 Cambodia. Within the receptor binding motif (RBM), four of the five amino acid
 250 residues critical for binding of SARS-CoV-2 to the ACE2 receptor (486, 493, 494 and
 251 501) are varied in RaTG15. Like most bat SARSr-CoVs, the polybasic (furin)
 252 cleavage site is absent at the S1-S2 junction of RaTG15 (Figure 2).

253

254 **Functional comparison of RBD from three lineages of SARSr-CoVs**

255 The sequence analysis indicated that the RaTG15 virus possibly uses ACE2 as an
 256 entry receptor, which was then experimentally confirmed by RBD-ACE2 binding
 257 studies using purified recombinant proteins. RBD proteins from SARS-CoV-2,
 258 SARS-CoV-1, RaTG13, pangolin-CoV-GD, pangolin-CoV-GX and RaTG15, as well
 259 as ectodomains of human and *R.affinis* ACE2 proteins were used (Figure S2A). We
 260 found that *R.affinis* derived RaTG13 and RaTG15 RBD proteins either show very
 261 weak or have no binding affinity to human ACE2 (HuACE2). In contrast, RBD
 262 proteins from the two pangolin SARSr-CoVs displayed much higher binding affinity
 263 to HuACE2, only slightly weaker than SARS-CoV-2 RBD but still higher than
 264 SARS-CoV-1 (Figure 3A-F and M). Furthermore, the binding affinity to HuACE2 of
 265 pangolin-CoV-GX is slightly weaker than pangolin-CoV-GD. Next, we wanted to
 266 find out whether bat CoVs RaTG13 and RaTG15 can use *R.affinis* ACE2 more
 267 efficiently than huACE2. Detectable binding was observed between RaTG15 RBD

268 and *R.affinis* ACE2 (RaACE2), though the affinity was still weaker than SARS-CoV-
269 2 and pangolin-CoV-GD/GX to RaACE2. RaTG13 RBD showed a very weak binding
270 to RaACE2, same as to HuACE2 (Figure 3G-M and Figure S2B).

271
272 To exclude the possibility that the ACE2 binding of RBD may not represent the
273 functionality of the full-length S protein, we also constructed a VSV-based
274 pseudovirus using previously published method [16]. We produced a list of SARSr-
275 CoVs pseudoviruses, or MERS-CoV pseudovirus as a negative control. HEK293T/17
276 cells overexpression HuACE2, RaACE2 or empty vector were infected with VSV-
277 based pseudoviruses, and the infection efficiency were determined 48 h after
278 infection. Consistent with the RBD-ACE2 protein binding assays, HuACE2 mediated
279 entry of all SARSr-CoVs except the RaTG15, whereas the *R.affinis* ACE2 supported
280 all SARSr-CoVs entry. Notably, RaTG13 pseudovirus infection of HuACE2 or
281 RaACE2-expression cells was minimal, if it is positive, compared to other groups. As
282 control, MERS-CoV pseudovirus failed to infect ACE2-expression cells, confirming
283 ACE2-independent infectivity of VSV backbone (Figure S3). Collectively, none of
284 the SARSr-CoV-2 lineage or the novel lineage virus from bats could efficiently bind
285 to HuACE2 [10,11], and it appears that whether there is deletion at RBD region
286 greatly affecting the binding capacity (Figure 3N). These results suggest that without
287 further adaptation, there is a limited zoonotic potential for bat-derived RaTG13,
288 RaTG15 and perhaps other SARSr-CoV-2 lineage or the novel lineage viruses. In
289 contrast, there is a high spillover potential of pangolin-CoV in the context of cell
290 receptor usage.

291

292 Discussion

Overall, we report the discovery of a novel lineage of SARSr-CoVs from bats that are closely related to SARS-CoV-2 in the RdRp region, but genetically distant to any known SARSr-CoVs at genome level. Although several SARS-CoV-2 related coronaviruses have been detected from wildlife, none of them shared >99% genetically identical to SARS-CoV-2 at the genome level. Recombination events happen commonly in coronaviruses and can be referred to as potential origin of the progenitor of SARS-CoV-1, as SARSr-CoVs discovered in a bat colony carried all the genomic fragments of SARS-CoV-1 [14,19]. The high sequence similarity to SARS-CoV-2 in some genomic regions detected from different wildlife species implies the recombination may happen during the virus evolution in cross-species or inter-species transmission. The new lineage virus we reported in this study showed weak binding affinity to bat but not human ACE2 though possess one deletion in the RBD of the spike which is different from the previously reported SARSr-CoVs in bat (Figure 2). These results suggested the SARSr-CoVs we discovered from bat now may be just the tip of the iceberg. These viruses may have experienced selection or recombination events in the animal hosts and render viral adaption to a new host then spread to the new species before they jumped into human society. So surveillance to this new lineage virus should be conducted to prevent future outbreaks, as viruses from the other two lineages of SARSr-CoV caused SARS and COVID-19, respectively [1,20]. Furthermore, none of the bat SARSr-CoV-2 lineage or the novel lineage viruses discovered so far could be isolated, or be capable of efficiently using human ACE2, thus pose little spillover potential to human without future adaptation [21]. In comparison, the ACE2 usage virus in bat SARSr-CoV-1 related lineage appears to be more dangerous in the context of cross-species transmission, which has been demonstrated in animal studies [22,23].

