SARS-like coronaviruses in horseshoe bats (*Rhinolophus* spp.) in Russia, 2020.

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Abstract

We found and genetically described two novel SARS-like coronaviruses in feces and oral swabs of the great (*R. ferrumequinum*) and the lesser (*R. hipposideros*) horseshoe bats in southern region of Russia. The viruses, named Khosta-1 and Khosta-2, together with related viruses from Bulgaria and Kenya, form a separate phylogenetic lineage. We found an evidence of recombination events in evolutionary history of Khosta-1, which involved the acquisition of structural proteins S, E, and M as well as nonstructural genes ORF3, ORF6, ORF7a, and ORF7b from a virus that is closely related to Kenyan isolate BtKY72. Examination of bats by RT-PCR revealed that 62,5% of great horseshoe bats in one of the caves were positive for Khosta-1 virus while its overall prevalence was 14%. The prevalence of Khosta-2 was 1,75%. Our results show that SARS-like coronaviruses circulate in horseshoe bats in the region and provide a new data on their genetic diversity.

Introduction

Horseshoe bats (*Rhinolophidae: Rhinolophus*) are considered as a main natural reservoir and source of zoonotic coronaviruses (CoV) which caused epidemic outbreak of severe acute respiratory syndrome (SARS) and COVID-19 pandemic in 2002 and 2019, respectively (1,2). These viruses, designated as SARS-CoV and SARS-CoV-2, together with related viruses found in bats and other animals (SARS-like coronaviruses or SARS-CoV-like viruses) belong to the subgenus *Sarbecovirus* of the genus *Betacoronavirus* of the family *Coronaviridae* (3). Horseshoe bats are wide distributed in Asia, Europe, and North Africa. In East Asia (in particular, in People's Republic of China (PRC)) SARS-CoV-like viruses circulate in multiple rhinolophid's species, however the Chinese rufous (*R. sinicus*), the greater (*R. ferrumequinum*), the

intermediate (*R. affinis*), and the king (*R. rex*) horseshoe bats seem to be of major importance (4). In Europe SARS-CoV-like viruses was found in the greater, the lesser (*R. hipposideros*), the Miditerranean (*R. euryale*), Mehely's (*R. mehelyi*), and Blasius's (*R. blasii)* horseshoe bats (5–8). The prevalence of SARS-like coronaviruses among bats in different caves-colonies can vary from 0% to 60% (4,7,9,10). In Russia three species of horseshoe bats (the greater, the lesser, and Miditerranean) are common in southern regions, lying below about 44º north latitude, mostly including North Caucasus and Crimea. In present work we hypothesized that SARS-like coronaviruses circulate in the region in local populations of horseshoe bats. To test this hypothesis, we examined the colonies of bats located in the southern macroslope of the Greater Caucasus on northern coast of the Black Sea in Russia. Using metagenomic analysis we found and genetically described two new SARS-like coronaviruses in feces and oral swabs of the greater and the lesser horseshoe bats. Further PCR analysis showed a high degree of infection of bats with discovered viruses in some locations.

Materials and methods

The samples from bats were collected in Sochi National Park (Sochi-Adler, Krasnodar krai, Russia) and surrounding areas in March-October 2020. The Sochi National Park is located on southern macroslope of the Greater Caucasus descending to the northern coast of the Black Sea (Figure 1). The park stuffs keep records of more than 300 karst formations (caves, breaks, mines, clefts, etc.) that are natural refuges for bats and other troglophilous animal species. Bats were caught by hand in eight locations including five caves as well as basements and attics of houses (Table 1). The bats were caught in the frame of ongoing surveillance of bats populations constantly carried out in the park. The species of the animals was determined based on their

morphological characters by an experienced park zoologist. The length of the forearm and weight of animals were measured. To collect bat oral swabs (saliva and buccal cells) an urological swab was placed in the bat's mouth for 10-15 sec and then placed in 250 µl of phosphate-buffered saline (PBS). To collect feces, an animal was placed in a small white cotton bag for 10-15 min. After that the animal was released, and the feces were collected from the walls of the bag into cryovials. No bats were harmed during sample collection. A total of 120 samples of oral swabs and 77 samples of feces from five species of bats were collected (Table 1). The samples were delivered to the laboratory on ice and stored at -70ºC until start of research.

Metagenomic analysis was conduct as described anywhere. Briefly, the feces were suspended and homogenized in 0,5 ml of PBS and pooled by 0,1 ml in three samples (faces from 20-30 animals in the pool). Pooled samples were clarified by centrifugation (10 000 g, 15 min) and treated with DNase I and RNase I_f (NEB, Great Britain) for removing of naked out-capsid nucleic acid. The viral particles were sedimented from treated samples by ultracentrifugation (30 000 g, 1 h) through 2 ml of 20% sucrose. Virus plaque was resuspended in 0,5 ml PBS. Total RNA was isolated from 0,25 ml of obtained solution with TRIzol LS reagent (ThermoFisher Scientific, USA).

Total RNA from oral swabs were isolated with TRIzol LS reagent from individual samples and pooled by 20 µl in five pooled samples (20-25 samples in the pool). The pooled RNA was precipitated by isopropyl alcohol with addition of glycogen followed by additional clarification with RNeasy MinElute Cleanup kit (Qiagen, Germany). NEBNext rRNA Depletion kit (NEB) was used to remove bacterial and eukaryotic rRNA from total RNA isolated from pooled samples. Treated RNA was used for cDNA library preparation by NEBNext Ultra II RNA library kit for Illumina (NEB).

The libraries were sequenced on a HiSeq4000 instrument (Illumina, USA) at the facility of Resource Center "BioBank" of the Research Park of Saint Petersburg State University (Saint-Petersburg, Russia). Reads were filtered by quality, trimmed to remove adapter's sequences, and assembled *de novo* using CLC Genomics Workbench 7.0 software (Qiagen). Obtained contig sequences were analyzed using blastx algorithm by DIAMOND software (11) against nr 'Viruses' database that included all reference viral sequences available in GenBank at December 2020. Nucleotide and deduced amino acid sequences were alignment by ClustalW implemented in MEGAX software (12). The best substitution model was evaluated for each alignment by the Model selection module in MEGAX software. Phylogenetic trees were inferred by 'maximum likelihood' method using appropriate model with 1000 bootstrap replicates by MEGAX software. Similarity plot analysis was conducted by SimPlot software (13). Possible recombination was analyzed by RDP5 software (14).

Primers and probes for specific detection of discovered coronaviruses were developed based on obtained sequences by Beacon 7.0 software (Premier Biosoft, USA). Real-time RT-PCR was conducted with TaqPath 1-Step Multiplex Master Mix (ThermoFisher Scientific, USA) and total RNA isolated by TRIzol LS reagent from individual oral swabs and faces samples.

