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Identification of a new alphacoronavirus (Coronaviridae: *Alphacoronavirus*) associated with the greater horseshoe bat (*Rhinolophus ferrumequinum*) in the south of European part of Russia

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Abstract

Introduction. Bats are recognized as primary natural reservoirs for alpha- and betacoronaviruses. The interspecies transmission of bat coronaviruses to other mammalian hosts, including livestock and humans, can lead to epidemics, epizootics, and global pandemics.

Objective. This study aims to describe coronaviruses associated with horseshoe bats (*Rhinolophus* spp.) in the southern regions of the European part of Russia.

Materials and methods. Fecal samples were collected from bats inhabiting caves on the southern macroslope of the Greater Caucasus (Sochi-Adler region) during 2020, 2021, and 2024. Viral genomes were detected and analyzed using high-throughput sequencing (NGS) and RT-PCR.

Results. A novel alphacoronavirus, designated Kudep virus (GenBank acc. # PQ649435), was identified in *R. ferrumequinum*. Presumably the Kudep virus represents a novel species within the subgenus *Decacovirus* of the genus *Alphacoronavirus.* The virus Showed 72% nucleotide identity to a Cardioderma bat coronavirus from Kenya and up to 67% nucleotide identity to the YN2012 virus group found in horseshoe bats in China. RT-PCR screening revealed active circulation of both Kudep virus and the previously described SARS-like betacoronavirus Khosta-1 in the study area. Infection rates in a single *R. ferrumequinum* colony during autumn 2021 reached 59.2% and 70.5% for Kudep and Khosta-1, respectively. Frequent co-infections with both viruses were observed in individual bats.

Conclusion. Our findings expand the understanding of the distribution of bat alphacoronaviruses and their genetic diversity. We demonstrate the presence of a persistent natural foci of two potentially zoonotic bat coronaviruses, ecologically associated with *R. ferrumequinum* in the southern European part of Russia.

Keywords: *bats; bat coronaviruses; Kudep virus; Khosta-1 virus; emerging and re-emerging infections; alphacoronaviruses; SARS-CoV-2; horseshoe bats*

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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
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Идентификация нового альфакоронавируса (Coronaviridae: *Alphacoronavirus*), ассоциированного с большим подковоносом (*Rhinolophus ferrumequinum*), на юге европейской части России

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Резюме

Введение. Летучие мыши рассматриваются как основной природный резервуар для альфа- и бетакоронавирусов. В результате преодоления межвидового барьера коронавирусы летучих мышей передаются другим видам млекопитающих, включая сельскохозяйственных животных и человека, что часто приводит к возникновению эпидемических или эпизоотических вспышек и пандемий.

Цель работы. Идентифицировать зоонозные коронавирусы, циркулирующие в популяции подковоносых летучих мышей (*Rhinolophus* spp.) в южных регионах европейской части России. Определить их генетические и экологические характеристики.

Материалы и методы. Материал для исследования (фекалии летучих мышей) был собран в пещерах на Южном макросклоне большого Кавказа (район Сочи-Адлер) в 2020, 2021 и 2024 гг. Использованы методы метагеномного анализа на основе NGS и ОТ-ПЦР.

Результаты. Идентифицирован новый альфакоронавирус (вирус Кудеп, GenBank № PQ649435), ассоциированный с большим подковоносом (*R. ferrumequinum*). Вирус Кудеп предположительно представляет новый вид подрода *Decacovirus* рода *Alphacoronavirus*. Максимальное генетическое сходство вирус Кудеп имеет с коронавирусом летучих мышей (от ложного вампира *Cardioderma cor*) из Кении (72% идентичных н.о.) и с группой вирусов YN2012, найденных у подковоносых летучих мышей в Китае (до 67% н.о.). Результаты ОТ-ПЦР-скрининга показывают, что вирус Кудеп и ранее описанный нами SARS-подобный бетакоронавирус Хоста-1 активно циркулируют на обследованной территории. Зараженность этими вирусами в одной из колоний большого подковоноса осенью 2021 г. достигала 59,2 и 70,5% соответственно. Выявлены частые случаи коинфекции отдельных особей одновременно двумя коронавирусами.

