

Accelerated Article Preview

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Received: 3 September 2021

Accepted: 8 February 2022

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Published online: 16 February 2022

Cite this article as: Temmam, S. et al. Bat coronaviruses related to SARS-CoV-2 and infectious for human cells. *Nature* <https://doi.org/10.1038/s41586-022-04532-4> (2022).

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The animal reservoir of SARS-CoV-2 is unknown despite reports of various SARS-CoV-2-related viruses in Asian *Rhinolophus* bats^{1–4}, including the closest virus from *R. affinis*, RaTG13^{5,6} and in pangolins^{7–9}. SARS-CoV-2 presents a mosaic genome, to which different progenitors contribute. The spike sequence determines the binding affinity and accessibility of its receptor-binding domain (RBD) to the cellular angiotensin-converting enzyme 2 (ACE2) receptor and is responsible for host range^{10–12}. SARS-CoV-2 progenitor bat viruses genetically close to SARS-CoV-2 and able to enter human cells through a human ACE2 pathway have not yet been identified, though they would be key in understanding the origin of the epidemics. Here we show that such viruses indeed circulate in cave bats living in the limestone karstic terrain in North Laos, within the Indochinese peninsula. We found that the RBDs of these viruses differ from that of SARS-CoV-2 by only one or two residues at the interface with ACE2, bind more efficiently to the hACE2 protein than the SARS-CoV-2 Wuhan strain isolated in early human cases, and mediate hACE2-dependent entry and replication in human cells, which is inhibited by antibodies neutralizing SARS-CoV-2. None of these bat viruses harbors a furin cleavage site in the spike. Our findings therefore indicate that bat-borne SARS-CoV-2-like viruses potentially infectious for humans circulate in *Rhinolophus* spp. in the Indochinese peninsula.

The origin of SARS-CoV-2, as its mode of introduction into the human population, is currently unknown. Since its emergence, numerous animal species have been studied to identify possible reservoirs and/or intermediate hosts of the virus, including a large diversity of insectivorous bats of the genus *Rhinolophus*. Despite the recent report of various SARS-CoV-2-related viruses in *Rhinolophus shameli* (isolated in Cambodia in 2010¹³), *R. pusillus* and *R. malayanus* (China, 2020 and 2019 respectively²), in *R. acuminatus* (Thailand, 2020³) and *R. cornutus* (Japan, 2013⁴), the closest SARS-CoV-2 bat-borne genome still remains the one from *R. affinis*, RaTG13 (China, 2013)^{5,6} with 96.1% similarity at the whole genome level. Several studies also suggested the involvement of pangolin coronaviruses in the emergence of SARS-CoV-2^{7–9}. Since its appearance in humans, SARS-CoV-2 has evolved through sporadic mutations and recombination events¹⁴, some of which correspond to

gains in fitness allowing the virus to spread more widely, or to escape neutralizing antibodies¹⁵.

To decipher the origin of SARS-CoV-2, it is therefore essential to ascertain the diversity of animal coronaviruses and more specifically of bat coronaviruses. Although the identification of SARS-CoV-2 in bats is a major goal, a more realistic objective is to identify the sequences that contribute to its mosaicism. The spike sequence appears essential, as it determines the binding affinity and accessibility of the receptor-binding domain (RBD) to the cellular angiotensin-converting enzyme 2 (ACE2) receptor and is therefore responsible for host range^{10–12}. The closest related bat strain identified to date (RaTG13) has a low RBD sequence similarity to SARS-CoV-2 and, with only 11/17 human ACE2 (hACE2) contact amino-acid residues conserved with SARS-CoV-2, its affinity to hACE2 is very limited¹⁶. Moreover, SARS-CoV-2 poorly infects bats

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and bat cells tested so far¹⁷. In addition, no bat SARS-CoV-2-like virus has been shown to use hACE2 to efficiently enter human cells, and none presents the furin cleavage site that is associated with an increased pathogenicity in humans¹⁸. SARS-CoV-2 RBD binds to *Rhinolophus macrotis* ACE2 with a lower affinity than to hACE2¹⁹. An essential piece of information, i.e. finding bat viruses with an RBD motif close to that of SARS-CoV-2 and capable of binding to hACE2 with high affinity, is therefore missing.

We hypothesized that this type of virus could be identified in bats living in the limestone karstic terrain common to China, Laos, and Vietnam in the Indochinese peninsula. We report here the presence of sarbecoviruses close to SARS-CoV-2 whose RBDs differ from that of SARS-CoV-2 by only one or two contact residues, that strongly bind to the hACE2 protein, and mediate a hACE2-dependent entry and replication into human cells. Despite the absence of the furin cleavage site, these viruses may have contributed to SARS-CoV-2's origin and may intrinsically pose a future risk of direct transmission to humans.

Diversity of bat and coronavirus species

A total of 645 bats belonging to six families and 46 species were captured (Table S1). Two hundred and forty-seven blood samples, 608 saliva, 539 anal/feces, and 157 urine swabs were collected from the northern part of Laos (Table S2). We first screened all 539 feces samples through a pan-coronavirus RT nested PCR approach²⁰. Overall, 24 individuals of 10 species were positive, and one individual (BANAL-27) was concomitantly infected by an alphacoronavirus and a betacoronavirus (Table S3). BLAST analysis of amplicons identified alphacoronavirus sequences of the *Decacovirus*, *Pedacovirus*, and *Rhinacovirus* subgenera and betacoronavirus sequences of the *Nobecovirus* and *Sarbecovirus* subgenera. Sequences of the *Sarbecovirus* subgenus were all identified from *Rhinolophus* individuals belonging to three different species i.e., *R. malayanus*, *R. marshalli*, and *R. pusillus*. Positive individuals were trapped in three different districts, and those infected with a sarbecovirus were all from the Fueng district in Vientiane Province (Fig. 1a, site 1).

NGS together with Sanger sequencing was used to obtain a complete genomic sequence of five of the seven sarbecoviruses (Fig. 1, Table S4). The coverage of the genome of the remaining two sarbecoviruses, i.e. BANAL-27 and BANAL-242 sampled from *R. pusillus* and *R. malayanus* bats respectively, was 90% and therefore they were not included in the final analyses. Phylogenetic analyses performed on the receptor-binding protein domain of lineages A and B human SARS-CoV-2²¹, and on representative bat and pangolin sarbecoviruses, placed the Laotian *R. malayanus* BANAL-52, *R. pusillus* BANAL-103, and *R. marshalli* BANAL-236 coronaviruses close to human SARS-CoV-2 and pangolin coronaviruses collected in 2019, while *R. malayanus* BANAL-116 and BANAL-247 coronaviruses belonged to a sister clade with other bat coronaviruses (RmYN02, RacCS203, RpYN06, and PrC31) from different *Rhinolophus* species. Pangolin coronaviruses sampled in 2017 displayed a basal position relative to these strains (Fig. 1b). Interestingly, one should note that very similar SARS-CoV-2-like viruses are shared by different bat species, suggesting a possible circulation of viruses between different species living sympatrically in the same caves. These results are consistent with the similarity plot analysis showing that RaTG13 and BANAL-52 bat coronaviruses exhibit high nucleotide identity with SARS-CoV-2 throughout the length of the genome (96.8% for BANAL-52 and 96.1% for RaTG13). Interestingly, BANAL-52 presents a higher level of nucleotide conservation than RaTG13 in the S1 domain of the spike, and especially in the spike's N-terminal domain (NTD) and RBD (Fig. 1c). These observations are congruent with amino-acid identities between human SARS-CoV-2 and representative bat and pangolin coronaviruses, which present a high level of conservation, except for ORF8 of bat BANAL-116, BANAL-247, Rc-o319 and RmYN02 (Supp. Figure 1). Interestingly, the S1 domain of the spike (and especially

the N-terminal domain) presents a lower degree of conservation in several bat coronaviruses, suggesting that this domain may reflect a relative degree of adaptation of the virus to its mammalian host (Fig. 1d, Extended Data Fig. 1).

Bat Sarbecovirus evolutionary history

Following GARD analysis, we identified 14 recombinant breakpoints during the evolutionary history of sarbecoviruses, which were further confirmed by phylogenetic analyses performed on the 15 fragments of sequences defined by the breakpoints (Fig. 2, Supp. Figure 2). No specific signature was identified in the breakpoints (Table S5). SARS-CoV-2 presents a mosaic genome, to which contributed more than five sequences close to sequences published or determined during this study: *R. malayanus* RmYN02 and *R. pusillus* RpYN06 viruses found in China in 2019, *R. affinis* RaTG13 coronavirus found in China in 2013, and *R. malayanus* BANAL-52 and *R. pusillus* BANAL-103 found in North Laos in 2020 (this study). No pangolin coronavirus sequence was immediately associated with a recombination event at the origin of SARS-CoV-2. Laotian *Rhinolophus* bat coronaviruses presented a lower degree of recombination compared to SARS-CoV-2. Such recombination events occurred between other BANAL viruses isolated from bats living sympatrically in caves in the same area.

Interestingly, the origin of several fragments of SARS-CoV-2 genomes could be assigned to several donor strains and not a unique donor sequence. For example, a breakpoint was identified 7 nucleotides upstream of the RBD region of S1: the downstream fragment of SARS-CoV-2, which comprises the RBD and the beginning of S2, could involve BANAL-52 *R. malayanus*, BANAL-103 *R. pusillus*, and BANAL-236 *R. marshalli* viruses, which formed a highly supported sister clade of SARS-CoV-2 (fragment 11, Supp. Figure 2). In a more basal position are *R. shameli* bat coronaviruses and pangolin-2019 coronaviruses, consistent with the conservation of RBD amino-acid sequences among SARS-CoV-2 and representative bat and pangolin coronaviruses (Extended Data Fig. 2). Among the 17 residues that interact with human ACE2, 16 are conserved between SARS-CoV-2 and BANAL-52 or -103 (one mismatch, H498Q), and 15/17 are conserved for BANAL-236 (two mismatches, K493Q and H498Q) while only 13/17 residues are conserved for the Cambodian bat *R. shameli* virus and 11/17 for the Chinese bat *R. affinis* RaTG13 virus. At the full spike protein level, bat *R. affinis* RaTG13 and pangolin-2017 P4L viruses looked closer to SARS-CoV-2 than bat *R. malayanus* BANAL-52, but this effect is due to a higher degree of conservation in S2. All these viruses shared the absence of a furin cleavage site and the conservation of the internal fusion peptide (Supp. Figures 1 & 3 and Extended Data Fig. 3).

Interaction of BANAL RBDs with ACE2

Biolayer interferometry experiments to measure the interaction between hACE2 and the RBDs of BANAL-52/103 (which have identical residues in the receptor-binding motif) (Extended Data Fig. 2), BANAL-236 and SARS-CoV-2 (residues 233-524), resulted in a dissociation constant K_d three times lower for the BANAL RBDs compared to SARS-CoV-2 (Fig. 3a). This higher affinity can be attributed to the Q498H mismatch that has been reported to increase the affinity of SARS-CoV-2 RBD for hACE2, and also to be involved in the host range expansion of SARS-CoV-2 and SARS-CoV-2 like viruses²²⁻²⁹.

To study the effect of the mutations at the interface between these RBDs and hACE2, we performed Molecular Dynamics (MD) simulations of the SARS-CoV-2, BANAL-236 and BANAL-52/103 RBD/hACE2 complexes initiated from the crystal structure and homology models of these systems, respectively (Table S6). Cluster analysis of the MD trajectories revealed that, at the RBD-hACE2 interface, both BANAL complexes were identical to the SARS-CoV-2 RBD/hACE2 complex within 2 Å backbone RMSD (Extended Data Fig. 4), except for one of

the BANAL-52/103 simulations that displayed larger conformational variability of the RBD residues S443-Y449 (Supp. Methods and Extended Data Fig. 5). Empirical scoring functions predicted a similar RBD/hACE2 binding energy in all three complexes (Extended Data Fig. 6).

The analysis of the persistence of hydrogen bonds and salt bridges provided further insights into the effect of the substitutions at the RBD/hACE2 interface (Fig. 3b and Extended Data Fig. 7). The H498Q mismatch present in both BANAL-52/103 and BANAL-236 RBDs disrupted the hydrogen bonds between RBD Q498 and both hACE2 K353 and Q42. However, these hydrogen bonds were only transiently formed in the SARS-CoV-2 complex: more persistent hydrogen bonds in this region (RBD T500 – hACE2 D355, RBD G502 – hACE2 K353, RBD Y505 – hACE2 E37) were not affected. The K493Q mismatch enabled the formation of two salt bridges between RBD and hACE2 that were not present in the SARS-CoV-2 complex (RBD K493 – hACE2 E35 and RBD K493 – hACE2 D38).

For further insight into the molecular details of these interactions, we determined the crystal structure of the complex BANAL-236 RBD with the hACE2 peptidase domain to 2.9 Å resolution (Table S7). The overall structure of this RBD is identical to that of the SARS-CoV-2 (RMSD 0.360 Å, 150 Cα). The only significant difference is in the region between amino acids D363 and S375 (Fig. 3c, arrow). In this region, BANAL spikes have the A372T mismatch, which converts the sequence ³⁷⁰NSA³⁷² to ³⁷⁰NST³⁷², an N-linked glycosylation sequon. The crystals indeed showed clear electron density for the first N-acetylglucosamine residue of glycan attached to N³⁷⁰. The main chain at residue T³⁷² makes a hydrogen bond with the glycan residue, altering the conformation of the main chain downstream which results in partial unwinding of helix H4 in RBD 236. The calculation of a simulated annealing composite omit map for the segment D363-S375 confirms the correct assignment of the structure for this polypeptide segment and the N-glycosylation at residue N370 (Extended Data Fig. 8).

As expected, most of the interactions observed in the SARS-CoV-2 RBD/hACE2 complex³⁰ are also present in the structure of the BANAL-236 RBD/hACE2 complex. In these interfaces, there are three main clusters of interactions as indicated in Fig. 3c (insets). The sequence mismatches are in clusters 2 (making the salt bridge RBD K493 – hACE2 E35) and 3 (hydrogen bond between RBD H498 and D38). Although the interaction K493-E35 contributes to stabilizing the complex, it does not seem to affect drastically the binding to hACE2 because both BANAL RBDs have similar K_d values.