Results

Results of sequencing of the samples. In total, 124 522 978 reads for three pooled fecal samples and 170 112 341 reads for five pooled oral swab samples were obtained. The reads were *de novo* assembled in contigs and analyzed by blastx algorithm for presence of viral sequences. The search results revealed two extended contigs with a length of approximately 29 Kb with open reading frames (ORFs) with similarity to members of the genus *Betacoronavirus* in the

pools one and three of fecal samples, respectively. With further analysis, a near-complete genome of two novel SARS-like coronaviruses was sequenced. Matching contigs have also been found in corresponding oral swab samples, but with smaller length and coverage. Two found SARS-like coronaviruses were named BtCoV/Khosta-1/Rh/Russia/2020 and BtCoV/Khosta-2/Rh/Russia/2020 and placed in GenBank by accession numbers MZ190137 and MZ190138, respectively. Bellow they are referred to as Khosta-1 and Khosta-2, respectively.

Genetic and phylogenetic analysis. The genomic organization of Khosta-1 and Khosta-2 is similar to that of other SARS-like coronaviruses (Figure 2). Approximately two thirds of the genome of coronaviruses is occupied by ORF1a and ORF1b genes which encode the proteins of the replicative complex and translated as ORF1ab polyprotein due to ribosomal shifting. The rest of the genome contains genes of structural proteins (S, E, M, and N), which form a virion, as well as several non-structural proteins (ORF3, ORF6, ORF7, ORF8, ORF9, and ORFX), the presence and structure of which varies in different viruses (3). Genome organization of Khosta-1 and Khosta-2 has the greatest similarity with BtCoV/BM48-31/2008 and BtKY72 viruses – two SARS-like coronaviruses found in horseshoe bats in Bulgaria and Kenya in 2008 and 2007, respectively (8,15). Their peculiarity is the absent of ORF8 gene which is common in bat SARS-like coronaviruses from East and Southeast Asia (Figure 2).

Pairwise alignments of the deduced proteins of Khosta-1 and Khosta-2 virus with those of other SARS-like coronaviruses showed its highest similarity with BtCoV/BM48-31/2008 and BtKY72 viruses (Table 2). Khosta-1 is closest related to BtCoV/BM48-31/2008 with 92,5% aa and 99% aa identity in the conservative ORF1a and ORF1b proteins, respectively. Similarity of Khosta-1 with SARS-CoV and related viruses from China are on average 81,5% aa identity in ORF1a protein and 96% in ORF1b protein. Comparison Khosta-1 with SARS-CoV-2 viruses

revealed 77,5% and 94,2% aa identity for ORF1a and ORF1b proteins, respectively. Despite the high similarity of Khosta-1 and BtCoV/BM48-31/2008 in ORF1a and ORF1b proteins, structural proteins S, E, and M of Khosta-1 are more similar to those of Kenyan virus BtKY72. Khosta-1 and BtKY72 share 89,1%, 98,7%, and 97,29% aa identity for S, E, and M proteins, whereas these values for Khosta-1 and BtCoV/BM48-31/2008 are 84,37%, 89,47, and 95%, respectively. N protein of Khosta-1 is more similar to that of BtCoV/BM48-31/2008 (96,64%aa identity) than BtKY72 (92,6% aa identity).

In contrast, Khosta-2 does not have such an increased similarity with some group of sarbecoviruses and has 79-81% aa identity with SARS-CoV viruses and 76-77% with SARS-CoV-2 viruses in ORF1a protein. ORF1b protein of Khosta-2 has 93,5-95% aa identities with all other bat SARS-like coronaviruses. Comparison of proteins of Khosta-1 and Khosta-2 showed that these viruses differ from each other at about the same level as Khosta-2 differs from other bat SARS-like coronaviruses (Table 2).

Recombination analysis. Genome sequence similarity between Khosta-1, Khosta-2, BtCoV/BM48-31/2008, and BtKY72 was analyzed by Simplot and RDP5 software (Figure 2A-2C). Simplot analysis showed high degree of similarity of Khosta-1 and BtCoV/BM48-31/2008 in the ORF1ab and N genes and a decrease in the similarity in the S-ORF7b region. Results of analysis carried out by RDP5 software using different methods implemented in the program showed clear signals of recombination events in evolutionary history of Khosta-1 (Figure 2C). Recombination events presumably included acquisition of S-ORF7b region by ancestor of Khosta-1 virus from a virus closely related to BtKY72.

Phylogenetic analysis. Phylogenetic analysis based on ORF1ab protein sequences showed that Khosta-1, Khosta-2, BtCoV/BM48-31/2008, and BtKY72 form a monophyletic lineage

located between SARS-CoV and SARS-CoV-2 lineages of the *Sarbecovirus* subgenus (Figure 3A). The separate cluster this group of viruses also formed on phylogenetic tree based on nucleotide sequences of S gene (Figure 3B). Topology of tree confirms the probable recombination event in evolutionary history of Khosta-1. In ORF1ab tree Khosta-1 is grouped together with BtCoV/BM48-31/2008, while in S gene tree with BtKY72.

Analysis of receptor binding motif (RBM) of S protein. Alignment of amino acid sequences of RBM of Khosta-1 and Khosta-2 with certain sarbecoviruses are presented in Figure 4. This is a highly variable region where multiple substitutions and deletions occur among SARS-CoV related viruses. Khosta-1 and Khosta-2 as well as BtCoV/BM48-31/2008 have a common deletion of four aa in the N-part of RBM. This deletion partially overlapping with the deletion that is characteristic of HKU3-1 and related strains of bat SARS-CoV-like viruses that unable to bind angiotensin-converting enzyme 2 (ACE2) receptor. We analyzed aa positions in RBM which are thought to be crucial for binding of ACE2 receptor and, therefore, important for adaptation of bat SARS-like coronaviruses to human (16,17). Only at position 442 Khosta-1 and Khosta-2 share a common amino acid (L), which is also inherent in SARS-CoV-2 and related viruses. Despite the significant genetic distance between Khosta-1 and BtKY72, crucial positions in RBM and their context coincide between them. In contrast, position 479, 480, and 487 of Khosta-2 coincides poorly with other groups of viruses (Figure 4).

PCR testing. We developed primers and probes for specific detection of Khosta-1 and Kosta-2 viruses in faces and oral swabs by real time RT-PCR (Appendix Table). Results of PCR testing of samples are presented in Table 1. RNA of Khosta-1 was detected mostly in the greater horseshoe bats collected in Kolokolnaya cave. All four found positive oral swabs belonged to animals with positive fecal samples. In other locations Khosta-1 virus was detected only in two

fecal samples – from the greater horseshoe bat from Khosta 1 cave and the lesser horseshoe bat from Partizanskaya cave, respectively. Also note that RNA of Khosta-1 was detected in feces much more often than in oral swabs and in a higher titer (based on Ct value, data not shown). RNA of Khosta-2 virus was detected in two lesser horseshoe bats collected in basement of the building at Research Institute of Medical Primatology. In one animal RNA of Khosta-2 was detected in both faces and oral swabs, and in another only swab.