Заключение. Полученные данные расширяют представления о распространении альфакоронавиурусов летучих мышей и их генетическом разнообразии. Показано наличие стойкого природного очага потенциально зоонозных коронавирусов (Хоста-1 и Кудеп), связанных с *R. ferrumequinum*, на юге европейской части России.

Ключевые слова: *летучие мыши; коронавирусы летучих мышей; вирус Кудеп; вирус Хоста-1; новые и возвращающиеся инфекции; альфакоронавирусы; SARS-CoV-2; подковоносы*

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Introduction

Bat coronaviruses of the genera *Alphacoronavirus* and *Betacoronavirus* (Coronaviridae: *Orthocoronavirinae*) have significant zoonotic potential [1–4]. Numerous studies have shown that most human coronaviruses, including the seasonal alphacoronaviruses (α-CoV) HCoV-229E and HCoV-NL63, as well as the betacoronaviruses (β-CoV) SARS-CoV, SARS-CoV-2, and MERS-CoV, originated from viruses associated with bats [5–7]. Coronaviruses closely related to bat viruses have also been identified in various wild and domestic animals [8, 9]. This underscores the role of bats as primary natural reservoirs of zoonotic coronaviruses, facilitating their transmission among different mammalian species.

Bats belong to the order Chiroptera, which comprises over 1,400 species, classified into 21 families and 234 genera. In terms of species diversity, Chiroptera ranks second after rodents among all mammals. In Russia, at least 45 bat species have been recorded, including three species of horseshoe bats (Rhinolophidae: *Rhinolophus*): the greater horseshoe bat (*R. ferrumequinum* (Schreber, 1774)), the lesser horseshoe bat (*R. hipposideros* (Bechstein, 1800)), and the Mediterranean horseshoe bat (*R. euryale* (Blasius, 1853)) [10]. Horseshoe bats are considered natural reservoirs of SARS-like coronaviruses, two of which, SARS-CoV and SARS-CoV-2, caused the SARS epidemic in 2002–2004 and the COVID-19 pandemic in 2019, respectively. In China and Southeast Asia, several groups of highly diverse alphacoronaviruses have been identified in horseshoe bats, such as *Rhinolophus bat coronavirus HKU32* (subgenus *Decacovirus*) or HKU2 group (subgenus *Rhinacovirus*), which includes the swine acute diarrhea syndrome coronavirus (SADS-CoV) responsible for major outbreaks in China between 2016 and 2019 [9, 11–13]. *R. ferrumequinum* is of particular interest due to its extensive range, spanning North Africa, southern and western Europe, Central Asia, China and South-East Asia. This raises the potential for associated viruses to spread across vast and distant regions. In Russia, horseshoe bats are found only in areas south of 44°N latitude, including the North Caucasus and the northern coast of the Black Sea. However, research on coronaviruses in Russian bat populations remains limited [14–17]. In a previous study we described here two novel SARS-like betacoronaviruses (Khosta-1 and Khosta-2), which are evolutionarily related to SARS-CoV, SARS-CoV-2, and other sarbecoviruses from Europe, Africa, and Asia [14]. Related viruses, closely resembling Khosta-1, have also been found in other areas of the Caucasus, including colonies of *R. ferrumequinum* in Dagestan [17] and *R. euryale* in Georgia [18]. These studies also detected a bat alphacoronavirus, tentatively classified within the *Decacovirus* subgenus based on its conserved RdRp domain (397 nt) [17]. In this study, we identified and sequenced the complete genome of a novel species of bat alphacoronavirus (designated «Kudep virus», after the Kudepsta River), associated with the species *R. ferrumequinum*. PCR screening revealed the co-circulation of alpha- and betacoronaviruses in the bat population in this region.