Virus replication in human cells

To assess whether the BANAL-236 spike protein could mediate entry into cells expressing human ACE2, we generated lentiviral particles pseudotyped with the Wuhan or the BANAL-236 spikes (Supp. Figure 4). We detected spike-mediated entry of the BANAL-236 spike-pseudotyped lentivirus in 293T-ACE2, contrarily to control cells not expressing hACE2 (Fig. 4a). Entry was blocked by human sera neutralizing SARS-CoV-2, but not by control non-neutralizing sera, demonstrating that neutralization of BANAL-236 was specific for epitopes shared with the spike of SARS-CoV-2 (Fig. 4b).

In order to isolate infectious viruses, rectal swabs were inoculated on VeroE6 cells. No CPE was observed 3 and 4 days after infection, but viral RNAs were detected for one of the 2 wells inoculated with the BANAL-236 sample ($C_T = 25.1$ at D3, $C_T = 21.7$ at D4). The culture supernatant (C1) formed plaques on VeroE6 and the titer was 3800 pfu/mL. A C2 viral stock was prepared by amplification on VeroE6 at a MOI of 10^{-4} . The culture supernatant was harvested on day 4 when CPE was observed and titrated on VeroE6 (Extended Data Fig. 9). The plaques' phenotype was small, but the titer reached $2.6 \cdot 10^6$ pfu/mL. The random NGS performed on the RNA extracted from this stock confirmed that the culture was pure and corresponded to the BANAL-236 virus, without any non-synonymous mutations between the original BANAL-236

genome and the C2 viral stock. Replication of BANAL-236 in VeroE6 was efficiently inhibited by soluble human ACE2, thus showing that entry and propagation was largely ACE2-dependent (Fig. 4c, Extended Data Fig. 9). Furthermore, BANAL-236 replicated in human cell lines expressing endogenous levels of ACE2, Calu-3 and Caco-2 (Fig. 4c). The kinetics of RNA synthesis were slower compared to Wuhan virus. Infectious viral particles were produced at D4 in the supernatant of Caco-2 and Calu-3 cells (respectively $10^{4.7}$ and $10^{2.9}$ pfu/mL).

Discussion

Many sarbecoviruses circulate in *Rhinolophus* colonies living in caves in China and probably also in neighboring countries further south^{31–33}. During the course of a prospective study in Northern Laos, we have identified, among other coronaviruses, five sarbecoviruses for which we obtained full-length sequences. Among these, three (BANAL-52, -103, and -236) were considered to be close to SARS-CoV-2 because of the similarity of one of the S1 domains (NTD, RBD) or S2 with SARS-CoV-2.

Because genomic regions subject to recombination are likely contributing to host-virus interactions, we compared SARS-CoV-2 strains from the two lineages identified at the very onset of the COVID-19 outbreak²¹ to these novel bat sarbecoviruses and to pangolin strains within the SARS-CoV-2 clade. Strains close to *R. pusillus* RpYN06, *R. malayanus* RmYN02, and *Rhinolophus* sp. PrC31 isolated in China in 2018–2019, along with *R. malayanus* BANAL-52, *R. pusillus* BANAL-103, and *R. marshalli* BANAL-236 isolated in Laos in 2020, contributed to the appearance of SARS-CoV-2 in different regions of the genome. No closer viral genome has yet been identified as a possible contributor, and pangolin coronaviruses appear as more distantly related than bat coronaviruses. We identified potential recombination sites, allowing for the reconstruction of the phylogenetic history of early isolated SARS-CoV-2 strains between homologous regions defined by recombination points. We identified a breakpoint at the beginning of the SARS-CoV-2 RBD, resulting in a downstream fragment key for the virus tropism and host spectrum composed of the RBD, the furin cleavage site, and ending in the N-terminal region of S2. Despite the absence of the furin site, phylogenetic reconstruction of this fragment revealed that Laotian *R. malayanus* BANAL-52, *R. pusillus* BANAL-103, and *R. marshalli* BANAL-236 coronaviruses are the closest ancestors of SARS-CoV-2 known to date. ORF8 was highly divergent between SARS-CoV-2 related genomes. ORF8 from strains BANAL-52, -103, -236, like that of RaTG13, were closer to SARS-CoV-2 than to pangolin strains. ORF8 encodes a protein that has been proposed to participate in immune evasion³⁴ and is deleted in many human SARS-CoV-2 strains that appeared after March 2020³⁵, which is reminiscent of the deletions identified during the 2003 SARS epidemic³⁶. Therefore, the presence of ORF8 is consistent with bats acting as a natural reservoir of early strains of SARS-CoV-2.

Structural and functional biology studies have identified the RBD domain that mediates the interaction with hACE2 and host range, as well as the major amino acids that are involved^{30,37,38}. The RBDs (BANAL-52, -103, and -236) are closer to SARS-CoV-2 than that of any other bat strain described so far, in particular that of RaTG13, the virus identified in *R. affinis* from the Mojiang mineshaft where pneumonia cases with clinical characteristics *a posteriori* interpreted as similar to COVID-19⁶ were recorded in 2012^{39,40}. Overall, one (H498Q (BANAL-103 and -52)) or two (K493Q and H498Q (BANAL-236)) amino acids interacting with hACE2 are substituted in these strains in comparison to SARS-CoV-2. These mutations did not destabilize the BANAL-236 / hACE2 interface, as shown by the BLI experiments (Fig. 3a) and analyzed by MD simulations.

Our results therefore support the hypothesis that SARS-CoV-2 could originally result from a recombination of sequences pre-existing in *Rhinolophus* bats living in the extensive limestone cave systems of South-East Asia and South China^{41,42}. Many species forage in the same

cave areas, including *R. malayanus* and *R. pusillus*⁴³. In addition, the distribution of *R. marshalli*, *R. malayanus*, and *R. pusillus* overlaps in the Indochinese sub-region (Supp. Figure 5), which means they may share caves as roost sites and foraging habitats⁴⁴. With the novel viruses here described, understanding the emergence of SARS-CoV-2 does not need to hypothesize recombination or natural selection for increased RBD affinity for hACE2 in an intermediate host like the pangolin before spillover⁴⁵, nor natural selection in humans following spillover⁴⁶. However, we found no furin cleavage site in any of these viruses on sequences determined from original fecal swab samples, devoid of any risk of counterselection of the furin site by amplification in Vero cells¹⁸. Lack of furin cleavage site may be explained by insufficient sampling in bats. Based on comparison of the sequences around the S1/S2 cleavage site (Extended Data Fig. 3), it has been suggested that the furin cleavage site present in SARS-CoV-2 could originate from recombination events between SARS-CoV-2-related coronaviruses co-circulating in bats^{2,47}, meaning that BANAL-116, BANAL-247, bat RmYN02² and bat RacCS203³ coronaviruses may share a common history with SARS-CoV-2. Alternatively, the furin cleavage site could have been acquired through passages of the virus in an alternate host or during an early poorly symptomatic unreported circulation in humans. Finally, the epidemiological link between these bat viruses and the first human cases remains to be established.

As expected from the high affinity for ACE2 of the S ectodomain of BANAL-236, pseudoviruses expressing it were able to enter efficiently human cells expressing endogenous hACE2 using an ACE2-dependent pathway. However, alternative routes of entry may still exist, especially in cells that do not express ACE2⁴⁸. Entry was blocked by a serum neutralizing SARS-CoV-2. The RaTG13 strain, the closest to SARS-CoV-2 known before, had never been isolated. In contrast, preliminary studies show that BANAL-236 replicated in primate VeroE6 cells with a small plaque phenotype compared to SARS-CoV-2. Further analysis may indicate more clearly which steps shape infectivity.

To conclude, our results pinpoint the presence of new bat sarbecoviruses that seem to have the same potential for infecting humans as early strains of SARS-CoV-2. Guano collectors, or certain ascetic religious communities who spend time in or very close to caves, as well as tourists visiting caves, are particularly at risk of being exposed. Further investigations are needed to assess if such exposed populations have been infected, symptomatically or not, by one of these viruses, and whether infection could confer protection against subsequent SARS-CoV-2 infections. In this context, it is noteworthy that SARS-CoV-2 with the furin site deleted replicates in hamsters and in transgenic mice expressing hACE2, but leads to less severe disease while protecting from rechallenge with wild-type SARS-CoV-2¹⁸.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-022-04532-4>.

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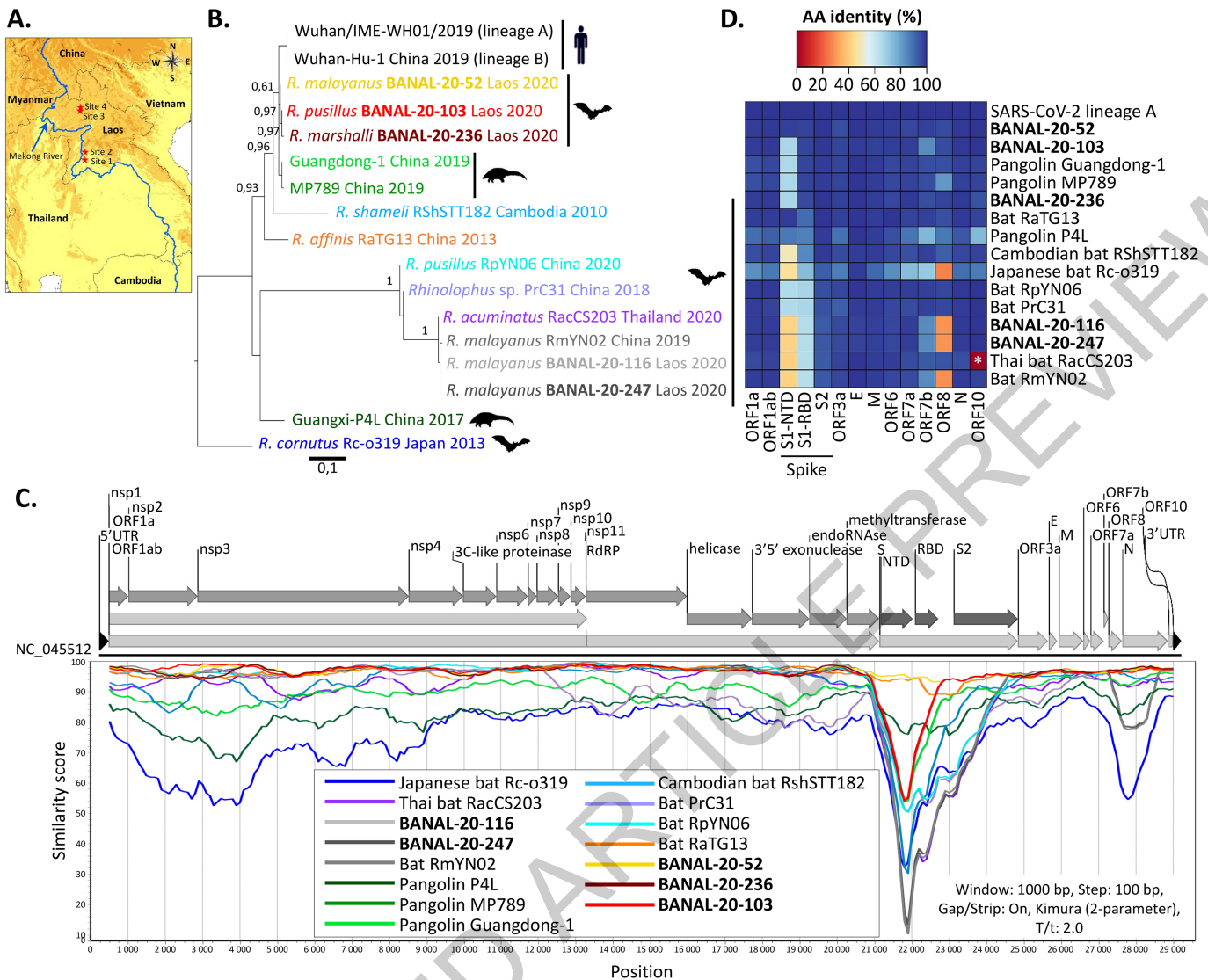


Fig. 1 | Genomic description of bat-borne sarbecoviruses identified in Laos.

(A) Map of sampling sites. All BANAL isolates were collected from the same site (site 1). Map was downloaded from <https://www.diva-gis.org/gdata>.

(B) Phylogenetic analysis of the protein sequence of the receptor-binding domain of Laotian and representative human, bat, and pangolin sarbecoviruses. Sequences were aligned with MAFFT⁴⁹ in “auto” mode, and maximum likelihood phylogenetic reconstruction was performed with PhyML implemented through the NGPhylogeny portal⁵⁰ with the LG+G substitution model. Branch support was evaluated with the aBayes parameter. Accession numbers and bat species are specified in the name of the sequences. Sequences are colored according to Fig. 1c. **(C)** Similarity plot analysis of Laotian and

representative bat and pangolin sarbecoviruses based on the full-length genome sequence of SARS-CoV-2 human prototype strain (NC_045512, Wuhan-Hu-1) used as reference. The analysis was performed with the Kimura-2 parameter model, a window size of 1,000 base pairs, and a step size of 100 base pairs with SimPlot program, version 3.5.1⁵¹. **(D)** Heatmap of identities at the protein level of representative human, bat, and pangolin sarbecoviruses compared to human SARS-CoV-2 lineage B (NC_045512). Spike protein has been divided into functional domains, and the sequences are ordered according to percentage of identity of the RBD domain. “*”: absence of a functional ORF10 in Thai bat RacCS203 (accession number MW251308). Heatmap was created using the gplots package in R (version 3.6.3).