Discussion

The emergence of SARS-CoV and SARS-CoV-2 viruses is a result of adaptation of SARS-CoV-like viruses, circulated in the horseshoe bats, to human (18,19). Horseshoe bats are widespread and, presumably, SARS-like coronaviruses circulate in all parts of their range including Asia, Europe and northern Africa. However, little information exists on the genetic diversity of bat SARS-like coronaviruses in the regions outside East and Southeast Asia. We described here two novel SARS-like coronaviruses circulated in the horseshoe bats in southern region of Russia. Khosta-1 and Khosta-2 viruses are closely related to viruses recently described in Bulgaria (strain BtCoV/BM48-31/2008) and Kenya (strain BtKY72) (8,15). Together they form a separate “western” (as they are found in the western part of the range of horseshoe bats) phylogenetic lineage of bat SARS-like coronaviruses. A feature of these viruses is the absence of ORF8 gene, which is common in SARS-CoV, SARS-CoV-2, and most of bat SARS-like coronaviruses of eastern lineages.

SASR-CoV and SARS-CoV-2 recognize host angiotensin-converting enzyme 2 (ACE2) as its receptor. Crucial for binding of ACE2 receptor amino acids (442, 487, 479, 487, and 491) are located in the RBM of the S protein (17,20). These amino acids and its context in RBM of

Khosta-1 and Khosta-2, like most other bat SARS-like coronaviruses, are quite different from SASR-CoV and SARS-CoV-2 viruses. The most bat SARS-like coronaviruses unable to bind ACE2 receptor of human and do not infect its cells (21). However, several strains of bat SARS-like coronaviruses that can use ACE2 receptor and whose proteins are highly similar to SARS-CoV or SARS-CoV-2 have been recently found in the Chinese rufous (*R. sinicus*) and the intermediate (*R. affinis*) horseshoe bats in People's Republic of China (22–25). In this vein, it is argued that direct progenitors of SARS-CoV and SARS-CoV-2 originated after sequential recombination events between bat SARS-like coronaviruses. Since the recombination is of great importance in the evolution of coronaviruses, we analyzed possible recombination events in the western lineage of bat SARS-like coronaviruses. Despite the small number of known full-length sequences (only four), we observe an evidence of recombination in evolutionary history of Khosta-1. The alleged recombination event involved the acquisition of structural proteins S, E, and M as well as nonstructural genes ORF3, ORF6, ORF7a, and ORF7b from a virus that is closer to the Kenyan isolate BtKY72 than to the European strain BtCoV/BM48-31/2008. Based on this, we can assume that genetic diversity of viruses in the region is significantly higher than is known today and there is constant exchange of genes between them. These findings require further investigation of the diversity of circulating variants, with particular emphasis on the diversity of the S gene.

Using RT-PCR we showed that 14% of tested horseshoe bats were carriers for Khosta-1 virus and 1,75% for Khosta-2 virus. However, most of the Khosta-1 positive samples were found in only one cave (Kolokolnaya cave) where infection rate of greater horseshoe bats reached 62,5%. This bias, together with the small number of samples from other locations, makes it difficult to accurately estimate the prevalence of Khosta-1 in the region and requires further

research. The closest European region where such studies have been carried out is Bulgaria: according to the data obtained by J. Drexler with coauthor (2010) SARS-like coronaviruses were detected in 13,3% of greater horseshoe bat, 15,9% of Blasius's horseshoe bat, 30,8% of Mehely's horseshoe bat, and 32,1% of Miditerranean horseshoe bat (8). Another studies found 38.8% positive lesser horseshoe bats in Slovenia and 37,9% positive greater horseshoe bats in France (5,6). All this data show that the prevalence of SARS-like coronaviruses in horseshoe bats in their western part of range can vary widely between different species, locations, and possibly the time of year of observation.

In conclusion, we have shown that SARS-like coronaviruses circulate in horseshoe bats in southern region of Russia and provide a new information on its genomics. Genetic diversity, prevalence, host range, as well as potential threat to human of these viruses remain to be determined.

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It includes arboviruses as well as zoonotic viruses of rodents, bats, and birds, distributed in Northern Eurasia.

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Table 1. The bat samples collected and results of RT-PCR testing for Kosta-1 and Khosta-2 viruses.

Location	Bat species	Number of samples collected		Khosta-1 virus positive samples (%*)		Khosta-2 virus positive samples (%*)	
		Oral swabs	Faces	Oral swabs	Faces	Oral swabs	Faces
Basement of the building at Research Institute of Medical Primatology (43°26'06.3"N 39°59'26.4"E)	Lesser horseshoe bat (*R. hipposideros*)	27	24	0	0	1 (3,7%)	2 (8,3%)
	Miditerranean horseshoe bat (*R. euryale*)	1	1	0	0	0	0
Museinaya cave (43°33'34.3"N 39°53'46.2"E)	Greater horseshoe bat (*R. ferrumequinum*)	4	2	0	0	0	0
	Lesser horseshoe bat (*R. hipposideros*)	3	2	0	0	0	0
Khosta 1 cave (43°33'49.5"N 39°53'57.2"E)	Greater horseshoe bat (*R. ferrumequinum*)	21	13	0	1 (7,7%)	0	0
	Common bent-wing bat *Miniopterus schreibersii*	3	1	0	0	0	0
Kolokolnaya cave (43°33'08.3"N, 39°56'02.4"E)	Greater horseshoe bat (*R. ferrumequinum*)	36	24	4 (11%)	15 (62,5%)	0	0

	Miditerranean horseshoe bat (*R. euryale*)	2	0	0	0	0	0
Partizanskaya cave (43°37'38.86"N, 39°54'46.06"E)	Greater horseshoe bat (*R. ferrumequinum*)	2	1	0	0	0	0
	Lesser horseshoe bat (*R. hipposideros*)	5	3	0	1 (33%)	0	0
Attic of house (44° 0'57.51"N, 39°15'3.63"E)	Lesser horseshoe bat (*R. hipposideros*)	6	4	0	0	0	0
Krasnoaleksandrovskaya cave (44° 0'57.21"N, 39°21'49.68"E)	Greater horseshoe bat (*R. ferrumequinum*)	1	0	0	0	0	0
	Lesser horseshoe bat (*R. hipposideros*)	6	0	0	0	0	0
	Myotis bat *Myotis* spp.	3	0	0	0	0	0
Attic of house, Izmaylovka village (43°37'51.72"N 39°49'45.38"E)	Lesser horseshoe bat (*R. hipposideros*)	0	2	0	0	0	0
Total		120	77	4 (4,6%**)	17 (14,9%**)	1 (0,89%**)	2 (1,75%**)

* the percentages quoted are indicative and not statistically reliable.

** value calculated only for horseshoe bats, common bent-wind bat and myotis bat were excluded from the calculation.

Table 2. Similarity (%) of deduced amino acid sequences of proteins of Khosta-1 and Khosta-2 viruses with certain representatives of *Sarbecovirus* subgenus (lineage B of betacoronaviruses).