Material and methods

Material for research. Sampling was conducted in caves located on the southern macro-slope of the Greater Caucasus along the northern coast of the Black Sea (Sochi-Adler region, Krasnodar Krai) during 2020–2024 (**Table**). Sampling took place either in late autumn, when bat colonies gather for hibernation (2020, 2021), or in early spring during their first flights after hibernation (2024). Bats were captured by hand from cave ceilings. Species identification was performed based on morphological features by an experienced zoologist. Additionally, the species identity of *R. hipposideros* and *R. ferrumequinum* was confirmed for selected samples by sequencing the mitochondrial cytochrome b gene. To collect fecal samples, individual bats were placed in clean cotton bags for 10–15 minutes, after which the animals were released, and fecal material from the bag walls was transferred to cryotubes. Samples were transported on ice and stored at −70 °C. No animals were harmed or sacrificed during the collection process. Sample collection was approved by the Scientific Council and Ethics Committee of the Sochi National Park.

RNA Extraction and RT-PCR. Samples were suspended in 0.2 mL of phosphate-buffered saline (PBS). Total RNA was extracted from 0.1 mL of the suspension using the RNA-Extran kit (Synthol, Russia) following the manufacturer's instructions. Screening for Khosta-1 and Khosta-2 viral RNA was performed as previously described [14] using specific primers and probes (Kh1_pr FAM-ACCTGTGCCTGTGAGTCCATT-BQ1, Kh1_F CACTGTTGGTGTAGGTTAC, and Kh1_R CTGGAATGACTGAAATCTCTTA for Khosta-1; Kh2_pr HEX-AAGCACACCAACGACACCAGCATCTC-BQ2, Kh2_F CGCCAAGCACTATTAAAGACAG, and Kh2_R CGAAGTCGTACCAGTTTCCA for Khosta-2) and TaqPath 1-Step Multiplex Master Mix (Thermo Fisher Scientific, USA). Briefly, 5 µL of RNA was added to 15 µL of reaction mix containing 1× TaqPath 1-Step Multiplex Master Mix, 400 nM of forward and reverse primers, and 200 nM of the corresponding probe. Thermal cycling conditions included a reverse transcription step at 50 °C for 30 minutes, initial denaturation at 95 °C for 30 seconds, followed by 45 PCR cycles of denaturation at 95 °C for 10 seconds and annealing/extension at 55 °C for 30 seconds with signal acquisition. RNA of the novel Kudep alphacoronavirus was detected using custom primers and probes (Alph_pr ROX-TGCCACAAGTTGCCACCGTCA-BQ2, Alph_F GCTTGCTGCTGAAGATCC, Alph_R CCACATCATTAACAGTGCGAATA) under equivalent conditions.

NGS Sequencing and Data Analysis. Sequencing of samples was performed as previously described [14]. Selected samples were pooled by species and location (10–12 samples per pool). PCR-positive samples were sequenced individually. Ribosomal RNA was depleted using the NEBNext rRNA Depletion kit (NEB, USA). cDNA libraries were prepared using the NEBNext Ultra II RNA Library Kit for Illumina (NEB, USA) according to the manufacturer's instructions. Library molarity was quantified by qPCR following the Sequencing Library

Table. Results of RT-PCR screening of horseshoe bats (*Rhinolophus* spp.) on coronaviruses Khosta-1, Khosta-2 (*Betacoronavirus*), and Kudep (*Alphacoronavirus*), collected in 2020, 2021 (autumn), and 2024 (spring), Northern coast of the Black Sea

Таблица. Результаты ОТ-ПЦР-скрининга подковоносых летучих мышей (*Rhinolophus* spp.) на наличие коронавирусов Хоста-1, Хоста-2 (*Betacoronavirus*) и Кудеп (*Alphacoronavirus*) в 2020, 2021 (осень) и 2024 (весна) гг., северное побережье Черного моря