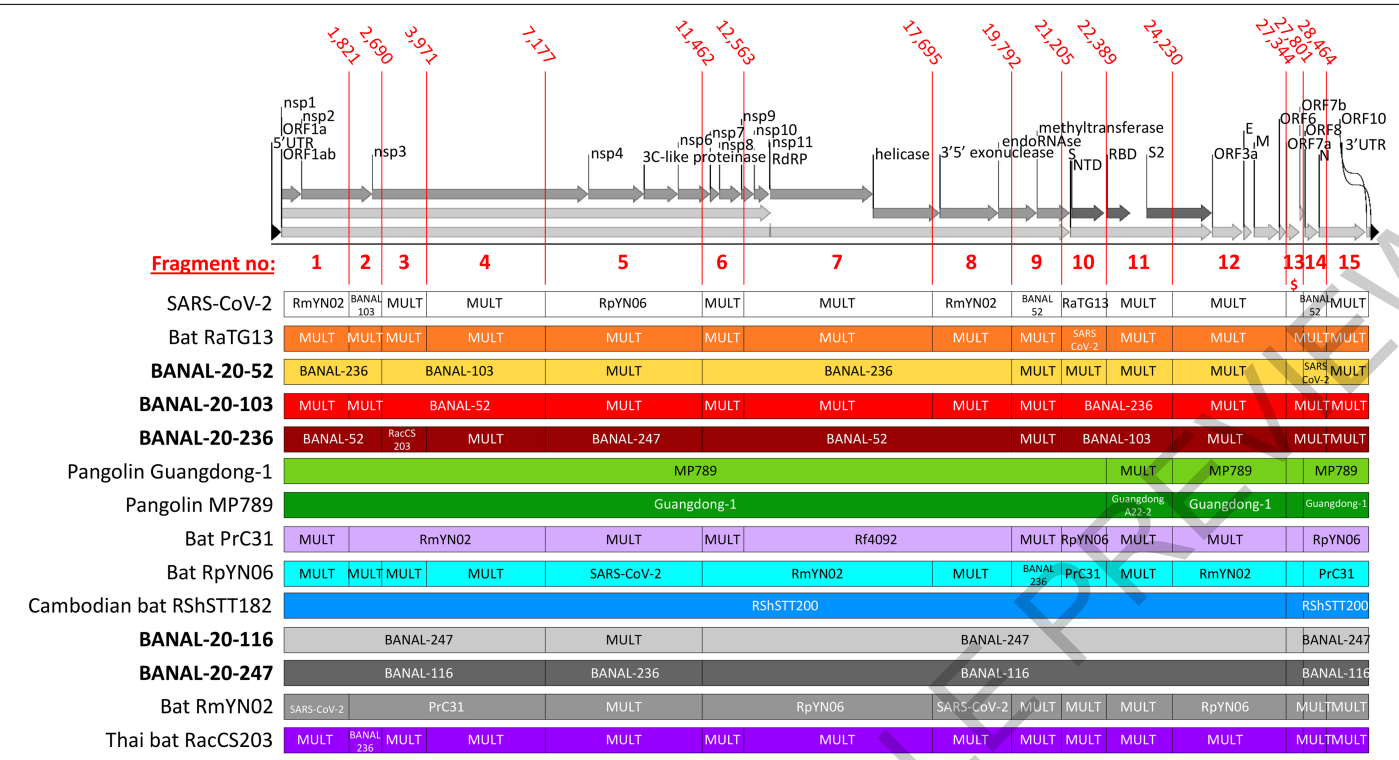


Fig. 2 | Recombination events in the evolutionary history of sarbecoviruses. Schematic representation of the 15 recombinant fragments of relevant *Sarbecovirus* genomes compared to SARS-CoV-2 human prototype strain (NC_045512). Breakpoint positions are referred to the position in the alignment. Where possible, the closest viral sequence is indicated for each

fragment. In other cases, “MULT” (group of multiple sequences) is mentioned. “\$”: unresolved fragment phylogeny (fragment 13, from positions 27344 to 27800 in the alignment). Sequences are colored as in Fig. 1. The complete phylogenetic analyses are presented in Supp. Figure 2.

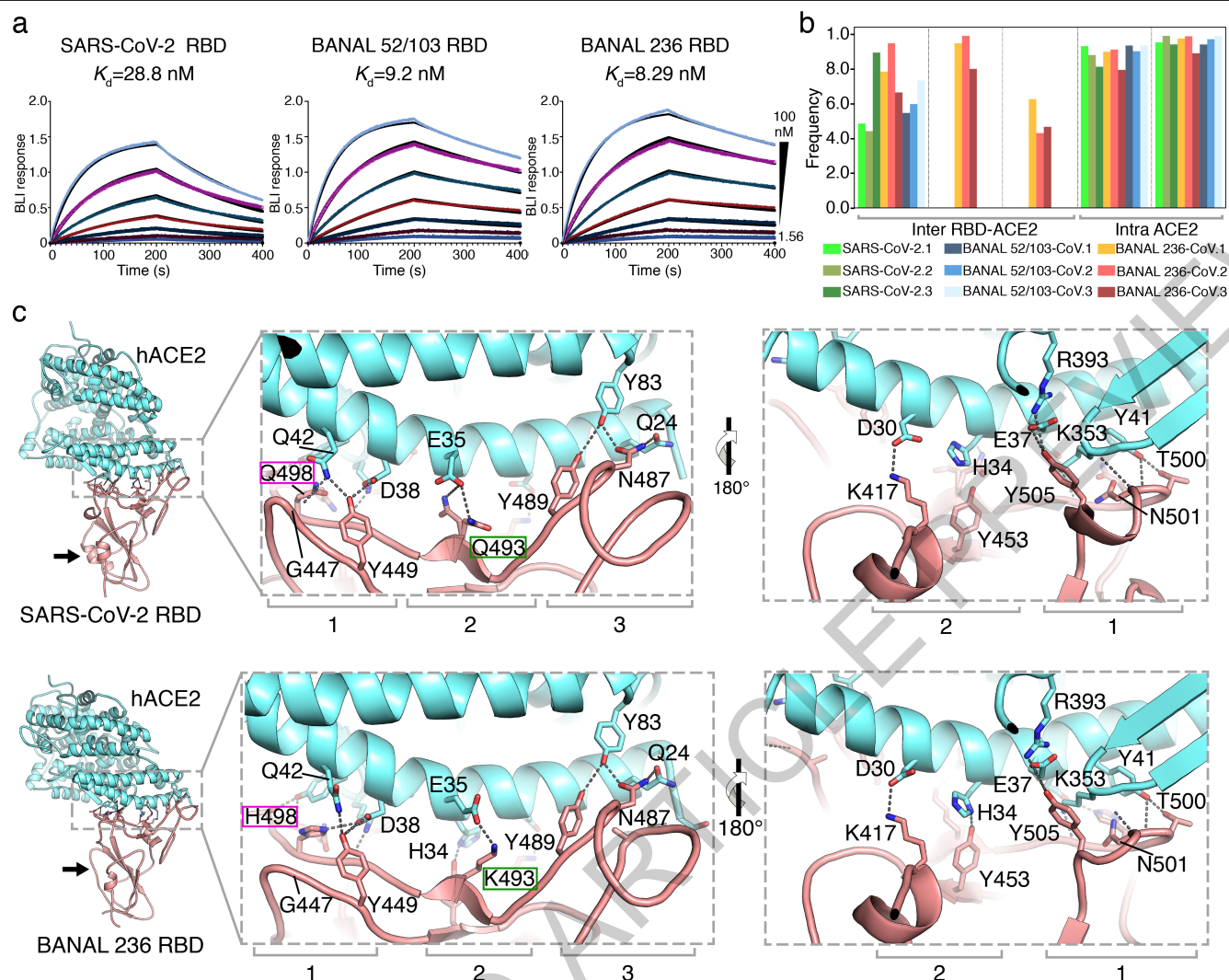


Fig. 3 | Dynamics of the binding of hACE2 to bat-sarbecovirus borne RBDs and structural insight of the complex. (A) Biolayer interferometry binding analysis of the hACE2 peptidase domain to immobilized BANAL52/103, BANAL-236 RBDs or SARS-CoV-2. Black lines correspond to global fit of the data using a 1:1 binding model. (B) Frequency of formation of salt bridges close to the RBD/ACE2 interface (from left to right: D30/K417, E35/K493, D38/K493, K31/E35, and D38/K353) during the course of the MD simulations. The analysis is performed for 9 different MD simulations (3 replicates for each complex) of

hACE2 in complex with SARS-CoV-2 (shades of green), BANAL-236 (shades of red) and BANAL-52/103 RBDs (shades of blue). (C) Ribbon representations of the crystal structures of hACE2 peptidase domain (cyan) in complex with SARS-CoV-2 (PDB 6M0J) or BANAL-236 (this study, PDB 7PKI) RBDs (pink). Black arrows in the overall structures indicate the structural difference between the two complexes at the level of helix H4. The insets show the main interactions in the ACE2-RBD interfaces. Residues in the RBM mutated between SARS-CoV-2 and BANAL-236 are indicated with colored boxes.

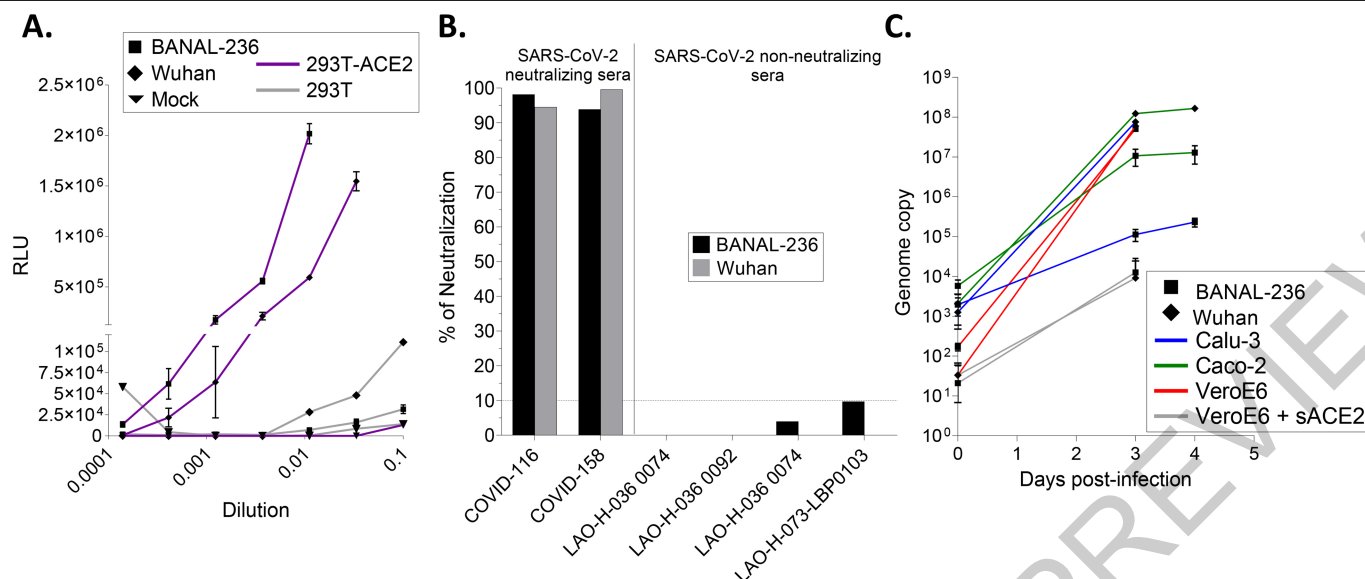


Fig. 4 | BANAL-236 entry and propagation in human cells. (A) Results of spike-pseudotyped BANAL-236 (square) and Wuhan-Hu-1 (diamond) pseudoviruses entry assay in HEK-293T cells expressing (purple) or not (grey) the hACE2 receptor, expressed in Relative Luminescence unit (RLU) produced by the firefly luciferase present in the lentiviral backbone and the Bright-Glo luciferase substrate. A single experiment performed in triplicate representative of two experiments is shown. Center values represent the average of the 3 replicates and error bars indicate SD. **(B)** Results of spike-pseudotyped BANAL-236 (black) and Wuhan-Hu-1 (grey) neutralization assay expressed in % of neutralization of luciferase activity in the absence of serum. SARS-CoV-2 neutralizing sera were from patients with confirmed

infections while non-neutralizing sera samples were collected before SARS-CoV-2 spread in Laos. Dashed line: neutralization threshold. A single experiment representative of three independent experiments is shown. **(C)** Human cell lines expressing endogenous ACE2, Calu-3 (blue line) and Caco-2 (green line), were infected at a MOI of 0.01 with BANAL-236 (square dots) and Wuhan (diamond dots) viruses. VeroE6 cells (red line) were infected at a MOI of 0.0001 with BANAL-236 and Wuhan viruses pre-incubated or not (grey line) with soluble human ACE2 (sACE2) at 25 $\mu\text{g}/\text{mL}$ for 30 min. Genome copy number was quantified by RT-qPCR in the supernatants recovered 3- and 4-days post-infection. A single experiment performed in triplicate is shown. Center values represent the average of the 3 replicates and error bars indicate SD.

Methods

Ethical and legal statements

The bat study was approved by the wildlife authorities of the Department of Forest Resource Management (DFRM), and the Ministry of Agriculture and Forestry, Lao PDR, No. 2493/DFRM, issued on May 21, 2020; No. 0755/MAF issued on June 2, 2020. All animals were captured, handled, and sampled following previously published protocols and ASM guidelines^{52,53}. Exportation from Lao and importation in France were conducted according to national regulations. Human serum samples used for neutralization assays were already available⁵⁴ and selected based on their status regarding seroneutralization of SARS-CoV-2. They were collected following a protocol approved by the Lao National Ethics Committee for Health Research (NECHR) (Ref #052/2020).

Biosafety

Both the Institut Pasteur du Laos (IPL) and the Faculty of Environmental Science have extensive experience in safely collecting bats (appropriate biosafety training and Personal Protective Equipment (PPE) for collectors). In this study, training prior to field work was organized once for field work participants. The aim was to teach participants on the transmission risks of infectious agents from bats and how to identify, assess, and mitigate these risks, as well to practice the use of PPE.