Protein	Viruses	Amino acid identity (%)								
		Bat SARS-CoV-	Bat SARS-CoV-	Bat SARS-CoV-like (China,	Civet SARS-CoV-	SARS-CoV	Bat SARS-CoV-2-	Pangolin SARS-	SARS-CoV-2	Khosta-1 vs Khosta-2

		like BGR/2008 (Bulgaria, 2008)	like BtKY72 (Kenya, 2007)	2005-2016)*	like SZ3 (China, 2003)	Urbani (2003)	like RatG13 (China, 2013)	CoV-2-like (China, 2017)	Wuhan-Hu-1 (2019)	
ORF1a	Khosta-1	92,95	84,6	81,53-81,6	81,67	81,53	77,2	77,89	77,32	82
	Khosta-2	81,1	80,9	79,4-79,6	79,5	79,4	76,3	77,1	76,45	
ORF1b	Khosta-1	99,07	96,3	95,82-96,3	96,15	96,15	94,22	94,22	94,21	94,75
	Khosta-2	94,7	93,7	94,9-95,17	95,02	94,9	93,44	93,47	93,5	
S	Khosta-1	84,37	89,11	75,5-76,2	75,7	75,7	73,0	72,4	72,22	82
	Khosta-2	79,54	79,7	73,03-73,9	73,2	73,0	72,5	71,74	72,54	
ORF3	Khosta-1	85,98	86,7	66,8-72,3	70,8	70,8	65,1	66,2	64,7	81,8
	Khosta-2	77,9	82,22	67,9-69,34	67,15	67,5	64,5	65,8	63,27	
E	Khosta-1	89,47	98,7	87,0	87	87	93,42	93,42	93,42	94,7
	Khosta-2	88,16	94,74	90,7	90,7	90,7	89,5	89,5	89,5	
M	Khosta-1	95,0	97,29	91,86-92,31	92,31	91,86	88,24	87,73	88,13	91
	Khosta-2	90,9	90,5	88,7-89,6	90,5	89,6	87,3	87,27	87,0	
ORF6	Khosta-1	68,25	63,0	49,21-52,38	49,21	49,21	50,82	50,82	50,82	58,73
	Khosta-2	58,1	58,1	44,4-47,6	46,03	46,03	46,7	46,7	46,7	
ORF7a	Khosta-1	69,7	70,6	58-59,7	61,34	61,34	58,5	59,32	58,5	73,5
	Khosta-2	63,25	70,34	58,2-59,26	60,0	60,0	60,0	58,3	59,13	
ORF7b	Khosta-1	86,05	81,4	71,8	71,8	71,8	61,5	71,8	74,4	70,7
	Khosta-2	71,4	73,1	64,2	64,2	64,2	64,2	64,2	64,2	
N	Khosta-1	96,64	92,6	88,36-88,9	89,1	89,1	87,9	87,6	87,4	91,85
	Khosta-2	91,13	90,21	85,75-86,73	86,5	86,5	85,5	86,4	85,24	

Figure 1. Map of the region where bat samples were collected. Location of Sochi National Park and surrounding area is shown in grey.

Figure 2. Scheme of the genome of Khosta-1 and Khosta-2 viruses with the designation of the main ORFs, and results of Simplot and recombination analysis. **A**. Khosta-1 was used as a query sequence and Khosta-2, BM48-31/BGR/2008, and BtKy72 viruses were used as reference sequences. **B**. Khosta-2 was used as a query sequence and Khosta-1, BM48-31/BGR/2008, and BtKy72 were used as reference sequences. The analysis was performing with Kimura (2-parametr) model, window size of 1000 bases and a step size of 100 bases. **C.** Results of bootstrap analysis of recombination events in Khosta-1 genome by RDP5 software.

Figure 3. Phylogenetic trees inferred by maximum likelihood method based on analysis of amino acid sequences of ORF1ab protein (**A**) and nucleotide sequences of S gene (**B**). HKU9-like strain Bt-BetaCoV/GX2018, belonged to subgenus *Nobecovirus* (lineage D), was used as an outer group for ORF1ab protein phylogeny. The viruses described in present work are marked by black circle.

Figure 4. Alignment of receptor binding motif (RBM) of receptor binding domain (RBD) of S protein of Khosta-1 and Khosta-2 with certain sarbecoviruses. Position (442, 472, 479, 480, 487, 491 numbering by SARS-CoV Urbani) in RBM which are thought to be important for adaptation SARS-CoV-like viruses to human ACE2 receptor (16,17) are bolded. Bat SARS-CoV-like viruses that are capable or not capable of utilizing ACE2 receptor are marked with "ACE2(+)" or "ACE2(-)", respectively.

Figure 1.

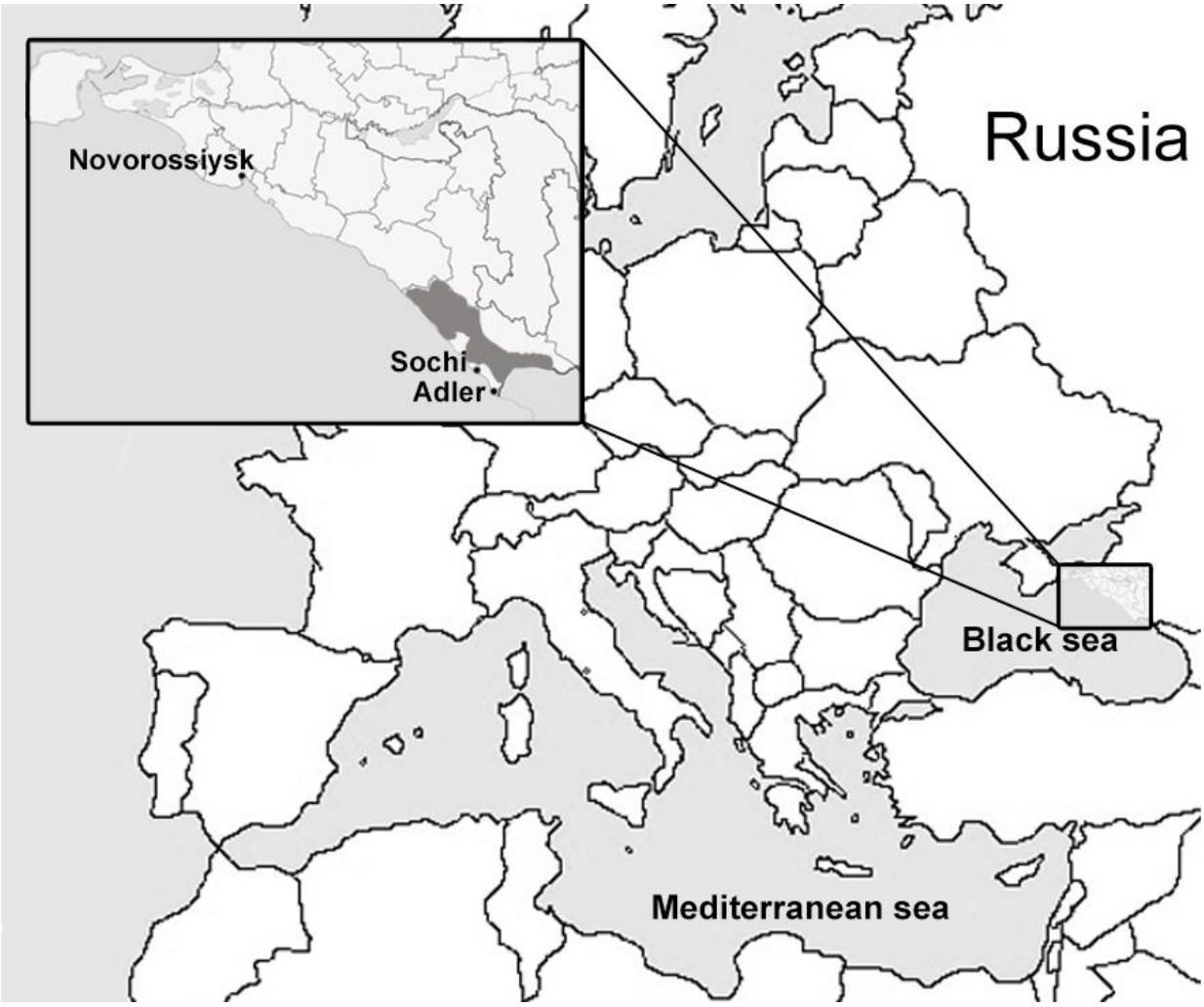
Russia
Novorossiysk
Sochi
Adler
Black sea
Mediterranean sea