Location Локация	Bat species Вид	2020 (autumn) 2020 (осень)			2021 (autumn) 2021 (осень)			2024 (spring) 2024 (весна)		
		Khosta-1 Хоста-1 (β-CoV)	Khosta-2 Хоста-2 (β-CoV)	Kudep Кудеп (α-CoV)	Khosta-1 Хоста-1 (β-CoV)	Khosta-2 Хоста-2 (β-CoV)	Kudep Кудеп (α-CoV)	Khosta-1 Хоста-1 (β-CoV)	Khosta-2 Хоста-2 (β-CoV)	Kudep Кудеп (α-CoV)
Institute of Medical Primatology Подвал НИИ мед. приматологии (43°26’06.3” N 39°59’26.4” E)	*R. hipposideros*	0/24	2/24 (8.3%)	0/24	0/13	1/13 (7.7%)	0/13	–	–	–
	R. euryale	0/1	0/1	0/1	–	–	–	–	–	–
Museinaya cave (Khosta 2 cave) Пещера Музейная (Хостинская 2) (43°33’34.3” N 39°53’46.2” E)	*R. ferrumequinum*	0/2	0/2	0/2	–	–	–	–	–	–
	R. hipposideros	0/2	0/2	0/2	–	–	–	–	–	–
Khosta 1 cave Пещера Хостинская 1 (43°33’49.5” N 39°53’57.2” E)	*R. ferrumequinum*	1/13 (7.7%)	0/13	2/13 (15,3%)	1/2	0/2	1/2	–	–	–
Kolokolnaya cave Пещера Колокольная (43°33’08.3” N, 39°56’02.4” E)	*R. ferrumequinum*	15/24 (62.5%) 1*	0/24	2/24 (8.3%) 1*	36/51 (70.5%) 21*	0/51	27/51 (52.9%) 21*	3/22 (13.6%)	0/22	3/22 (13.6%)
	R. euryale	0/2	0/2	0/2	–	–	–	–	–	–
	R. hipposideros	–	–	–	–	–	–	0/2	0/2	0/2
Partizanskaya cave Пещера Партизанская (43°37’38.86” N, 39°54’46.06” E)	*R. ferrumequinum*	0/1	0/1	0/1	–	–	–	–	–	–
	R. hipposideros	1/3 (33%)	0/3	0/3	–	–	–	–	–	–
Всего Total		17/70 (14.9%)	2/70 (1.75%)	4/70 (3.5%)	37/66 (56.0%)	1/66 (1.5%)	28/66 (42.4%)	3/24 (12.5%)	0/24	3/24 (12.5%)

Note. The number of positive samples/number of samples is shown for each species and location. The percentage value (%) is indicative and is not confirmed by statistical methods. * – number of samples simultaneously positive for Khosta-1 virus and Kudep virus.

Примечание. Указано число положительных проб/общее число обследованных проб для каждого вида и локации. Указанное значение долей (%) является индикативным и не подтверждено статистическими методами. * – число проб, одновременно положительных по вирусу Хоста-1 и вирусу Кудеп.

qPCR Quantification Guide (Illumina, USA). Sequencing was conducted in 2 × 100 format on a NovaSeq 6000 platform (Illumina, USA) at the Genetico Center (Moscow). Pooled samples yielded 80–110 million paired-end reads on average, while individual samples generated 25–40 million paired-end reads. Reads were quality-filtered, adapters were removed, and assemblies were generated *de novo* using CLC Genomics Workbench 7.0 (Qiagen, Germany). Contigs were analyzed using the blastx algorithm with DIAMOND software against the Coronaviridae protein database retrieved from GenBank using a custom Python script (BioPython module).