During field collection, sampling stations were selected to minimize potential exposure to infectious agents and stress on the animals during the handling time by selecting (1) an area easy to disinfect, (2) out of view of the general public, (3) a location that would not expose the general population (like a picnic area), and (4) procedures that reduce time and stress on bats caused by handling. For handling bats and bat samples the following minimum PPE was required: eye protection, an N95 respirator, long clothing/cover all, and latex gloves (2pairs). All wastes were disposed of in biohazard bags and were transported to a disposal site of IPL in Vientiane capital. Each sample box containing 81 cryovial tubes was put inside individual plastic ziplock and transported from field to IPL using dry ice by packing them in a polystyrene foam box. At IPL, samples were stored in a specific -80 °C freezer until analysis.

For initial sample analysis at IPL, samples were transferred to BSL-3 room for nucleic acid extraction. Initial Coronavirus screening by RT-PCR was then performed under internal regulation on biosafety and security of IPL. Sample extracted products (50 µl) were stored in NucleoSpin 8 sample boxes (8x12) and sent to IP-Paris by packing them in the individual plastic ziplock and using dry ice. Aliquots of anal swab samples were sent in a separated box by individually triple packaging in dry ice.

All experiments on potentially infectious samples done at Institut Pasteur (Paris) were conducted in BSL-3 laboratories according to procedures adapted to respiratory viruses.

Bat sampling areas and sample collection

Trapping sessions were conducted on four sites, in Fueng and Meth Districts, Vientiane Province, and in Namor and Xay Districts, Oudomxay Province, between July 2020 and January 2021 (Fig. 1a, Table S1 and S2). Bats were captured using four-bank harp traps⁵⁵ and mist nets set in forest patches between rice fields/orange/banana plantations and karst limestone formations, for 5-8 nights depending on accessibility. Harp traps were set across natural trails in patches of forest understory. Mist nets were set across natural trails, at the edges of forests, at entrances of caves, and in areas near cave entrances, as well as in open areas or those with high forest canopy. Bats were morphologically identified following morphological criteria⁵⁵⁻⁵⁷. Other data such as forearm length (FA), sex, developmental stage (adult or juvenile) and reproductive condition (pregnant or lactating) were also recorded.

Bats were sampled for saliva, feces and/or urine, and blood before release at the capture site. Species identification of PCR-positive individuals was confirmed by sequencing the mitochondrial Cytochrome oxidase I³².

Initial Coronavirus screening

Total RNA was extracted from feces samples using the NucleoSpin 8 virus kit (Macherey Nagel). cDNA was synthesized using the Maxima H minus first strand cDNA synthesis kit (Thermo Scientific) and random hexamers following the manufacturer's instructions. The presence of coronaviruses was tested by a nested PCR approach using PCR master mix (Promega) and by targeting the RNA-dependent RNA Polymerase gene using combinations of degenerate and non-degenerate consensus primers as previously described²⁰. PCR products of the expected size were directly sequenced on both strands by Sanger sequencing using the nested PCR primers. The sequences obtained were confirmed by similarity analysis using the NCBI BLASTn search (<http://www.ncbi.nlm.nih.gov/BLAST>).

Primer design for *Betacoronavirus* enrichment before Next-Generation Sequencing

Betacoronavirus enrichment was performed at the genus level by adapting a previously described protocol⁵⁸ based on k-mers for targeted-sequence enrichment prior to NGS. Briefly, 2,000 complete *Betacoronavirus* genomes were downloaded from the GenBank and GISAID databases and then clustered to a 95% sequence identity using CD-HIT-EST⁵⁹. Overall, 185 representative sequences of all betacoronaviruses were used for further analysis. Due to the high diversity within the genus *Betacoronavirus*, the full genomes belonging to the subgenera *Sarbecovirus*, *Nobecovirus*, and *Merbecovirus* plus *Embecovirus* were separately aligned using MAFFT multiple sequence alignment software and used to design 13-mer spiked primers per cluster. The genomic position of the full set of primers was extracted from some representative subgenera sequences and close primers were removed. Finally, 416 spiked primers were synthesized by Eurofins Genomics Germany GmbH.

Sample preparation for sequencing

Reverse transcription was performed using the mix of spiked primers and random hexamers at a 10:1 ratio using the SuperScript™ IV First-Strand Synthesis System (Invitrogen). After a denaturation step at 95 °C for 3 min in the presence of dNTPs (500 µM), DTT (5 mM), and RNaseOUT inhibitor, the RT reaction was incubated for 10 thermal cycles consisting of 6 steps at 8 °C for 12 s, 15 °C for 45 s, 20 °C for 45 s, 30 °C for 30 s, 35 °C for 2 min and 42 °C for 3 min, followed by a final incubation step at 42 °C for 20 min, as previously described⁶⁰. Double-stranded cDNA was generated using the Sequenase2.0 DNA Polymerase (Applied Biosystems™) in presence of dNTPs and then purified using the Beckman Coulter™ Agencourt AMPure XP.

For samples with a low nucleic acid content, a random amplification step was performed using the MALBAC Single Cell WGA kit (Yikon Genomics, Promega). The amplified product was then purified using AMPure XP Beads, eluted in a final volume of 20 µL of low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA), and quantified with the Qubit™ DNA HS Assay (Life Technologies, Thermo Fisher Scientific Inc.).

Libraries were generated using the NEBNext Ultra II DNA Library Prep kit (New England Biolabs) after a fragmentation step using the Covaris M220 Focused-ultrasonicator using microTUBE-15 (Peak Incident power (W)=18, Duty Factor=20%, Cycles per Burst=50, Treatment time (sec)=60). The PCR amplified libraries were cleaned up using 0.9X AMPure XP Beads and checked on the 2100 Bioanalyzer system with the High-Sensitivity DNA kit (Agilent Technologies, France) and quantified with the Qubit™ DNA HS Assay. Finally, the dual-multiplexed libraries were pooled (six samples/pool) and run on the Illumina NextSeq500 platform with High Output Kit v2.5 (150 Cycles).

Amplicon sequencing

In addition to enrichment-based sequencing, cDNA was amplified using the AmpliSeq for Illumina SARS-CoV-2 Research Panel (cat# 20020496), applying twenty-six amplification cycles in PCR1 and nine cycles in PCR2. Primers at the end of the amplicons were partially digested during the library preparation, following manufacturer's instructions. Libraries were barcoded individually using the Illumina UD dual-indexes and normalized with the AmpliSeq Library Equalizer for Illumina (cat# 20019171), then pooled and sequenced on the Illumina NextSeq500 instrument using a Mid Output v2.5 kit (SR 150 cycles). To eliminate residual PCR primer sequences, raw reads were trimmed by 15 bases at each end and special attention was paid to checking that internal sequences corresponding to primer regions in overlapping amplicons did not derive from primer sequences of the multiplex PCR. As an internal control, we verified that sequences of the complete genome of sample BANAL-236 obtained from the enrichment-based sequencing approach and from the AmpliSeq approach were identical.

Genome assembly and finishing

Raw reads from the enrichment-based sequencing were processed with an in-house bioinformatics pipeline (Microseek; Bigot et al., submitted) comprising quality check and trimming, reads normalization, *de novo* assembly, and ORF prediction of contigs and singletons, followed by 3 levels of taxonomic assignment⁶¹. Sequences identified as *Sarbecovirus* were then mapped onto appropriate reference sequences using CLC Genomics Workbench 20.0 (Qiagen). Trimmed reads from the amplicon sequencing were mapped to the SARS-CoV-2 genome first, then mapped again (refined mapping) to the closest genome relative. When needed, complete genomes were obtained by conventional PCR and Sanger sequencing. Briefly, viral RNA was reverse transcribed using the SuperScript IV reverse transcriptase (Invitrogen, USA) and cDNA was subsequently used to fill the gaps in the genomes using the Phusion High Fidelity DNA polymerase (New England Biolabs, France) and specific primers flanking the missing regions. Positive PCR products were further purified and sequenced by Sanger sequencing at Eurofins Genomics.

Recombination and phylogenetic analyses

Identification of recombination events occurring during the evolutionary history of bat sarbecoviruses was performed using the IDPlot package⁶², a web-based workflow that includes multiple sequence alignment and phylogeny-based breakpoints prediction using the GARD algorithm from the HyPhy genetic analysis suite⁶³. First, a comprehensive analysis comprising 106 sequences and covering all non-human *Sarbecovirus* and *Sarbecovirus*-related complete genomes available in GenBank and GISAID databases was performed, including prototype strains of SARS-CoV-2 isolated in 2019. Special attention was paid to including bat-borne and pangolin-borne viral sequences in order to maximize the ability to capture a large diversity of the sarbecoviruses. Then, a reduced set of 36 sequences was chosen because of their phylogenetic proximity with SARS-CoV-2, and the GARD algorithm was run to identify recombination breakpoints in Laotian and representative human, bat, and pangolin sarbecoviruses. Breakpoint coordinates were confirmed by performing phylogenetic analyses on the corresponding fragments using PhyML implemented through the NGPhylogeny portal⁵⁰. Branch support was evaluated with the aBayes parameter.

Generation of lentiviral pseudoviruses

The BANAL-236 and Wuhan synthetic spike genes were cloned into the pVAX1 vector with a cytoplasmic tail truncation of 19 amino acids. Pseudotyped lentiviral particles were prepared using HEK-293T cells (ATCC CRL-3216) seeded in 10-cm dishes. 293T cells were co-transfected with a 5 µg of spike-encoding plasmid, 10 µg of lentiviral

backbone plasmid expressing the firefly luciferase (pHAGE-CMV-Luc 2-IRES-ZsGreen-W), and 3.3 µg of each lentiviral helper plasmid expressing HIV Gag-Pol (HDM-Hgpm2), Tat (HDM-tat1b), and Rev (pRC-CMV-Rev1b) using calcium-phosphate precipitation⁶⁴. The medium was replaced 5 h post-transfection by 6 mL of DMEM without fetal calf serum (FCS) and phenol red. Pseudotyped particles were harvested 48 h post-transfection, clarified by centrifugation at 2500 g for 5 min and frozen at -80 °C. Mock pseudotyped lentivirus was generated as above but in the absence of an S-expressing plasmid.

Spike-pseudotyped lentivirus entry assays

HEK-293T cells stably expressing human ACE2 (293T-ACE2) were transduced in suspension by mixing 50 µL of 3-fold serial dilutions of S-pseudotyped lentiviruses with 50 µL of cells at 4.10^5 cells/mL in 96-well white culture plates⁶⁵. At 60-72 h post-transduction, 100 µL of Bright-Glo luciferase substrate (Promega) were added to the wells and luminescence was measured using a Berthold Centro XS luminometer.

Neutralization assays

Sera neutralizing or not SARS-CoV-2 were described in⁵⁴. They were decomplexed at 56 °C for 30 min and 2.5 µL were incubated with 0.5 µL of S-pseudotyped lentiviruses in a final volume of 50 µL of DMEM-10% FCS without phenol red in 96-well white culture plates. After 30 min at room temperature, 50 µL of 293T-ACE2 cells in suspension at 4.10^5 cells/mL were mixed to the wells. Luminescence was measured at 60-72 h post-transduction as described above. Neutralization was calculated using the following formula: $1 - (\text{RLU in presence of serum} / (\text{mean of RLU in absence of serum determined in 12 wells} - 3 \times \text{STD}))$.

Virus isolation and multiplication

Rectal swabs were inoculated in duplicate in 24-well plates containing VeroE6 cells (ATCC CRL-1586) (1/5 dilution in 100 µL of DMEM without FCS supplemented with 1% Penicillin-Streptomycin, 1% Fungizone, and 1 µg/mL TPCK-treated trypsin). After 1 h of adsorption at 37 °C, inoculum was removed and 1 mL of the medium described above was added. Three and 4 days after infection, cytopathic effect (CPE) was monitored and 100 µL of supernatant was collected for RNA extraction. RT-qPCR targeting a conserved sequence in the E gene was performed as described⁶⁶. Culture supernatant (C1) was harvested at day 4 and titrated by plaque assay on VeroE6 overlaid with 0.5% carboxymethylcellulose containing 1 µg/mL TPCK-treated trypsin. A viral stock was prepared by amplification on VeroE6 cells at a MOI of 10^{-4} . Culture supernatant (C2) was harvested at day 4 when massive CPE was observed and titrated by plaque assay on VeroE6 as described above. A C3 viral stock was produced by a subsequent viral amplification at a MOI of 10^{-4} for 3 days on VeroE6. RNA was extracted from the viral stock and submitted to random NGS analysis using the SMARTer® Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian (Takara Bio USA, Inc.). Raw reads were processed with Microseek pipeline, as described above.

For infection experiments, VeroE6 or human cell lines Calu-3 (lung cells, ATCC HTB-55) and Caco-2 (intestinal cells, ATCC HTB-37) were infected in triplicate in 24-well plates with BANAL-236 (C3) or Wuhan viral stocks at a MOI of 0.0001 and 0.01 respectively. Human soluble ACE2 was pre-incubated at 25 µg/mL⁶⁷ for 30 min with the viral inoculum before VeroE6 infection. Infections were carried out without TPCK in the medium described above for 3 and 4 days. Supernatants were recovered at 0-, 3- and 4-days post-infection and genome copy number and viral titers were quantified as described above.

Protein expression and purification

BANAL-52/103, BANAL-236 and SARS-CoV-2 RBDs (residues 233-524, with C-terminal 8xHis-Strep and AVI tags) and hACE2 peptidase domain (residues 19-615, with C-terminal 8xHis tag) were expressed in Expi293F cells at 37 °C and 8% CO₂ (GnTi- Expi293™, ThermoFisher Scientific). Cell culture supernatants were collected five days post transfection

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and purified by affinity chromatography followed by size exclusion chromatography (SEC) using a 200 10/300 GL column pre-equilibrated in 20 mM Tris-HCl pH 8.0, 100 mM NaCl.

For crystallization experiments, the same constructs were expressed in Expi293F GnTI cells. The protein tags were cleaved overnight with thrombin and deglycosylated with EndoH. The RBD was mixed with a 1.3 molar excess of hACE2 and the complex was purified by SEC.