318

319 The closest bat CoV to SARS-CoV-2 at this stage, RaTG13 only showed very weak
320 binding affinity to HuACE2. Albeit there is a speculation claiming the possible
321 leaking of RaTG13 from lab that caused SARS-CoV-2, the experiment evidence
322 cannot support it. In contrast, the pangolin-CoV shows strong binding capacity to
323 human or bat ACE2, posing high cross-species potential to human or other species. In
324 the context of SARS-CoV-2 animal origin, there could either be a bat SARS-CoV
325 closer than RaTG13 that is capable of using HuACE2, or be a pangolin-CoV that
326 obtained higher genome similarity other than spike gene. In future, more systematic
327 and longitudinal sampling of bats, pangolins or other possible intermediate animals is
328 required to better understand the origin of SARS-CoV-2.

329

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337

338 **Declaration of Interests**

339 The authors declare no competing interests.

340

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405

406 **Figure legends**

407 **Figure 1. Discovery of a novel lineage of bat SARSr-CoVs.** (A) Similarity plot
408 analysis based on the full-length genome sequence of bat SARSr-CoV RaTG15. Full-
409 length genome sequences of SARS-CoV-1, SARS-CoV-2, bat and pangolin CoVs
410 related to SARS-CoV-2 were used as reference sequences. The analysis was
411 performed with the Kimura model, a window size of 1500 base pairs and a step size
412 of 150 base pairs. (B) Phylogenetic tree based on complete genome sequences of
413 betacoronaviruses. The trees were constructed by the Neighbour-joining method using
414 the Jukes-Cantor model with bootstrap values determined by 1000 replicates.
415 Bootstraps > 50% are shown. The scale bars represent 0.1 substitutions per nucleotide
416 position. The novel SARSr-CoVs characterized in this study are shown in bold. Ra,
417 *Rhinolophus affinis*; Rst, *Rhinolophus stheno*; Rsh, *Rhinolophus shameli*; Rs,
418 *Rhinolophus sinicus*; Rac, *Rhinolophus acuminatus*; Rm, *Rhinolophus malayanus*; Rc,
419 *Rhinolophus cornutus*; MHV, murine hepatitis virus.

420

Figure 2. Comparison of receptor-binding domain (RBDs) of SARSr-CoVs. The RBM is shown in pink and the five key residues that contact ACE2 directly are highlighted in green. Comparison of the five critical residues of these SARSr-CoVs are listed in the table. Two deletions in the RBM, aa 444-447 (deletion 1) and aa 473-486 (deletion 2) are indicated by red boxes. GenBank or GISAID entries for each virus can be found in Methods.

Figure 3. Binding affinity of SARSr-CoV RBDs to ACE2 from human and *R.affinis* bat. (A-F) Binding of different RBD proteins to human ACE2. (G-L) Binding of different RBD proteins to *R.affinis* ACE2. (M) Comparison of dissociation constants (KD) between different RBD to human and *R.affinis* ACE2. Relative binding is analyzed by comparing with SARS-CoV-2 RBD to human ACE2. (N) Summary of the binding efficiency of different RBD to human or bat ACE2. Y, yes; ND, not determined. Evidences for WIV16-CoV, Rc-o0319, RmYN02 and RacCS213 were from previous reports [10,11,21]. The presence of deletion in RBM (related to Figure 2) is indicated. Binding assay of human or *R.affinis* ACE2 to different RBD proteins was measured by Bio-layer interferometry. The parameters of KD value (M), Kon (1/M.s), Koff (1/s) are shown on the upper right side of the picture. Different RBD proteins were immobilized on the sensors and tested for affinity with graded concentrations of human or *R.affinis* ACE2s. The Y-axis shows the real-time binding response. Values reported representing the global fit to all data. The coefficient of determination (R^2) for these interactions was close to 1.0 (Figure S2B).

Figure S1. Phylogenetic tree base on the complete S gene sequences (A) or complete RdRp gene sequences (B) of betacoronaviruses. The trees were

constructed by the Maximum-likelihood method using the Jukes-Cantor model with bootstrap values determined by 1000 replicates. Bootstraps > 50% are shown. The scale bars represent 0.1 and 0.05 substitutions per nucleotide position, respectively. The novel SARSr-CoVs characterized in this study are shown in bold. Ra, *Rhinolophus affinis*; Rst, *Rhinolophus steno*; Rsh, *Rhinolophus shameli*; Rs, *Rhinolophus sinicus*; Rac, *Rhinolophus acuminatus*; Rm, *Rhinolophus malayanus*; Rc, *Rhinolophus cornutus*; MHV, murine hepatitis virus.

Figure S2. Binding affinity of SARSr-CoVs RBD proteins to ACE2 from human and *R.affinis*. (A) The purity of different CoV-RBD and ACE2 proteins used for binding assay were analyzed by SDS-PAGE. (B) Binding assay of human or *R.affinis* ACE2 to different RBD proteins measured by Bio-layer interferometry, Related to **Figure 3**. The Y-axis shows the real-time binding response. Values reported representing the global fit to all data. The coefficient of determination (R^2) for these interactions was shown on the upper right.