Genetic and Phylogenetic Analysis. Nucleotide and amino acid sequences of alphacoronaviruses were aligned using the ClustalW algorithm implemented in MEGAX (https://www.megasoftware.net/). A total of 50 genomes of alphacoronaviruses representing nine of the 16 known subgenera were included in the analysis. Amino acid alignments for individual proteins (ORF1a, ORF1b, S, ORF3, E, M, and N) were used to calculate sequence identity percentages between viruses. Phylogenetic analyses were conducted by selecting the best substitution model using the «Model Selection» module in MEGAX. Phylogenetic trees were constructed based on amino acid sequences of RdRp, spike protein S, and nucleocapsid protein N using the selected models (LG + G + I + F for RdRp, LG + G + F for S and N) with bootstrap testing (1000 replicates) in MEGAX. For taxonomic analysis, the criteria established by the International Committee on Taxonomy of Viruses (ICTV) [19] were used. Specifically, amino acid sequences of conserved domains within the *ORF1ab* gene (nsp5 (3CLpro), NiRAN, nsp12 (RdRp), ZBD, and nsp13 (Hel1)) from each virus analyzed were concatenated into a single sequence, aligned using the ClustalW algorithm, and analyzed to calculate the percentage of amino acid differences. According to ICTV (https://ictv.global/report/chapter/coronaviridae/coronaviridae) viruses classified as different species must share no more than 92% amino acid identity in these conserved domain sequences.

Results

Identification and Genetic Characterization of a Novel Bat Alphacoronavirus. The genome of the novel alphacoronavirus was initially identified as a 28,142-nucleotide (nt) contig in the *de novo* assembly of sample F2, collected from *R. ferrumequinum* in Kolokolnaya Cave in 2020. Subsequent PCR screening, using specific primers and probes, enabled the sequencing of individual positive samples with high viral RNA titers (Ct < 25). This analysis yielded nearly complete genomic sequences for seven Kudep virus strains, including one strain from 2020 (Kudep/F2/2020) and six from 2021 (Kudep/2021/59, Kudep/2021/71, Kudep/2021/80, Kudep/2021/86, Kudep/2021/100, and Kudep/2021/107). All strains were obtained from *R. ferrumequinum* collected in Kolokolnaya cave.

Genomic analysis revealed 100% nucleotide identity among strains 59, 71, and 86 (2021), as well as between strains 80 and 107 (2021). Comparisons between these groups showed 27 nucleotide substitutions. Strain 100 exhibited seven substitutions compared to group 59/71/86 and 34 substitutions compared to group 80/107. The prototype strain F3/2020 differed by 8, 19, and 32 substitutions from groups 80/107, 59/71/86, and strain 100, respectively. Notably, strains F3, 80, and 107 contained a unique 9-nt insertion in the *N* gene, resulting in the insertion of amino acids 164QNN166 in the nucleocapsid protein, absent in strains 59, 71, and 86.

The Kudep virus genome exhibits the typical structure of alphacoronaviruses. Approximately two-thirds of the genome comprises the *ORF1ab* region, which encodes a large polyprotein that is first processed into polyproteins ORF1a and ORF1b, and then into 16 nonstructural proteins (nsp1–nsp16). The RNA-dependent RNA polymerase (RdRp, nsp12), used for coronavirus classification, is located within the ORF1b polyprotein at amino acid positions 1–927. The remaining part of the genome includes structural protein genes (S, E, M, and N) and several nonstructural accessory proteins genes. A total of eight genes (open reading frames, ORFs) were identified: ORF1ab-S-ORF3a-ORF3b-E-M-N-ORFx. Kudep virus displays the division of ORF3 into two genes, *ORF3a* (encoding a 120-aa protein with no viral homologs in BLASTp) and *ORF3b*, encoding a nonstructural protein with 55–78% amino acid identity to those of other bat alphacoronaviruses. Kudep virus lacks genes of accessory proteins commonly found in bat coronaviruses (e.g., ORF4, ORF7, ORF8, and ORF9) but includes *ORFx* gene encoding a 107-aa protein of unknown function.