Biolayer Interferometry

Purified Avi-tagged RBD was biotinylated using a BirA biotin-protein ligase kit according to manufacturer's instructions (Avidity). The biotinylated RBDs at 100 nM were immobilized to SA sensors. A 1:2 dilution series of hACE2 starting at 100 nM in PBS-BSA buffer was used in cycles of 200 s association followed by 200 s dissociation steps to determine protein-protein affinity. The data were baseline-subtracted and the plots fitted using the Pall FortéBio/Sartorius analysis software (version 12.0). Data were plotted in Prism 9.1.0.

Crystallization and data collection

Crystals of complex BANAL-236 RBD/hACE2 were obtained at 4 °C in sitting drops by mixing 200 nl of the protein complex at 8 mg/mL with 200 nl of reservoir solution containing 0.2 M lithium sulfate, 0.1 M Tris 8.5, 30 % w/v PEG 4000. The crystals were soaked in reservoir solution containing 20% glycerol as cryoprotectant before being flash-frozen in liquid nitrogen. X-ray diffraction data was collected on the beamline PROXIMA 1 at the SOLEIL synchrotron (St Aubin, France) and reduced using the XDS package⁶⁸. The structure of the complex was determined by molecular replacement with Phaser software⁶⁹ using the coordinates of SARS-CoV-2 RBD in complex with hACE2 as search template (Protein Data Bank (PDB) 6MOJ). The model was manually corrected in COOT⁷⁰ and refined with phenix.refine⁷¹. The final coordinates were deposited in the PDB with the entry code 7PKI.

Molecular Dynamics simulations of RBD/hACE2 complexes

Generation of homology models. Homology models of the BANAL-236 and BANAL-52/103 RBD/hACE2 complexes were constructed using the X-ray structure of the SARS-CoV-2 RBD/hACE2 complex (PDB code 6MOJ; resolution 2.45 Å) as template using MODELLER v. 10.1⁷². These models included the fragments S19-D615 and T333-G526 of hACE2 and RBD, respectively, which were the regions resolved in the template. In these regions, BANAL-236 and BANAL-52/103 RBDs have a sequence identity to SARS-CoV-2 RBD equal to 96.9% and 97.4%, respectively. The alignment reported in Extended Data Fig. 3 was used. The zinc and chloride atoms present in the template were retained during homology modeling; *N*-Acetylglucosamine (NAG) and water residues were removed. 7 disulfide bonds were detected by MODELLER in the template (3 in hACE2 and 4 in the RBD) and enforced in the generation of the homology models using CHARMM-like distance and dihedral angles restraints. For each construct, 100 homology models were built and ranked based on the normalized DOPE score⁷³. The top 3 scoring models of each complex were used as starting points of 3 independent MD simulations, as described in the following section.

Details of the MD simulations: setup, equilibration, and production.

The 6 homology models described in the previous section along with the X-ray structure of the SARS-CoV-2 RBD/hACE2 complex (PDB code 6MOJ) were used as input to the CHARMM-GUI server⁷⁴. In the case of the X-ray structure, for consistency with the homology models, the zinc and chloride atoms present in 6MOJ were retained while the NAG and water residues were removed. The 7 systems were solvated in a triclinic box of initial x-y-z dimensions of -13.5 nm * 9.2 nm * 8.3 nm. Potassium and chloride ions were added to ensure charge neutrality at a salt concentration of 0.15 M. The total number of atoms was ~104000. Additional details of the systems are reported in Table S4. The CHARMM36m force field⁷⁵ was used for the protein and ions and the

TIP3P model⁷⁶ was used for the water molecules. CHARMM36m force field parameters for the 7 pairs of cysteines linked by disulfide bonds were used. The CHARMM-GUI models were first energy-minimized using the steepest descent algorithm. After minimization, the systems were equilibrated using a 1-ns long simulation in the NPT ensemble followed by a 1-ns long simulation in the NVT ensemble. The temperature *T* was set at 300 K and the pressure *P* at 1 atm using the Bussi-Donadio-Parrinello thermostat⁷⁷ and the Berendsen barostat⁷⁸, respectively. During equilibration, harmonic restraints on the positions of the protein backbone and sidechains heavy atoms were applied. For each system studied, production simulations were performed in the NVT ensemble for 1 μ s. A time step of 2 fs was used together with LINCS constraints on h-bonds⁷⁹. The van der Waals interactions were gradually switched off at 1.0 nm and cut off at 1.2 nm; the particle-mesh Ewald method was used to calculate electrostatic interactions with cutoff at 1.2 nm⁸⁰. Production simulations were performed at room temperature for consistency with the BLI experiments.

Details of the analysis. To evaluate the stability of the starting model during the production simulations, we calculated the backbone Root Mean Square Deviation (RMSD) with respect to the energy-minimized structure for each frame of the trajectories. The RMSD was calculated separately for the residues in: RBD (Extended Data Fig. 5A), hACE2 (Extended Data Fig. 5B), RBD-hACE2 interface (Extended Data Fig. 5C), and in the entire complex (Extended Data Fig. 5D). Interfacial residues were defined as the residues in one subunit closer than 0.8 nm to the residues in the other subunit in the X-ray structure of the SARS-CoV-2 RBD/hACE2 complex. RMSD calculations were performed using the *driver* utility of PLUMED v. 2.7⁸¹. To characterize conformational heterogeneity at the RBD-hACE2 interface, we performed a cluster analysis of the MD trajectories using as similarity metrics the backbone RMSD of the interfacial residues and the GROMOS clustering approach⁸² with a cutoff equal to 0.2 nm. The 9 trajectories were first concatenated, clustering was then performed, and finally the population of each cluster was calculated separately for each trajectory (Extended Data Fig. 5E). To estimate the binding energy between RBD and hACE2, we used the *InterfaceAnalyzer* tool in ROSETTA v. 3.11⁸³ (Extended Data Fig. 6A) and the *AnalyseComplex* tool in FoldX v. 4⁸⁴ (Extended Data Fig. 6B). To identify relevant interactions at the RBD and hACE2 interface that could contribute to the binding affinity of the complex, we quantified the frequency of formation of inter-subunits salt bridges (Fig. 3b) and hydrogen bonds (Extended Data Fig. 7) during the course of the MD simulations. For each frame of the trajectories, we used PLUMED to calculate the distances between the sidechain charged groups of aspartic acids (OD1/OD2), glutamic acids (OE1/OE2), lysines (NZ), and arginines (NH1/NH2). An inter-subunits salt bridge was then defined as formed if the distance between groups with opposite charge was lower than 0.32 nm. We confirmed this calculation using the *Salt Bridges* tool available in VMD⁸⁵. To monitor the formation of inter-subunits hydrogen bonds, we used the *Hydrogen Bond Analysis* module of the MDAnalysis library v. 1.0.0⁸⁶. A donor-acceptor distance and angular cutoffs of 0.3 nm and 150° were used to define the formation of a hydrogen bond. We confirmed this calculation using the *Hydrogen Bonds* tool available in VMD.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

Sequences data that support the findings of this study have been deposited in the GenBank and GISAID databases with the following accession numbers: MZ937000/EPI_ISL_4302644 (BANAL-52), MZ937001/EPI_ISL_4302645 (BANAL-103), MZ937002/EPI_ISL_4302646 (BANAL-116),

MZ937003 / EPI_ISL_4302647 (BANAL-236), and MZ937004 / EPI_ISL_4302648 (BANAL-247). Raw sarbecovirus NGS reads and Sanger sequencing .ab1 files were deposited onto the NCBI SRA repository under the BioProject PRJNA796968 (SAMN24959173-77). The crystal structure presented in this manuscript was deposited in the Protein Data Bank (PDB) with accession code 7PKI. The GROMACS topology and input files as well as the analysis scripts used are freely available on PLUMED-NEST (www.plumed-nest.org) under accession ID plu-mID:21.037. Source data are provided with this paper.

Code availability

MODELLER v. 10.1 (<https://salilab.org/modeller/>) and GROMACS v. 2020.4 (<http://www.gromacs.org>) were used for MD simulations. XDS (v. feb 5 2021), CCP4 (v.7.0), COOT (v. 0.8.6) and PHENIX (v. 1.19.2-4158) softwares were used for X-ray diffraction data processing, model building and refinement of BANAL 236 RBD-hACE2 complex. PyMOL (v 2.4.2) was used for structural image rendering. PLUMED v. 2.7 (<https://www.plumed.org>); ROSETTA v. 3.11 (<https://www.rosetta-commons.org/>); FoldX v. 4 (<http://foldxsuite.crg.eu/>); MDAnalysis v. 1.0.0 (<https://www.mdanalysis.org/>); MDTraj v. 1.9.5 (<https://www.mdtraj.org/1.9.5/index.html>); and in-house scripts available at <https://github.com/maxbonomi/bat-MD> were used for MD simulations. hyphy 2.5.31; PhyML & MAFFT implemented through NGPhylogeny (<https://ngphylogeny.fr/>); Simplot 3.5.1 were used for phylogenetic and recombination analyses. MSSPE 1 was used for *Betacoronavirus* primer enrichment design and is available at <https://github.com/chilulab/MSSPE-design>. MICROSEEK is an in-house pipeline that uses ALIEN TRIMMER v. 2.0 (<https://gitlab.pasteur.fr/GIPhy/AlienTrimmer>) for read trimming / clipping, BBNORM from BBMAP v. 38.86 package (<https://sourceforge.net/projects/bbmap/>) for coverage normalization, MEGAHIT v. 1.2.9 (<https://github.com/voutcn/megahit>) for assembly, a in-house ORF-finder (https://figshare.com/articles/code/translateReads_py/7588592), and then DIAMOND v. 2.0.4 (<https://github.com/bbuchfink/diamond/>) and NCBI BLAST v. 2.12.0+ (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.12.0/>) both for sequence searching.

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Acknowledgements The authors want to thank Souand Mohamed Ali, Nicolas Da Rocha, Angela Brisebarre, Thonglakhone Xaybounsou, and Somsanith Chonephetsarath for their help at the bench. Next-generation sequencing was performed with the help of Biomics Platform, C2RT, Institut Pasteur, Paris, France, supported by France Génomique (ANR-10-INBS-09-09), IBISA, and the Illumina COVID-19 Projects' offer. The work was granted access to the HPC resources of IDRIS under the allocation 2020-101592 made by GENCI. We thank the Ministry of Health and the Ministry of Natural Resources and Environments, Lao PDR, for their authorization of the field work and the Faculty of Environmental Science for its authorization of the field research collaboration. The work was funded by an Institut Pasteur "Covid Taskforce" and in part by the H2020 project 101003589 (RECOVER) and Labex IBEID (ANR-10-LABX62-IBEID) grants. Field and laboratory work at IP-Laos was also funded by a UK embassy grant (Grant No. INT 2021/LOV C19 02) and Luxembourg Development special grant (Grant No. LAO/030-202324).

Author contributions ST, KV, PTB, ME conceived the study design. KV, BD, KL, NPDS, VX, PPA carried out sample procurement and bat species identification. SM, FD performed virus isolation, entry, and neutralization assays. BR, DC, PP prepared the NGS libraries and carried out the next-generation sequencing. ST, BR, TB carried out the genome assembly, recombination, and phylogenetic analyses. VL, SS, KL, NP implemented the pan-coronavirus PCR testing. EBS and FR performed and analyzed the structure and binding studies. MB, YK, MN performed and analyzed the molecular dynamic simulations. ME and ST wrote the manuscript with inputs from all other authors.

Competing interests All authors of the manuscript are cited as inventors of a U.S. Provisional patent application number 63/240,535 filed on September 03, 2021 entitled: NEW ISOLATED BAT SARS-COV ABLE TO INFECT HUMAN CELLS AND HARBORING RECEPTOR-BINDING DOMAINS CLOSE TO SARS-COV-2. The patent covers medical applications (diagnostic, vaccination) of the sequences described in the manuscript, more specifically in the part "Diversity of bat and coronavirus species"

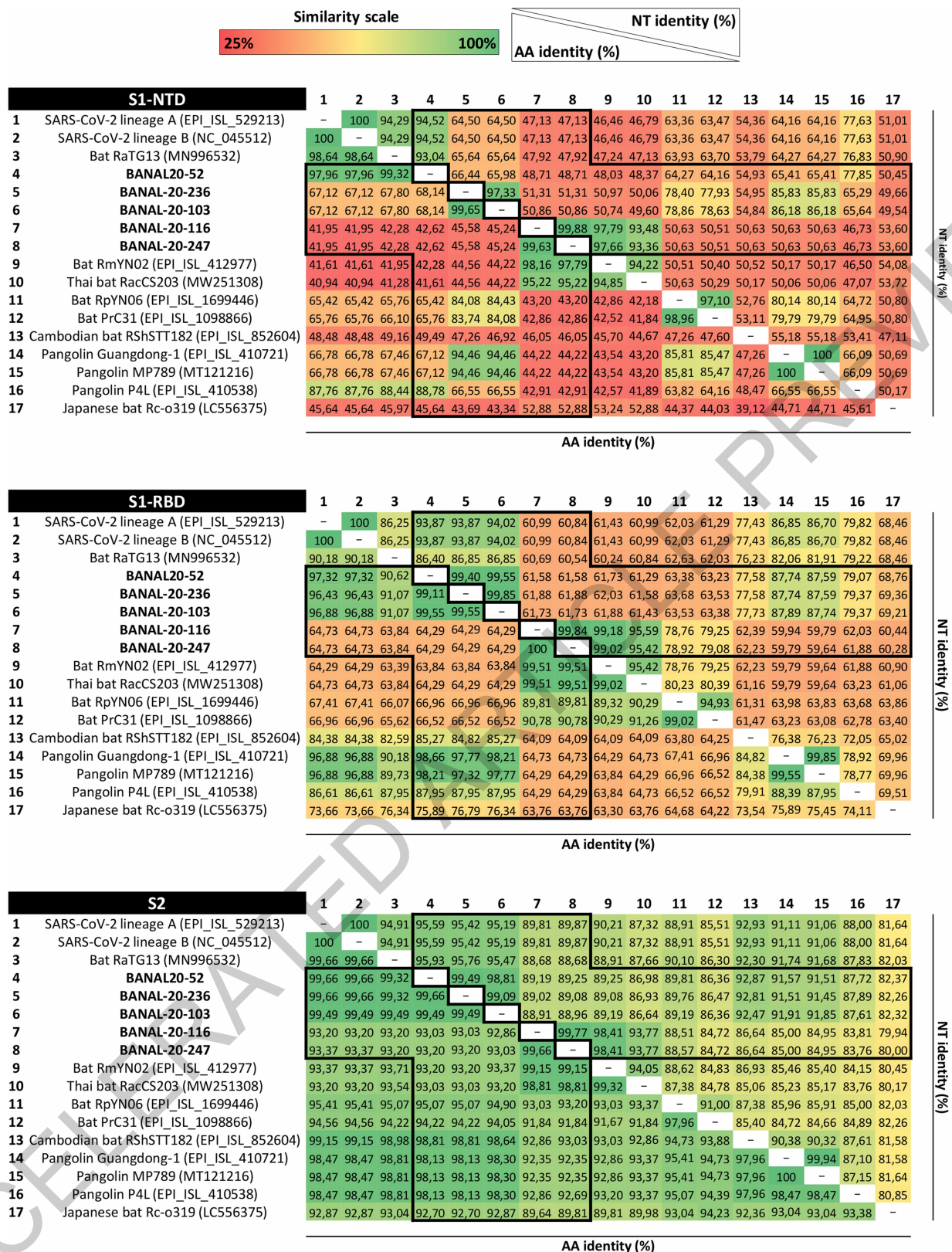
Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41586-022-04532-4>.