Figure 2A

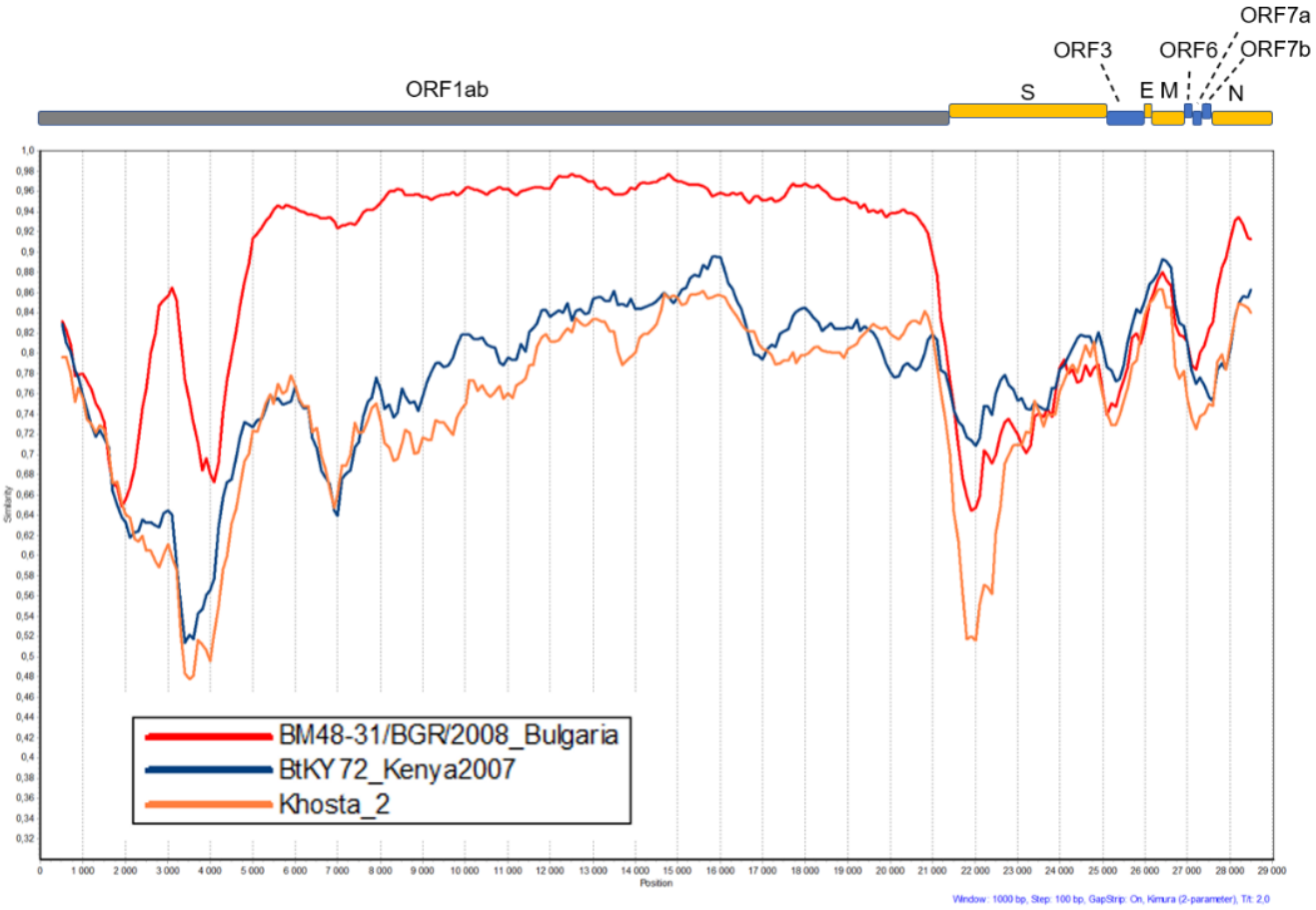
ORF7a
ORF3
ORF6
ORF7b
ORF1ab
S
E
M
N
BM48-31/BGR/2008_Bulgaria
BtKY72_Kenya2007
Khosta_2
Position
Window: 1000 bp, Step: 100 bp, GapStrip: On, Kimura (2-parameter), Tt: 2,0

Figure 2B.