Comparative analysis revealed that the Kudep virus genome is most closely related to Cardioderma bat coronavirus/Kenya/KY43/2006 (BtKY43) isolated from *Cardioderma cor* in Kenya in 2006, sharing 72% nt identity [20]. The genome also showed up to 67% nt identity to some Rhinolophus-associated viruses from China, such as YN2012 [10]. RdRp amino acid (aa) identity was 92% with BtKY43 and 87–90% with viruses from China and Southeast Asia. Among structural proteins, E, M, and N showed the highest similarity to BtKY43, with 85%, 92%, and 81% amino acid identity, respectively. When compared to viruses from the Asian region, the highest aa identity values are 83% for the E protein (strain HuB2013, *R. ferrumequinum*, 2013, China), 90% for the M protein (strain PH20, *R. shameli*, 2010, Cambodia), and 69.7% for the N protein (strain RfLN20, *R. ferrumequinum*, 2020, China), respectively.

The spike (S) protein of Kudep virus shows 58–73% amino acid identity with known alphacoronaviruses, with the closest matches being the BtCoV/Rh/YN2012_Rs4125 and BtCoV/Rh/YN2012_Rs4259 viruses, isolated from horseshoe bats in China in 2012–2013. In contrast, it shares only 62% amino acid identity with BtKY43, which is the closest match to Kudep virus in terms of RdRp. Two non-structural accessory proteins, ORF3b and the putative ORFX, exhibit 77 and 55% amino acid identity, respectively, with BtKY43.

Phylogenetic Analysis. Phylogenetic analyses based on the RdRp (**Figure** *a*), spike protein S (Fig. *b*), and nucleoprotein N (Fig. *c*) place the Kudep virus lineage within the *Decacovirus* subgenus. In the RdRp and N protein trees, the Kudep virus formed a sister clade to BtKY43. In the S protein tree, the Kudep virus was more closely related to strains associated with horseshoe bats from China.

Taxonomic Analysis. Pairwise comparison of the conserved domains concatomer 3CLpro-NiRAN-RdRp-ZBD-Hel1 of the Kudep virus with other alphacoronaviruses showed the highest similarity (91% amino acid identity) with BtKY43. When compared to the closest strains identified in Chinese horseshoe bats, the similarity was 88%.

RT-PCR Screening Results. Table summarizes RT-PCR screening results for Rhinolophus bats, including detection of Kudep virus, and the betacoronaviruses Khosta-1 and Khosta-2, that were recently described [13]. Khosta-1 was predominantly associated with *R. ferrumequinum* and the only positive sample was from *R. hipposideros,* collected in Partizanskaya cave. Khosta-1 showed high prevalence in the Kolokolnaya cave colony – 62,5 and 70% in autumn 2020 and autumn 2021, respectively. In the autumn of 2024, the infection rate in the colony was found to be 13%. The overall infection rate of horseshoe bats in the region for Khosta-1 ranged from 12.5% (2024) to 56% (2021). The Khosta-2 was detected only in the *R. hipposideros*, and at a single location throughout the entire observation period.

The novel alphacoronavirus Kudep was detected only in *R. ferrumequinum* at two relatively closely located Khostinskaya-1 and Kolokolnaya caves. The infection rate in Kolokolnaya Cave reached 52.9% in autumn 2021. In the spring of 2024, this rate was 13.6%. The overall infection rate of horseshoe bats with the Kudep virus ranged from 3.5% (2020) to 42.4% (2021). Notably, 21 samples from Kolokolnaya Cave were simultaneously positive for both Khosta-1 virus and Kudep alphacoronavirus in 2021. In 2020, only one such sample was found in this cave (out of two positives for Kudep virus). At the second site, Khostinskaya-1 Cave, where the Kudep alphacoronavirus was also detected, co-infection with Khosta-1 virus was found in one sample