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Peer review information Nature thanks Michael Worobey and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Extended Data Fig. 1 | Spike identity matrices at the genus level of representative sarbecoviruses. Amino-acid (lower) and nucleotide (upper) identity matrices of Laotian and representative human, bat, and pangolin sarbecoviruses. Spike N-terminal (NTD), Receptor-binding (RBD) and S2

nucleotide and amino-acid sequences were aligned with MAFFT, and identity matrices were constructed using CLC Main Workbench 21.0.4 (Qiagen). Matrices were colored according to the identity scale, from 25% (red) to 100% (green) of nucleotide or amino-acid identity.

SARS-CoV-2 lineage A 319 RVQPTESIVRFPNITNLCPFGVEVFNATRFASVYAWNKRKISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFT 430
SARS-CoV-2 lineage B 319 RVQPTESIVRFPNITNLCPFGVEVFNATRFASVYAWNKRKISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIADYNYKLPDDFT 430
BANAL-20-52 319T.....T.....V..... 430
Bat RaTG13 319D.....T.....T.....T..... 430
Pangolin P4L 317I.....SK.....T.....VK.....V..... 428
BANAL-20-103 315D.....T.....T.....V..... 426
BANAL-20-236 315D.....T.....T.....V..... 426
Pangolin Guangdong-1 315T.....T.....V.....R..... 426
Pangolin MP789 315T.....T.....T.....V.....R..... 426
Bat PrC31 315Q.V.....V.....HK.....P.....E.TK.....D.I.....T.F.....T.....S.I.....S.....T.L.....FS.....V.....V..... 426
Bat RpYN06 315Q.V.....V.....HN.....P.....E.TK.....D.I.....T.F.....T.....S.I.....S.....T.L.....FS.....V.....V..... 426
Cambodian bat RShSTT182 306 .TS..TQV.....T.....R.....TT.....V.....M 417
BANAL-20-116 298 ..L.STEV.....F...DK.....PN...Q.TK..D.I...T...T.....S.I.....S.....T.L.....FS.....E..V..... 409
BANAL-20-247 298 ..L.STEV.....F...DK.....PN...Q.TK..D.I...T...T.....S.I.....S.....T.L.....FS.....E..V..... 409
Bat RmYN02 298 ..L.STEV.....F...DK.....PN...Q.TK..D.I...T...T.....S.I.....S.....T.L.....FS.....E..V..... 409
Thai bat RacCS203 298 ..L.STEV.....F...DK.....PN...Q.TK..D.I...T...T.....S.I.....S.....T.L.....FS.....E..V..... 409
Japanese bat Rc-o319 293QDTV.....S.....T.....R.....T.....Q.....S.....V.....V..... 404

SARS-CoV-2 lineage A 431 GCVIAWNSNNLDSKVGGNYLYRLFRKSNLKPFFERDISTEIYQAGSTPCNGVEGFNCYFPLOSYGFOPTNGVGYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFN 541
SARS-CoV-2 lineage B 431 GCVIAWNSNNLDSKVGGN.NYLYRLFRKSNLKPFFERDISTEIYQAGSTPCNGVEGFNCYFPLOSYGFOPTNGVGYPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVNFN 541
BANAL-20-52 431H.....N.....I..... 541
Bat RaTG13 431KHI.A.E...F.....A.....K.....QT.L...Y.YR...Y.D...H.....N..... 541
Pangolin P4L 429VKQ.ALT...G.....K.....QV.L...Y.ER...H.T..N...F.....NG.....L.T...D..... 539
BANAL-20-103 427H.....N.....I..... 537
BANAL-20-236 427K.....H.....N.....I..... 537
Pangolin Guangdong-1 427H.....N.....Q..... 537
Pangolin MP789 427H.....K.....Q..... 537
Bat PrC31 427TAKQ.VG---S.F..SH.STK.....L.SDE-----R-----T.ST.D.N.NVPLE..AT.....N.....L.Q.....Q..... 518
Bat RpYN06 427TAKQ.VG---S.F..SH.STK.....L.SDE-----R-----T.ST.D.N.NVPLE..AT.....N.....L.Q.....Q..... 518
Cambodian bat RShSTT182 418IS..A---S.Y...V.....QD.....DK..S---PD.....Y..S.....N.....H.V..... 523
BANAL-20-116 410 ..L...TAQQ.IG---S.F..SH.AVK.....L.SDE-----R-----T.ST.D.N.NVPLD..AT.....N.....L.Q.....R..... 501
BANAL-20-247 410 ..L...TAQQ.IG---S.F..SH.AVK.....L.SDE-----R-----T.ST.D.N.NVPLD..AT.....N.....L.Q.....R..... 501
Bat RmYN02 410 ..L...TAQQ.IG---S.F..SH.AVK.....L.SDE-----R-----T.ST.D.N.NVPLD..AT.....N.....L.Q.....R..... 501
Thai bat RacCS203 410 ..L...TAQQ.IG---S.F..SH.AVK.....L.SDE-----R-----T.ST.D.N.NVPLD..AT.....N.....L.Q.....R..... 501
Japanese bat Rc-o319 405 ..L...R.Q.ASTS..F..Y..IW.SEK.R.....AHYD..V.TQFKS-----S.KN...YSSA.DSH.....N.....Q..E.I..... 506

Extended Data Fig. 2 | Alignment of spike RBD domain. Protein alignment of the Receptor Binding Domain (RBD) of Laotian and representative human, bat and pangolin sarbecoviruses. Sequences were aligned with MAFFT in G-INS-I

mode. Residues interacting with human ACE2 receptor are highlighted in grey. The domain used for interactions modeling, based on the X-ray structure 6M0J (residues T333 to G526), is highlighted by a black line.

A.

	Y	Q/N	T/S	Q	T	N	S	P	R	R	A	R	S/R	V	A/T	S/T	Q/N	S
1. SARS-CoV-2_Wuhan-1	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
2. BANAL-20-52	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
3. BANAL-20-103	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
4. BANAL-20-236	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
5. Rhinolophus_affinis_Yunnan_RaTG13_2013	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
6. Rhinolophus_shameli_Cambodia_RShSTT182_2010	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
7. Rhinolophus_shameli_Cambodia_RShSTT200_2010	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
8. BANAL-20-116	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
9. BANAL-20-247	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
10. Rhinolophus_malayanus_Yunnan_RmYN02_2019	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t
11. Rhinolophus_acuminatus_Thailand_2020_RacCS203	t	a	t	c	a	g	a	c	t	c	a	a	c	t	a	a	t	t

B.

	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
1. SARS-CoV-2_Wuhan-1	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
2. BANAL-20-52	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
3. BANAL-20-103	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
4. BANAL-20-236	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
5. Rhinolophus_affinis_Yunnan_RaTG13_2013	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
6. Rhinolophus_shameli_Cambodia_RShSTT182_2010	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
7. Rhinolophus_shameli_Cambodia_RShSTT200_2010	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
8. BANAL-20-116	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
9. BANAL-20-247	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
10. Rhinolophus_malayanus_Yunnan_RmYN02_2019	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T
11. Rhinolophus_acuminatus_Thailand_2020_RacCS203	T	A	T	C	A	G	A	C	T	C	A	A	C	T	A	A	T	T

C.

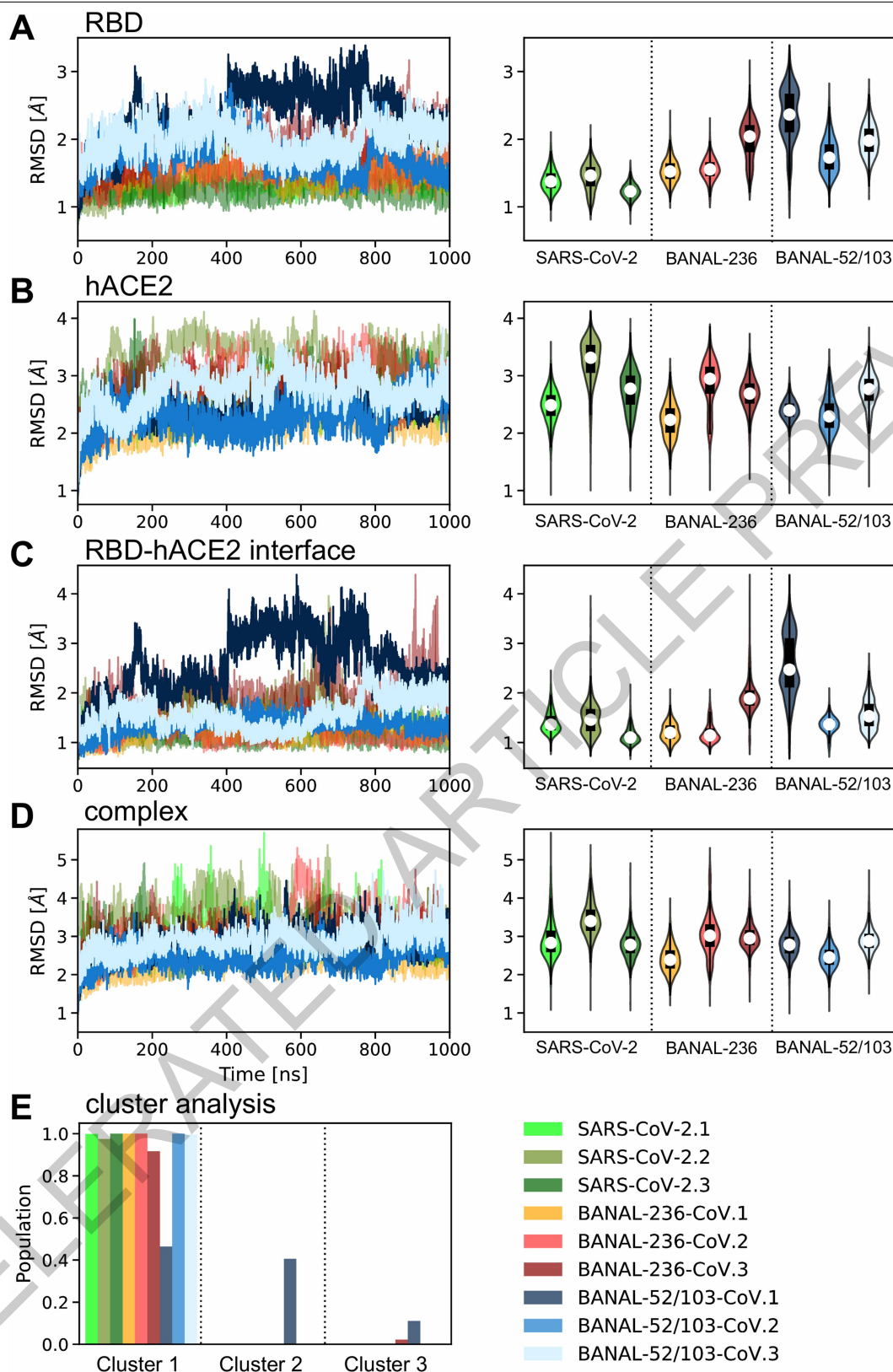
	Y	Q	T	N	S	P	R	R	A	R	S/R	V	A/T	S/T	Q/N	S
1. SARS-CoV-2_Wuhan-1	Y	Q	T	N	S	P	R	R	A	R	S	V	A	S	Q	S
2. BANAL-20-52	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
3. BANAL-20-103	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
4. BANAL-20-236	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
5. Rhinolophus_affinis_Yunnan_RaTG13_2013	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
6. Rhinolophus_shameli_Cambodia_RShSTT182_2010	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
7. Rhinolophus_shameli_Cambodia_RShSTT200_2010	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
8. BANAL-20-116	Y	N	S	P	A	-	-	-	A	R	V	G	T	N	S	S
9. BANAL-20-247	Y	N	S	P	A	-	-	-	A	R	V	G	T	N	S	S
10. Rhinolophus_malayanus_Yunnan_RmYN02_2019	Y	N	S	P	A	-	-	-	A	R	V	G	T	N	S	S
11. Rhinolophus_acuminatus_Thailand_2020_RacCS203	Y	N	S	P	V	-	-	-	A	R	V	G	T	N	S	S

D.