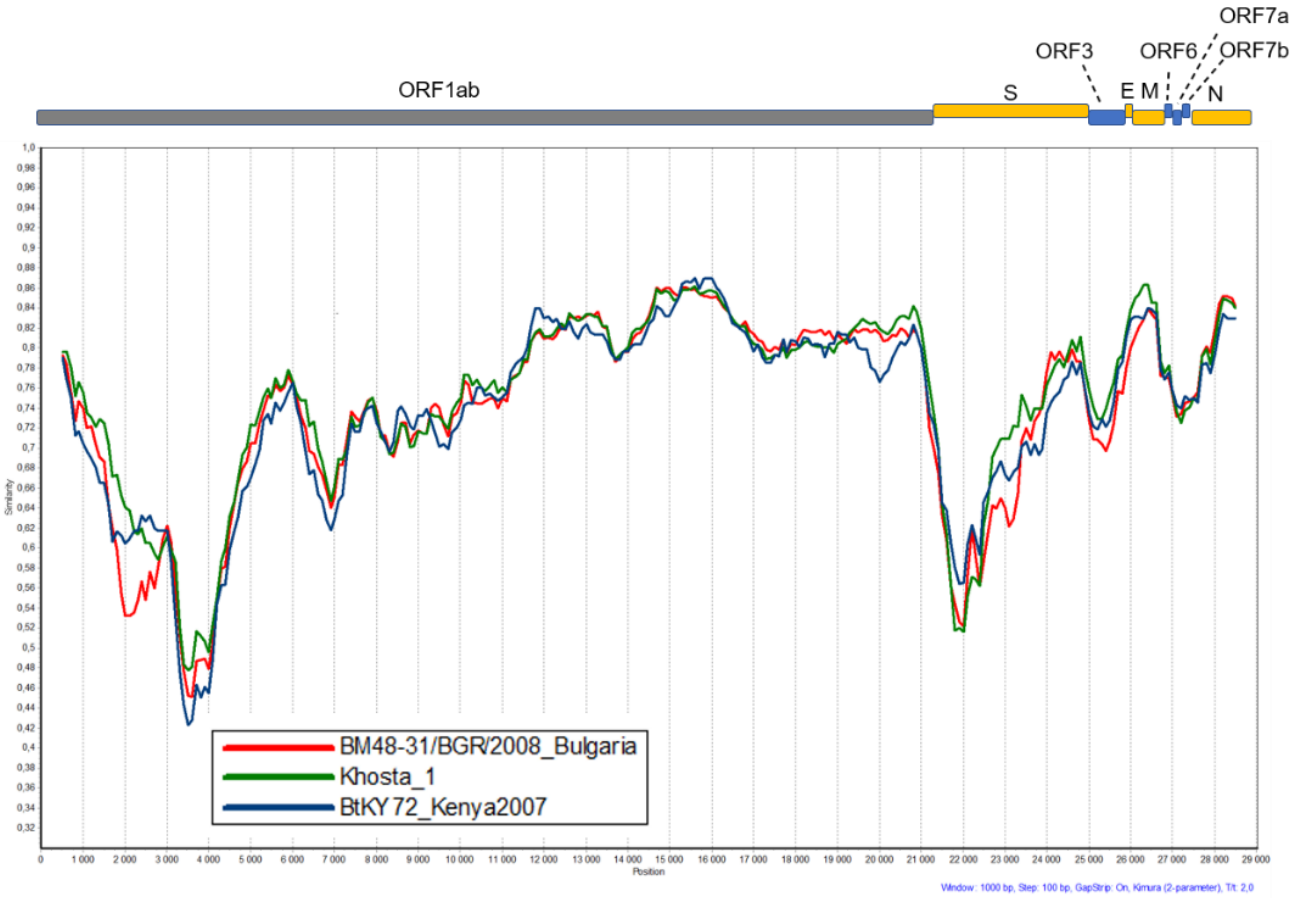
ORF7a
ORF3
ORF6
ORF7b
ORF1ab
S
E M
N
BM48-31/BGR/2008_Bulgaria
Khosta_1
BtKY72_Kenya2007
Position
Window: 1000 bp, Step: 100 bp, GapStrip: On, Kimura (2-parameter), Tt: 2,0

Figure 2C.

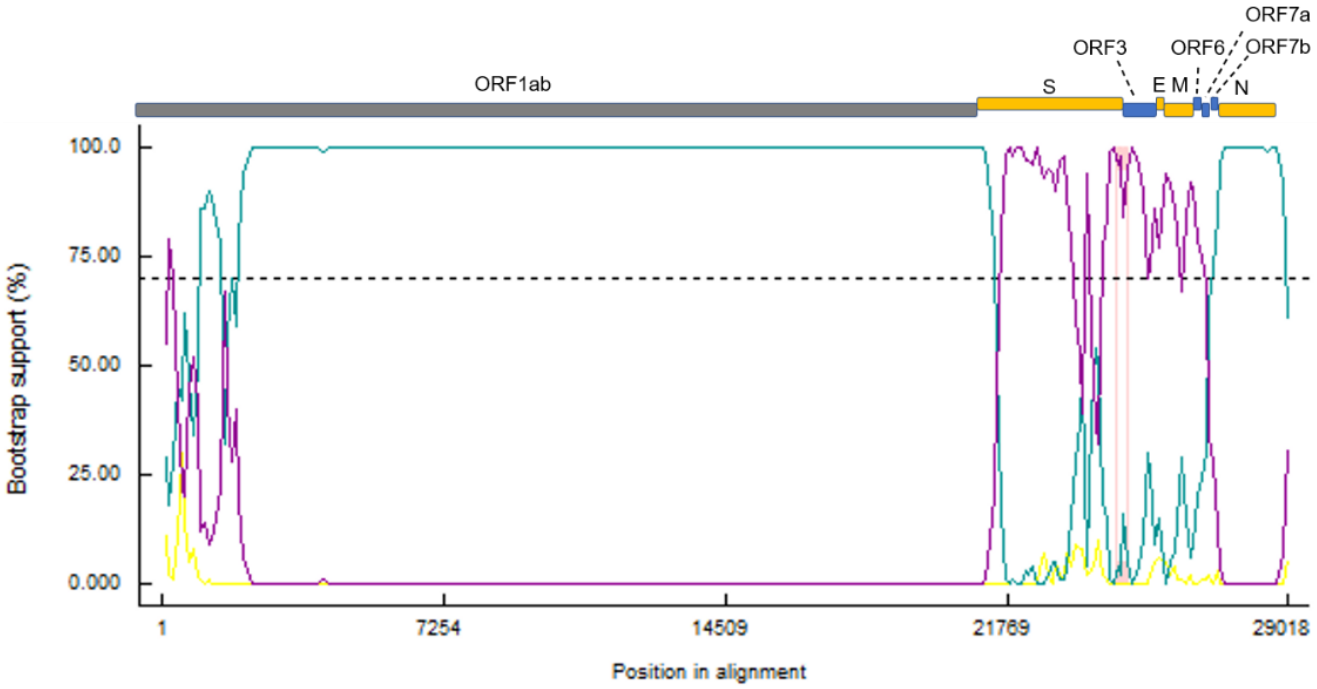
ORF7a
ORF3
ORF6
ORF7b
ORF1ab
S
E
M
N
100.0
75.00
50.00
25.00
0.000
Bootstrap support (%)
1
7254
14509
21769
29018
Position in alignment