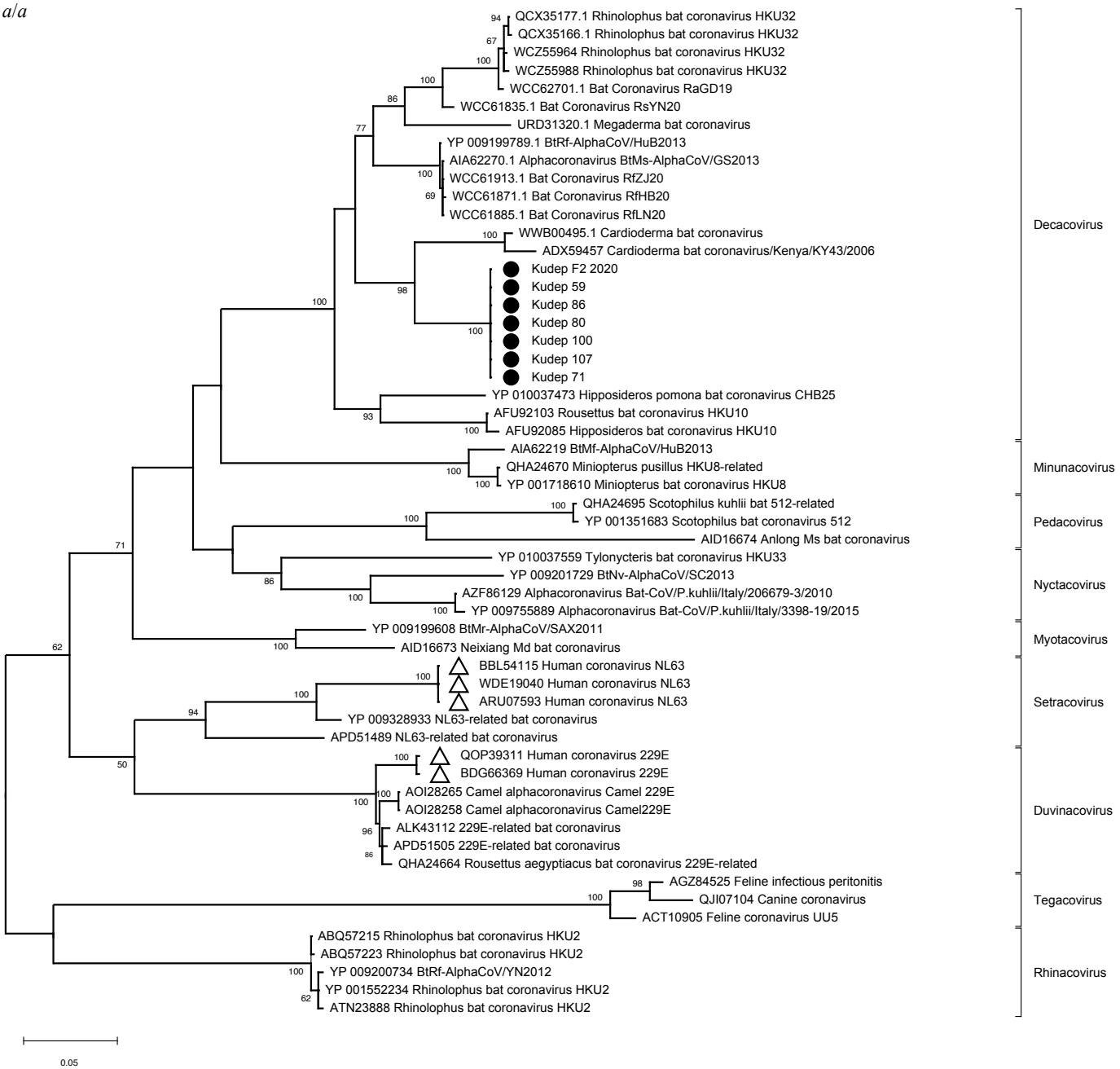
in 2020 (out of two positive samples) and in one sample in 2021.