	Y	Q	T	N	S	P	R	R	A	R	S/R	V	A/T	S/T	Q/N	S
1. SARS-CoV-2_Wuhan-1	Y	Q	T	N	S	P	R	R	A	R	S	V	A	S	Q	S
2. BANAL-20-52	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
3. BANAL-20-103	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
4. BANAL-20-236	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
5. Rhinolophus_affinis_Yunnan_RaTG13_2013	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
6. Rhinolophus_shameli_Cambodia_RShSTT182_2010	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
7. Rhinolophus_shameli_Cambodia_RShSTT200_2010	Y	Q	T	N	S	-	-	-	R	S	V	A	S	Q	S	S
8. BANAL-20-116	Y	-	-	-	N	S	P	A	-	A	R	V	G	T	N	S
9. BANAL-20-247	Y	-	-	-	N	S	P	A	-	A	R	V	G	T	N	S
10. Rhinolophus_malayanus_Yunnan_RmYN02_2019	Y	-	-	-	N	S	P	A	-	A	R	V	G	T	N	S
11. Rhinolophus_acuminatus_Thailand_2020_RacCS203	Y	-	-	-	N	S	P	V	-	A	R	V	G	T	N	S

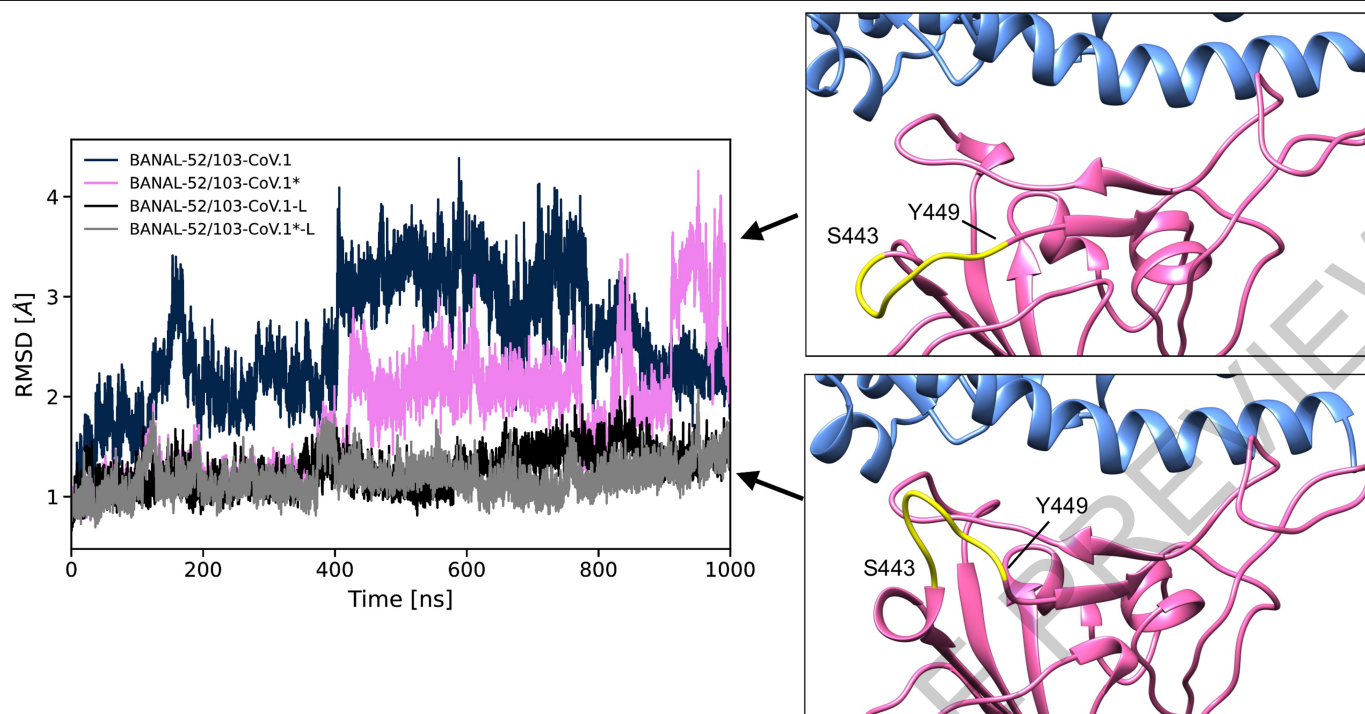
Extended Data Fig. 3 | Nucleotide and amino-acid alignments of the furin cleavage site region. Complete nucleotide and amino-acid spike sequences of representative bat SARS-CoV-2-like coronaviruses were downloaded from GenBank and GISAID and aligned with MAFFT (G-INS-I parameter) (A & C).

Alignments were manually edited as proposed by Zhou² and Lytras⁴⁷ with CLC Main Workbench (Qiagen) (B & D). Alignments of the furin cleavage region are presented at the nucleotide (A & B) and the amino-acid (C & D) level, respectively.



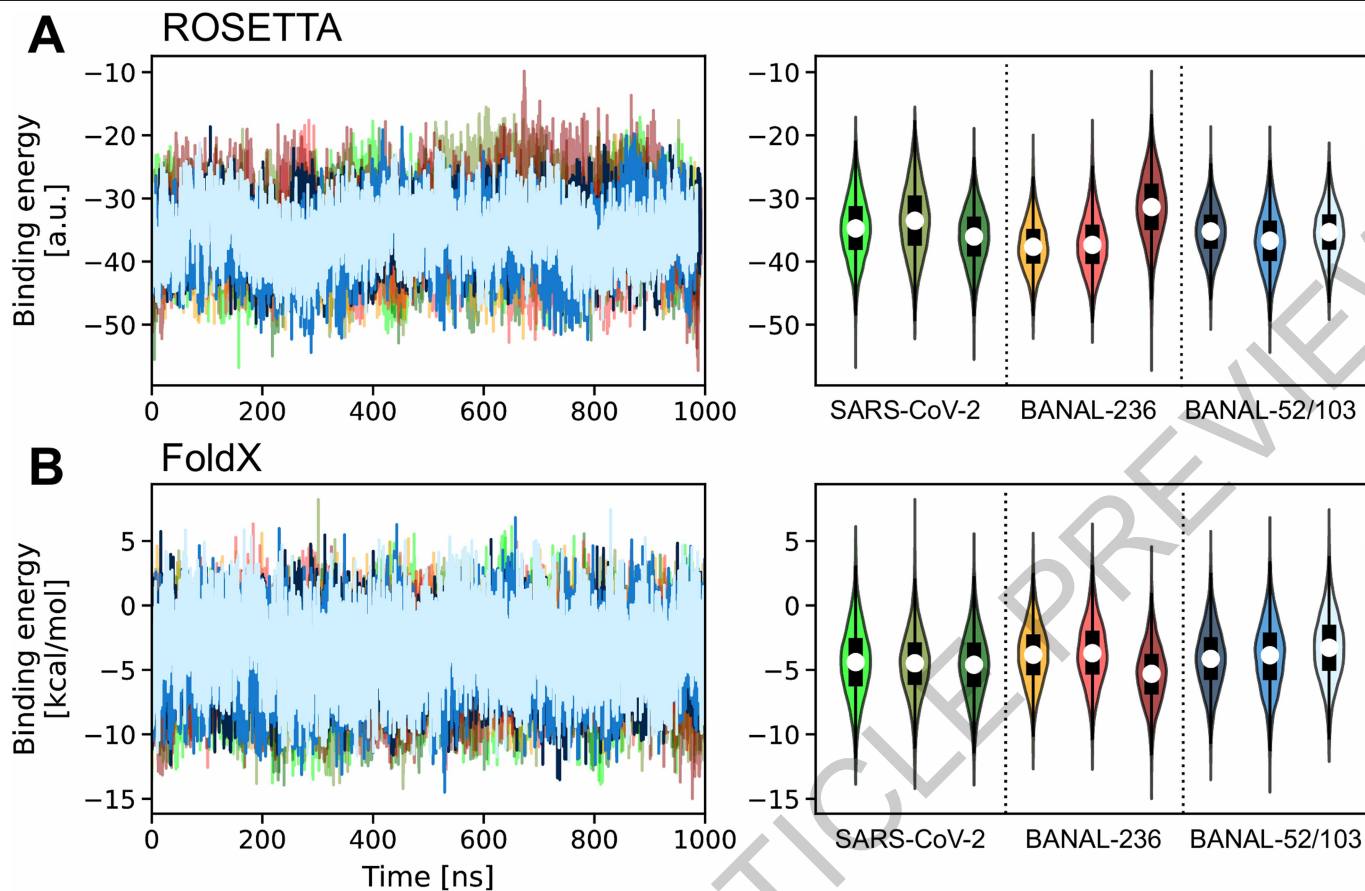
Extended Data Fig. 4 | Analysis of the stability and conformational heterogeneity of RBD/hACE2 complexes. Time series (left column) and violin plots (right column) of backbone Root Mean Square Deviation (RMSD) from the initial, energy-minimized model calculated on the residues in RBD (A), hACE2 (B), at the interface of RBD and hACE2 (C), and on the entire complex (D). In the violin plots, the white circle corresponds to the median value, the black

rectangle extends from the first to the third quantiles, and the thin black line represents the 95% confidence intervals. Population of the 3 most significant clusters visited during the course of the MD simulations (E). The analysis is performed for 9 different MD simulations: 3 replicates of the SARS-CoV-2 (shades of green), BANAL-236 (shades of red), and BANAL-52/103 (shades of blue) RBD/hACE2 complexes.



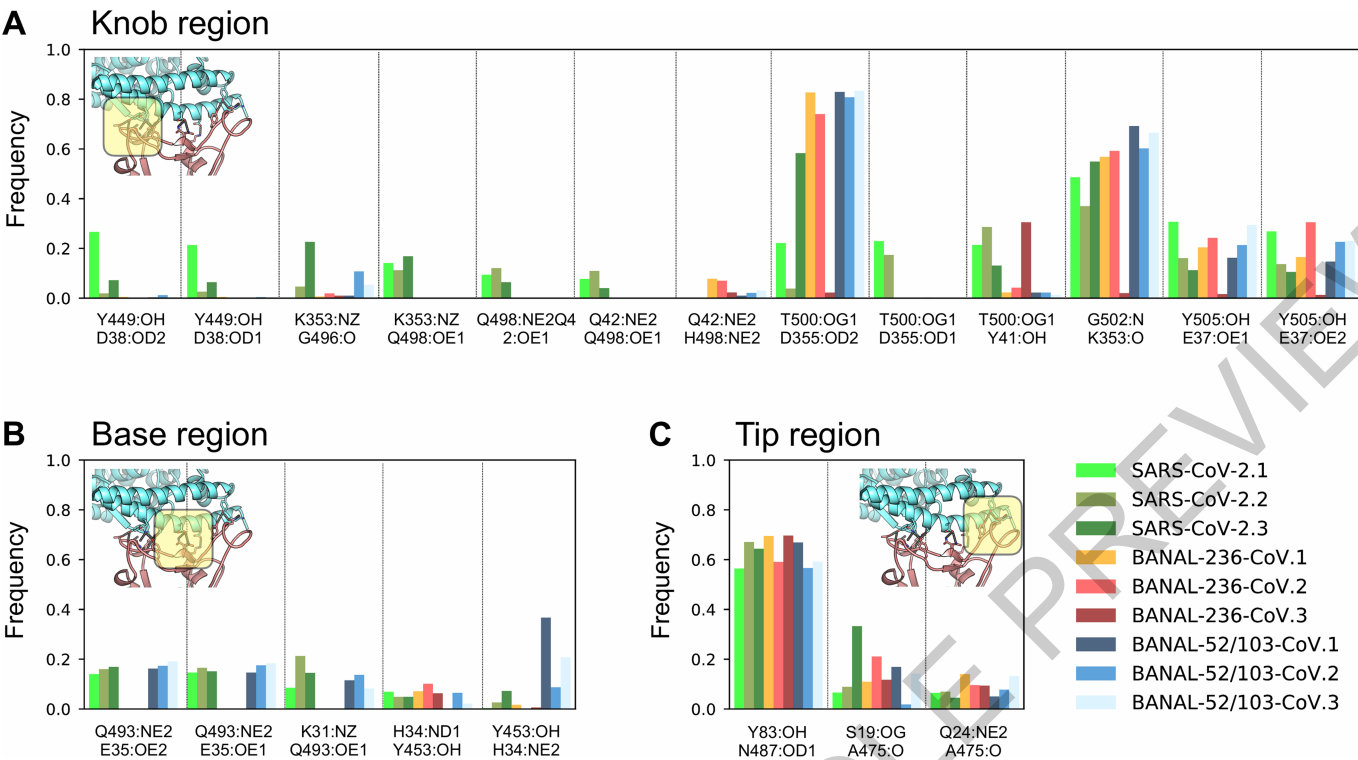
Extended Data Fig. 5 | Additional analysis of the MD simulations of the BANAL-52/103 RBD/hACE2 complex. Comparison of the time series of interface RMSD during the course of two MD simulations of the BANAL-52/103 RBD/hACE2 complex with short (BANAL-52/103-CoV.1) and long (BANAL-52/103-CoV.1*) equilibration phase. The large fluctuations of the interface

RMSD are due to the flexibility of the RBD loop between residues S443 and Y449 (insets, in yellow). When these residues were not included in the calculation of the interface RMSD, the time series displayed a more stable behavior (BANAL-52/103-CoV.1-L and BANAL-52/103-CoV.1*-L).