Figure 3A

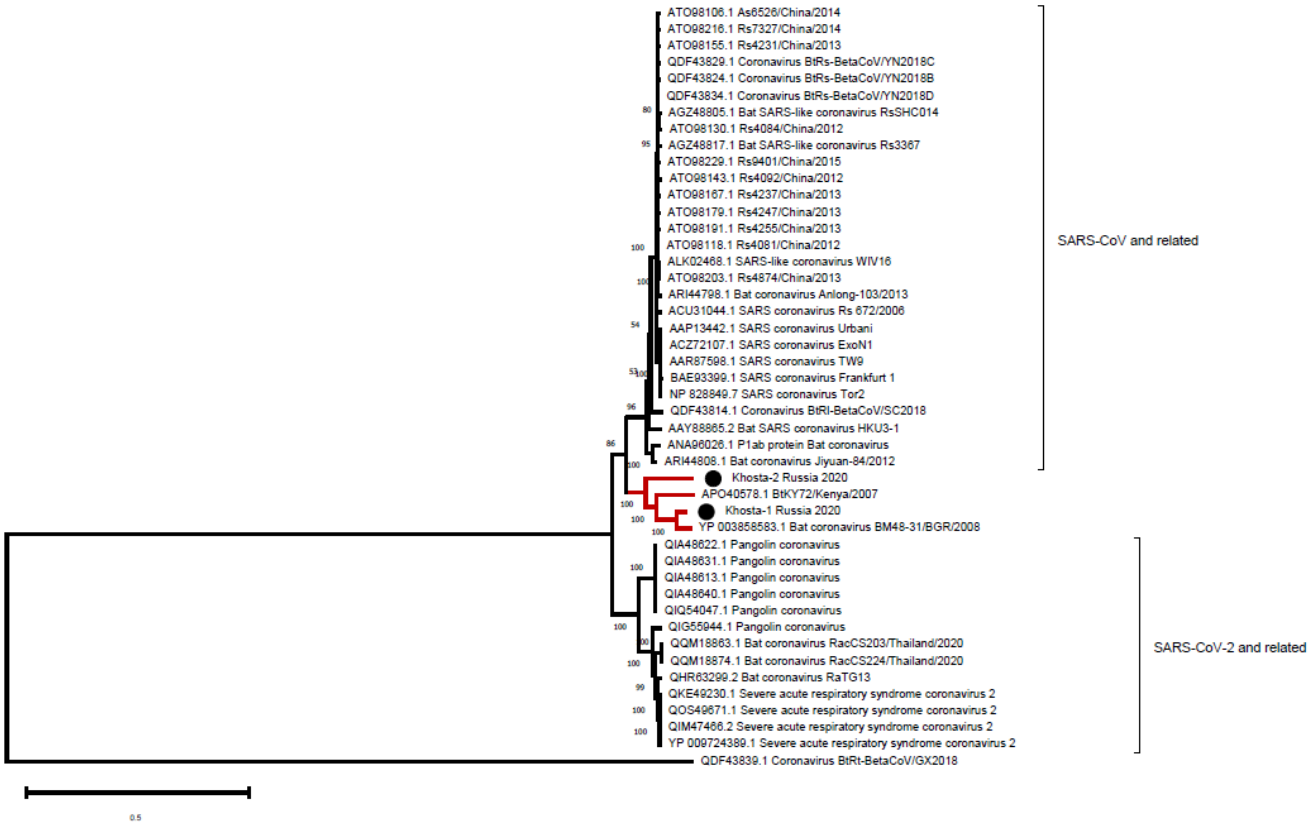
ATO98106.1 As6526/China/2014
ATO98216.1 Rs7327/China/2014
ATO98155.1 Rs4231/China/2013
QDF43829.1 Coronavirus BtRs-BetaCoV/YN2018C
QDF43824.1 Coronavirus BtRs-BetaCoV/YN2018B
QDF43834.1 Coronavirus BtRs-BetaCoV/YN2018D
AGZ48805.1 Bat SARS-like coronavirus RsSHC014
ATO98130.1 Rs4084/China/2012
AGZ48817.1 Bat SARS-like coronavirus Rs3367
ATO98229.1 Rs9401/China/2015
ATO98143.1 Rs4092/China/2012
ATO98167.1 Rs4237/China/2013
ATO98179.1 Rs4247/China/2013
ATO98191.1 Rs4255/China/2013
ATO98118.1 Rs4081/China/2012
ALK02468.1 SARS-like coronavirus WIV16
ATO98203.1 Rs4874/China/2013
ARI44798.1 Bat coronavirus Anlong-103/2013
ACU31044.1 SARS coronavirus Rs 672/2006
AAP13442.1 SARS coronavirus Urbani
ACZ72107.1 SARS coronavirus ExoN1
AAR87598.1 SARS coronavirus TW9
BAE93399.1 SARS coronavirus Frankfurt 1
NP 828849.7 SARS coronavirus Tor2
QDF43814.1 Coronavirus BtRl-BetaCoV/SC2018
AAY88865.2 Bat SARS coronavirus HKU3-1
ANA96026.1 P1ab protein Bat coronavirus
ARI44808.1 Bat coronavirus Jiyuan-84/2012
Khosta-2 Russia 2020
APO40578.1 BtKY72/Kenya/2007
Khosta-1 Russia 2020
YP 003858583.1 Bat coronavirus BM48-31/BGR/2008
QIA48622.1 Pangolin coronavirus
QIA48631.1 Pangolin coronavirus
QIA48613.1 Pangolin coronavirus
QIA48640.1 Pangolin coronavirus
QIQ54047.1 Pangolin coronavirus
QIG55944.1 Pangolin coronavirus
QQM18863.1 Bat coronavirus RacCS203/Thailand/2020
QQM18874.1 Bat coronavirus RacCS224/Thailand/2020
QHR63299.2 Bat coronavirus RaTG13
QKE49230.1 Severe acute respiratory syndrome coronavirus 2
QOS49671.1 Severe acute respiratory syndrome coronavirus 2
QIM47466.2 Severe acute respiratory syndrome coronavirus 2
YP 009724389.1 Severe acute respiratory syndrome coronavirus 2
QDF43839.1 Coronavirus BtRt-BetaCoV/GX2018
SARS-CoV and related
SARS-CoV-2 and related
0.5

Figure 3B.