Figure S3. Infectivity analysis of SARSr-CoV spike VSV-pseudoviruses in human and *R.affinis* ACE2 expression cells. HEK293T/17 cells expression human/*R.affinis* ACE2 were infected with SARS-CoV-2 and SARSr-CoV spike-pseudotyped viruses. The infected cell lysis was analyzed by measuring luciferase activities. All results were performed in triplicate from three independent experiments. Error bars indicate mean $\pm$ SEM. Statistical significance was tested by one-way ANOVA with Dunnett posttest. (A) SARS-CoV-2, (B) RaTG15, (C) pangolin-CoV-GD, (D) pangolin-CoV-GD, (E) RaTG13, (F) SARS-CoV-1, (G) MERS-CoV. (H) Genome copies of VSV-CoV-S pseudotyped particles. Viral copy

471 numbers were calculated according to the standard curve of VSV P protein gene. A
 472 representative result is shown. (I) ACE2 expression was detected using mouse anti-S-
 473 tag monoclonal antibody followed by HRP-labelled goat anti-mouse IgG antibody. β -
 474 actin was detected with mouse anti- β -action monoclonal antibody by HRP-labelled
 475 goat anti-mouse IgG antibody.
 476

	455	486	493	494	501
SARS-CoV-2 WIV04	L	F	Q	S	N
SARS-CoV-2 B.1.1.7	L	F	Q	S	Y
Bat CoV RaTG13	L	L	Y	R	D
Bat CoV RshSTT182	L	P	Q	S	N
Pangolin-CoV-GD	L	F	Q	S	N
Pangolin-CoV-GX	L	L	E	R	T
Bat CoV RaTG15	L	A	L	K	V
Bat CoV RmYN02	S	-	S	T	V
Bat CoV RacCS203	S	-	S	T	V
SARS-CoV-1	Y	L	N	D	T

Furin-like cleavage site	
SARS-CoV-2	675 QTQTNSPERRARSVASQS 691
Bat RaTG15	658 DITKA---RTSSTPA 669
Bat RmYN02	635 ----NSP-AAR-VGTNS 645
Bat RacCS203	635 ----NSP-VAR-VGTNS 645
Bat RaTG13	675 QTQTNS---RSVASQS 687
Bat RshSTT182	657 QTQTNS---RSVTSQS 669
Bat ZC45	652 HTASIL---RSTSQA 664
Bat Rc-o319	640 HTPSML---RSANNK 652
Pangolin-CoV-GD	671 QTQTNS---RSVSSQA 683
Pangolin-CoV-GX	675 HSMSSF---RSVNQRS 687
SARS-CoV-1	661 HTVSL---RSTSQKS 673
Bat WIV1	662 HTVSSL---RSTSQKS 674
Bat HKU3-1	648 HTASVL---RSTGQKS 660

319	433
SARS-CoV-2 WIV04	RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNKRKISNCVADYSVLNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFTGCV
SARS-CoV-2 B.1.1.7	
Pangolin-CoV-GD	
Bat RaTG13	
Pangolin-CoV-GX	
Bat RaTG15	
Bat RshSTT182	
SARS-CoV-1	
Bat WIV1	
Bat Rc-o319	
Bat ZC45	
Bat RmYN02	
Bat RacCS203	
Bat HKU3-1	

434	455	486	493/494	501	541
SARS-CoV-2 WIV04	IAWNSNNLDSKVGGNYYNLYRLFRKSNLKPFFERDISTEIYQAGSTPCNGVEGFNCYFPLOS	Y	Y	Y	Y
SARS-CoV-2 B.1.1.7					
Pangolin-CoV-GD					
Bat RaTG13					
Pangolin-CoV-GX					
Bat RaTG15					
Bat RshSTT182					
SARS-CoV-1					
Bat WIV1					
Bat Rc-o319					
Bat ZC45					
Bat RmYN02					
Bat RacCS203					
Bat HKU3-1

M

	ACE2	Human	<i>R. affinis</i>
	RBD		
SARS-CoV-2		1.00	>1.09
RaTG15		Not detectable	3.90
Pangolin-CoV-GD		2.28	1.13
Pangolin-CoV-GX		3.26	1.30
RaTG13		Low affinity, >3.53	16.57
SARS-CoV-1		9.07	6.19

N

		Human ACE2	Bat ACE2	Deletion 1	Deletion 2
SARS-CoV-1 related lineage	SARS-CoV-1	✓	✓		
	WIV16-CoV	✓	✓		
	Rp3-CoV	×	×	Y	Y
SARS-CoV-2 related lineage	SARS-CoV-2	✓	✓		
	RaTG13	weak	weak		
	Rc-o0319	×	✓		Y, partial
	RmYN02	×	ND	Y	Y
	RacCS213	×	ND	Y	Y
	Pangolin-CoV-GX	✓	✓		
Novel lineage	Pangolin-CoV-GD	✓	✓		
	RaTG15	×	✓	Y	