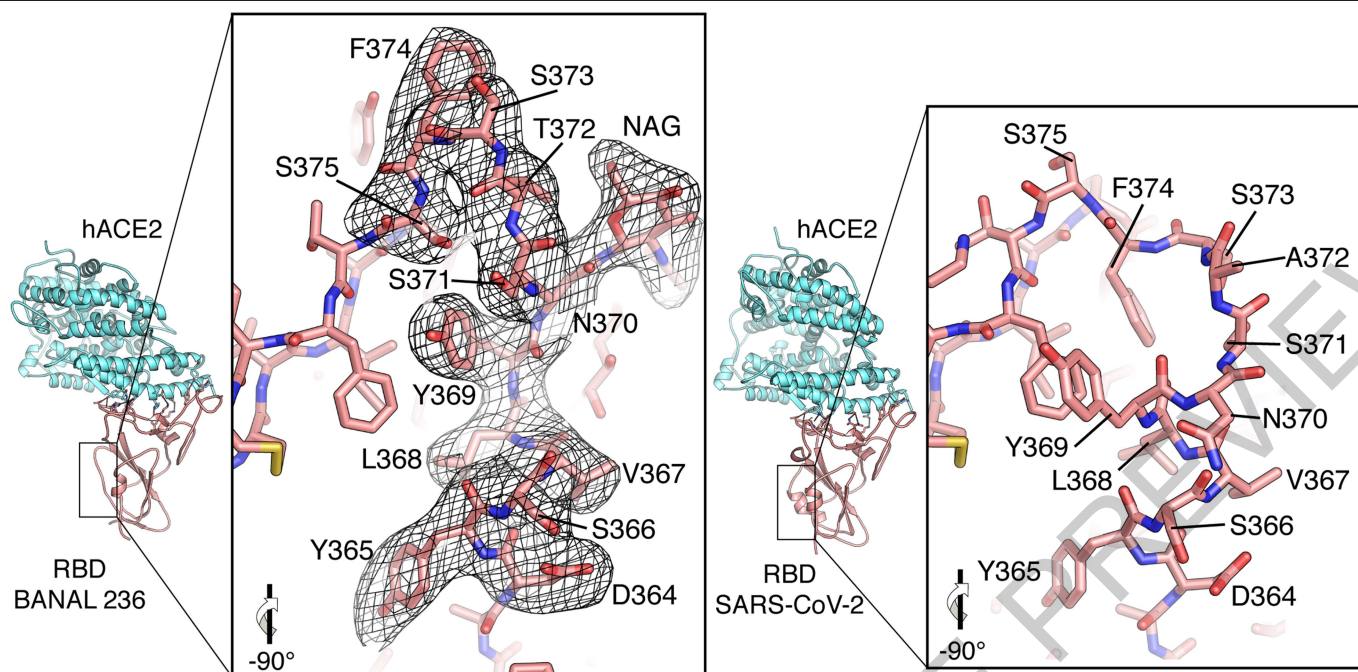
Extended Data Fig. 6 | Estimation of RBD-hACE2 binding energy. Time series (left column) and violin plots (right column) of the RBD-hACE2 binding energy estimated using ROSETTA (A) and FoldX (B). In the violin plots, the white circle corresponds to the median value, the black rectangle extends from

the first to the third quantiles, and the thin black line represents the 95% confidence intervals. The analysis is performed for 9 different MD simulations: 3 replicates of the SARS-CoV-2 (shades of green), BANAL-236 (shades of red), and BANAL-52/103 (shades of blue) RBD/hACE2 complexes.

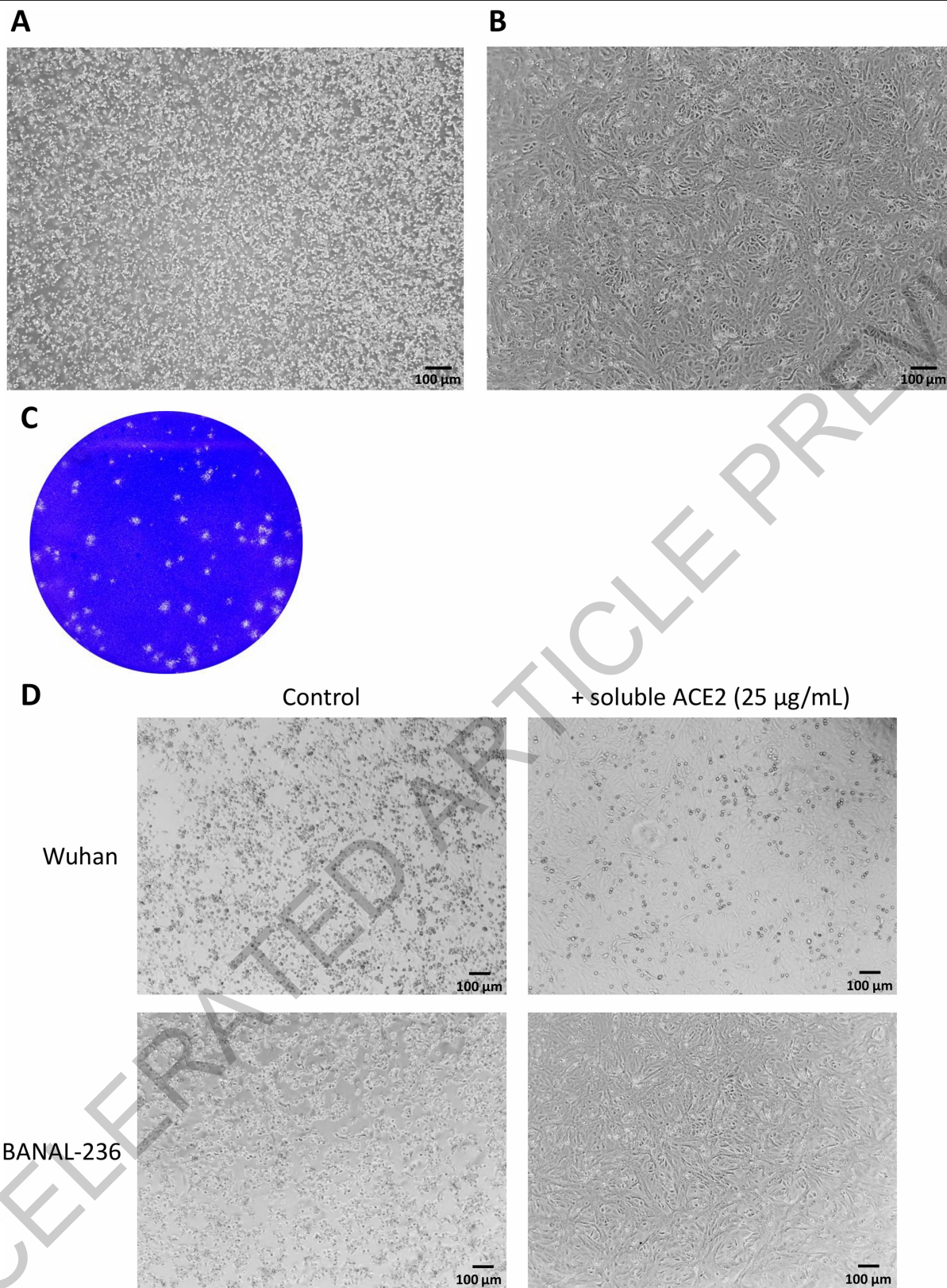


Extended Data Fig. 7 | Analysis of the inter-subunits hydrogen bonds at the interface of RBD and hACE2. Frequency of formation of hydrogen bonds at the interface of RBD and hACE2 in the knob (A), base (B), and tip regions (C).

The analysis is performed for 9 different MD simulations: 3 replicates of the SARS-CoV-2 (shades of green), BANAL-236 (shades of red), and BANAL-52/103 (shades of blue) RBD/hACE2 complexes.



Extended Data Fig. 8 | Sticks representation. of segments D364-S375 of BANAL 236 (left panel) and SARS-CoV-2 (right panel) RBDs. A 2Fo-Fc composite omit map (contoured at 3σ is shown for this region in BANAL 236 RBD.



Extended Data Fig. 9 | Isolation of BANAL-236 on VeroE6 cells. (A) CPE observed on VeroE6 4 days after inoculation at an MOI of 10^{-4} from the C1. (B) Uninfected VeroE6 cells layer. (C) Plaque assay performed from the C2 stock on VeroE6 cells. (D) Comparative CPE observed on VeroE6 cells infected with

Wuhan (top panel) or BANAL-236 (bottom panel) in absence (left) or in presence (right) of soluble ACE2. Photos were taken with a EVOS XL Core microscope at $\times 10$. A single experiment performed in triplicate is shown for each cell line.

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☒ ☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☒ ☐ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☒ ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☒ ☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☒ ☐ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection MODELLER v. 10.1 (<https://salilab.org/modeller/>) and GROMACS v. 2020.4 (<http://www.gromacs.org>) were used for MD simulations.

Data analysis XDS (v. feb 5 2021), CCP4 (v 7.0), COOT (v. 0.8.6) and PHENIX (v. 1.19.2-4158) softwares were used for X-ray diffraction data processing, model building and refinement of BANAL 236 RBD-hACE2 complex. PyMOL (v 2.4.2) was used for structural image rendering.

PLUMED v. 2.7 (<https://www.plumed.org>); ROSETTA v. 3.11 (<https://www.rosettacommons.org/>); FoldX v. 4 (<http://foldxsuite.crg.eu/>); MDAnalysis v. 1.0.0 (<https://www.mdanalysis.org/>); MDTraj v. 1.9.5 (<https://www.mdtraj.org/1.9.5/index.html>); and in-house scripts available at <https://github.com/maxbonomi/bat-MD> were used for MD simulations.

hyphy 2.5.31; PhyML & MAFFT implemented through NGPphylogeny (<https://ngpphylogeny.fr/>); Simplot 3.5.1 were used for phylogenetic and recombination analyses.

MSSPE 1 was used for Betacoronavirus primer enrichment design.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The sequences generated through the study were deposited into the GISAID database (<https://www.gisaid.org/>) under the accessions EPI_ISL_4302644 - 48. Raw reads used to assemble the genomes were deposited onto SRA database (BioProject PRJNA796968 : BioSamples SAMN24959173-77).

The crystal structure presented in this manuscript was deposited in the Protein Data Bank (PDB) with accession code 7PKI.

The GROMACS topology and input files as well as the analysis scripts used are freely available on PLUMED-NEST (www.plumed-nest.org) under accession ID plumID:21.037

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	description of the fecal virome of bats from Laos, with a focus on sarbecoviruses
Research sample	A total of 645 bats belonging to six families and 46 species were captured . Two hundred and forty-seven blood samples, 608 saliva, 539 anal/feces, and 157 urine swabs were collected from the northern part of Laos
Sampling strategy	No calculation of sample size
Data collection	A total of 645 bats belonging to six families and 46 species were captured. Bats were collected using four-banks harp traps and mist nets. Captured bats were held individually in cloth bags and transport in polystyrene foam box to field station for ID and sampling. After samples were taken, bats were marked with wing bands for individual identification and released at the captured point within 12 hours. Those infected with a sarbecovirus were all from the Fueng district in Vientiane Province
Timing and spatial scale	First quarter 2021
Data exclusions	no
Reproducibility	without object
Randomization	without object
Blinding	without object
Did the study involve field work?	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No

Field work, collection and transport

Field conditions	All samples were stored at -20°C in the field using portable mini freezer (-20°C). The samples were then transported with dry ice to IPL and stored at -80°C till laboratory analysis.
Location	The GPS coordinates of sampling are provided below 18° 32.879'N 101° 58.938'E 18° 32.914'N 101° 58.459'E 18° 34.018'N 101° 58.338'E 18° 31.743'N 101° 58.648'E 18° 30.495'N 101° 57.421'E 18° 54.426'N 101° 58.789'E 18° 54.898'N 101° 59.048'E 18° 53.717'N 101° 56.957'E

18° 54.787'N 101° 58.292'E
 18° 55.386'N 101° 57.723'E
 20° 43.071'N 101° 45.811'E
 20° 43.111'N 101° 46.577'E
 20° 41.647'N 101° 47.179'E
 20° 44.143'N 101° 46.770'E
 20° 52.396'N 101° 46.973'E
 20° 51.907'N 101° 46.926'E

Access & import/export	The bat study was approved by the wildlife authorities of the Department of Forest Resource Management (DFRM), and the Ministry of Agriculture and Forestry, Lao PDR, No. 2493/DFRM, issued on May 21, 2020; No. 0755/MAF issued on June 2, 2020. Importation in France was approved (import permit 2021-75-13530)
Disturbance	no disturbance

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> Antibodies
<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input type="checkbox"/>	<input checked="" type="checkbox"/> Animals and other organisms
<input type="checkbox"/>	<input checked="" type="checkbox"/> Human research participants
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data
<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern

Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	GnTI- Expi293™ cell lines for recombinant protein production were obtained from ThermoFisher Scientific. Calu-3 (ATCC HTB-55), Caco-2 (ATCC HTB-37), 293T (ATCC CRL-3216), VeroE6 (ATCC CRL-1586) were obtained from ATCC. 293T-ACE2 were developed at Institut Pasteur and described in PMID: 33259646
Authentication	Cells were authenticated by the provider
Mycoplasma contamination	All cell lines tested negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals	The study did not involve samples from laboratory animals.
Wild animals	A total of 645 bats belonging to six families and 46 species were captured. Bats were collected using four-banks harp traps and mist nets. Captured bats were held individually in cloth bags and transport in polystyrene foam box to field station for ID and sampling. After samples were taken, bats were marked with wing bands for individual identification and released at the captured point within 12 hours.
Field-collected samples	All samples were stored at -20°C in the field using portable mini freezer (-20°C). The samples were then transported with dry ice to IPL and stored at -80°C till laboratory analysis.
Ethics oversight	The bat study was approved by the wildlife authorities of the Department of Forest Resource Management (DFRM), and the Ministry of Agriculture and Forestry, Lao PDR, No. 2493/DFRM, issued on May 21, 2020; No. 0755/MAF issued on June 2, 2020.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics	Serum samples were previously tested by ELISA and seroneutralization The study protocol was approved by the Lao National Ethics Committee for Health Research (NECHR) (Ref #052/2020). Oral and written informed consent was obtained from people aged 15 years and older. Parental consent was taken for children aged between 5 and 12 years, and assent from children aged between 12 and 14 years in addition to parental consent, before the survey
Recruitment	Sera were already available annd selected based on their status regarding seroneutralization of SARS-CoV-2
Ethics oversight	Lao National Ethics Committee for Health Research (NECHR) (Ref #052/2020)

Note that full information on the approval of the study protocol must also be provided in the manuscript.