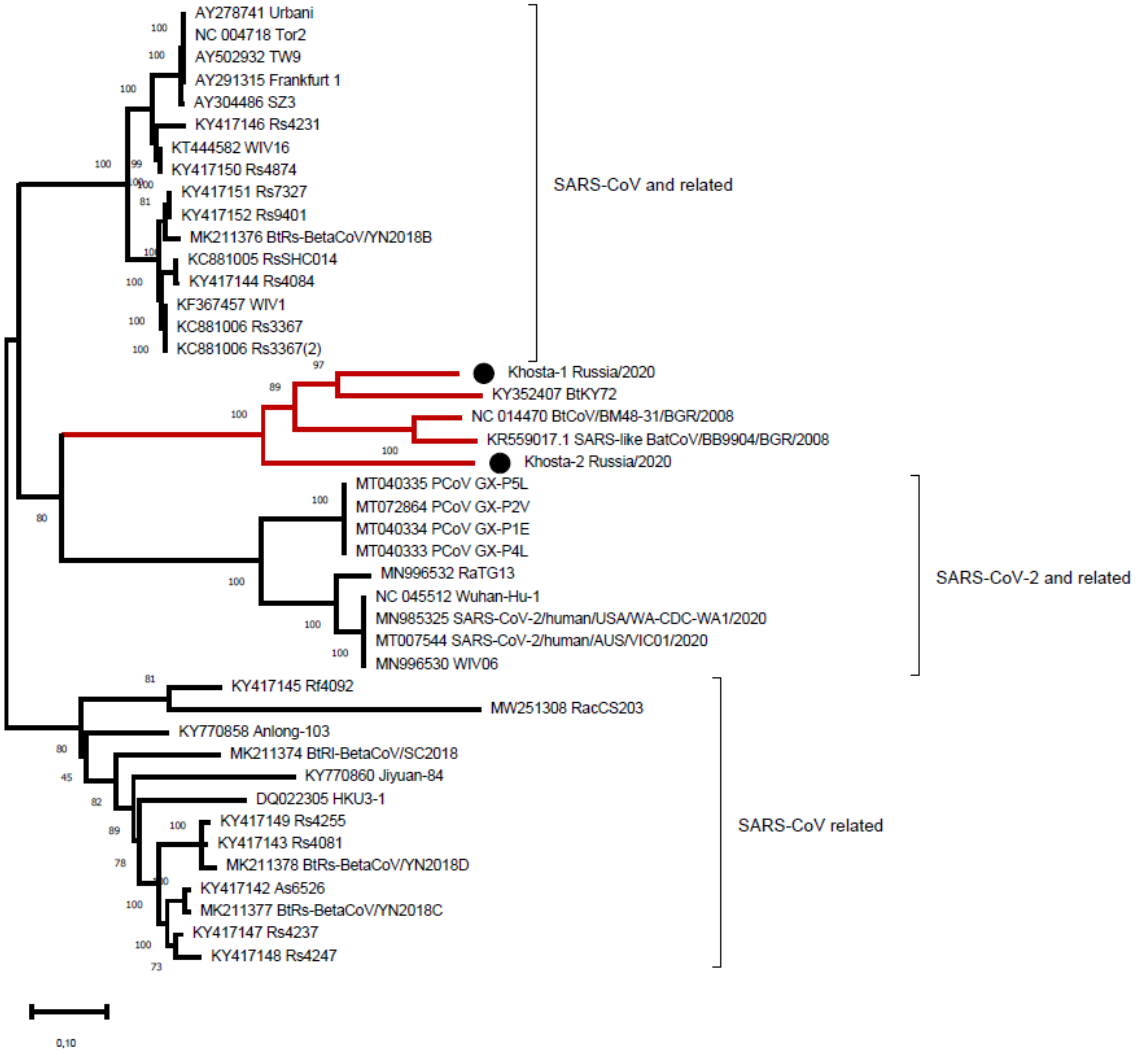
AY278741 Urbani
NC 004718 Tor2
AY502932 TW9
AY291315 Frankfurt 1
AY304486 SZ3
KY417146 Rs4231
KT444582 WIV16
KY417150 Rs4874
KY417151 Rs7327
KY417152 Rs9401
MK211376 BtRs-BetaCoV/YN2018B
KC881005 RsSHC014
KY417144 Rs4084
KF367457 WIV1
KC881006 Rs3367
KC881006 Rs3367(2)
SARS-CoV and related
Khosta-1 Russia/2020
KY352407 BtKY72
NC 014470 BtCoV/BM48-31/BGR/2008
KR559017.1 SARS-like BatCoV/BB9904/BGR/2008
Khosta-2 Russia/2020
MT040335 PCoV GX-P5L
MT072864 PCoV GX-P2V
MT040334 PCoV GX-P1E
MT040333 PCoV GX-P4L
MN996532 RaTG13
NC 045512 Wuhan-Hu-1
MN985325 SARS-CoV-2/human/USA/WA-CDC-WA1/2020
MT007544 SARS-CoV-2/human/AUS/VIC01/2020
MN996530 WIV06
SARS-CoV-2 and related
KY417145 Rf4092
MW251308 RacCS203
KY770858 Anlong-103
MK211374 BtRl-BetaCoV/SC2018
KY770860 Jiyuan-84
DQ022305 HKU3-1
KY417149 Rs4255
KY417143 Rs4081
MK211378 BtRs-BetaCoV/YN2018D
KY417142 As6526
MK211377 BtRs-BetaCoV/YN2018C
KY417147 Rs4237
KY417148 Rs4247
SARS-CoV related
0,10

Figure 4.

```
                                                   442                                472   479-80   487  491
Khosta-1                    NTKSIDK----GQGFYYRLFRHGKIKPYERDTSNVPYNAQGGTCTDTSQLNCYQPLKSYDFTDTVGVGYQPY
Khosta-2                    NTRTIDS----KRGFYYRLFRHGNIRPYERDTSNVPYNAAGGTCNQPGTHNCYEPLQDYGFTSTSGVGYQPF
NC_014470 BM48-31/BGR/2008  NTNSLDS----SNEFFYRRFRHGKIKPYGRDLSNVLFNPSG-GTCSAEGLNCYKPLASYGFTQSSGIGFQPY
KR559017 BB9904/BGR/2008    NTNALDS----NKDFYYRLFRHGKIKPYGRDLSNIPYSPSG-TCSTINNLNCFAPLKSYGFTQSSGISFQPY
KY352407 BtKY72             NTNSVDSKS--GNNFYYRLFRHGKIKPYERDISNVLYNSAGGTCSSISQLGCYEPLKSYGFTPTVGVGYQPY
AY278741 SARS Urbani        NTRNIDATSTGNYNYKYRYLRHGKLRPFERDISNVPFSPDGKPCTPP-ALNCYWPLNDYGFYTTTGIGYQPY
AY304486 Civet SZ3          NTRNIDATSTGNYNYKYRYLRHGKLRPFERDISNVPFSPDGKPCTPP-ALNCYWPLKDYGFYTTSGIGYQPY
MT040335 Pangolin SARS-2    NSVKQDALTGGNYGYLYRLFRKSKLKPFERDISTEIYQAGSTPCNGQVGLNCYYPLERYGFHPTTGVNYQPF
MN996532 RaTG13             NSKHIDAKEGGNFNYLYRLFRKANLKPFERDISTEIYQAGSKPCNGQTGLNCYYPLYRYGFYPTDGVGHQPY
NC_045512 SARS-CoV-2        NSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPY
KT444582 WIV16              NTRNIDATQTGNYNYKYRSLRHGKLRPFERDISNVPFSPDGKPCTPP-AFNCYWPLNDYGFYITNGIGYQPY
KF367457 WIV1               NTRNIDATQTGNYNYKYRSLRHGKLRPFERDISNVPFSPDGKPCTPP-AFNCYWPLNDYGFYITNGIGYQPY
KC881005 RsSHC014           NTNSKDSSTSGNYNYLYRWVRRSKLNPYERDLSNDIYSPGGQSCSAV-GPNCYNPLRPYGFFTTAGVGHQPY
KY417151 Rs7327             NTRNIDATSTGNYNYKYRSLRHGKLRPFERDISNVPFSPDGKPCTPP-AFNCYWPLNDYGFFTTNGIGYQPY
DQ022305 HKU3-1             NTAKHDTGN-----YYYRSHRKTKLKPFERDLSSDDG-------------NGVYTLSTYDFNPNVPVAYQAT
KY417145 Rf4092             NTAKQDVGS-----YFYRSHRSSKLKPFERDLSSDE--------------NGVRTLSTYDFNPNVPLDYQAT
KY417147 Rs4237             NTAKQDQGQ-----YYYRSSRKTKLKPFERDLSSDE--------------NGVRTLSTYDFYPTVPIEYQAT
KY417148 Rs4247             NTAKQDTGH-----YYYRSHRKTKLKPFERDLSSDDG-------------NGVYTLSTYDFNPNVPVAYQAT
```

SARS-CoV

SARS-CoV-2

Bat SARS-CoV ACE2(+)

Bat SARS-CoV ACE2(-)