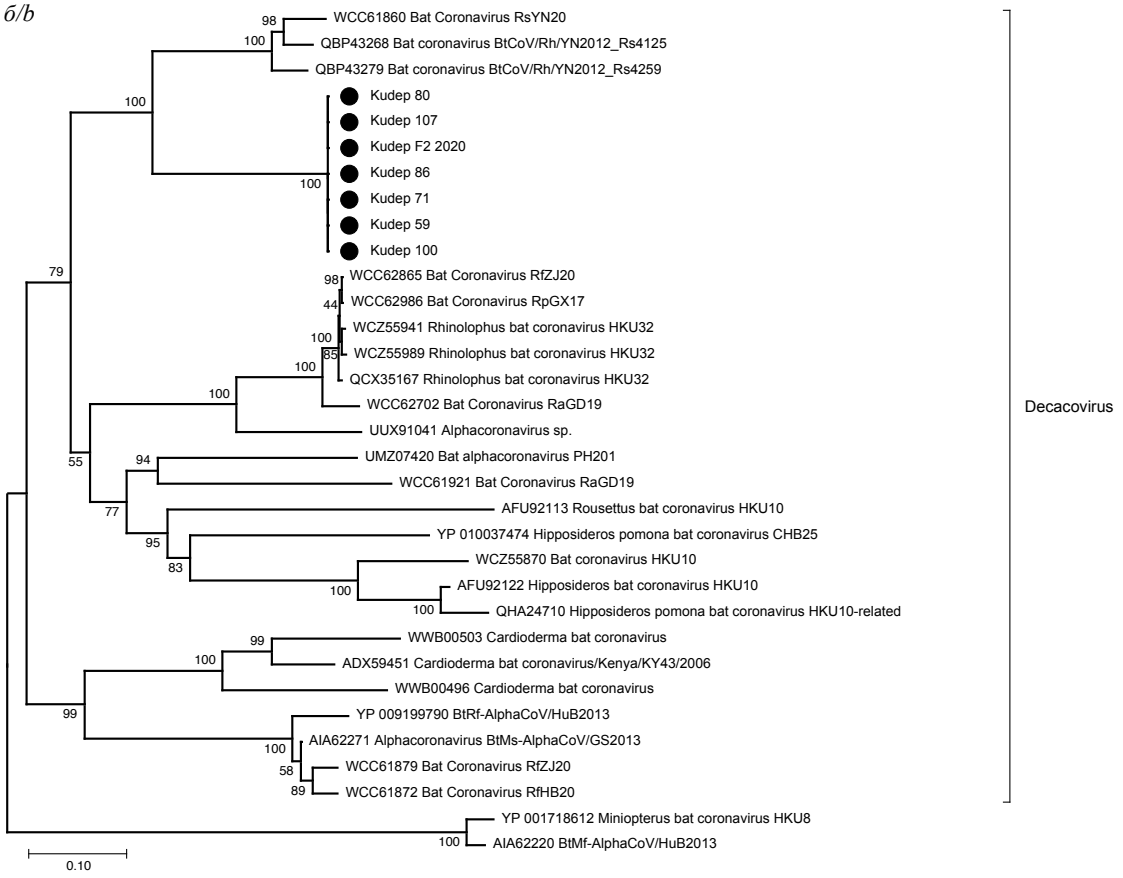
Discussion

Bats are recognized as primary natural reservoirs for both alpha- and betacoronaviruses. Through interspecies transmission, bat coronaviruses can cross species barriers, infecting other mammals, including domestic animals and humans, often leading to outbreaks, epidemics, or pandemics. Numerous studies have shown that bat coronaviruses are widely distributed across their habitats, including Europe, Africa, China, Southeast Asia, and the Americas [21–24]. In this study, we identified a novel alphacoronavirus, Kudep virus, circulating in the greater horseshoe bat (*R. ferrumequinum*) in the southern regions of the European part of Russia (the subtropical region of Krasnodar Krai, Sochi-Adler). We characterized seven complete genome sequences of Kudep virus: one from 2020 and six from 2021. Although all sequenced strains were obtained from a single location within a one-year period, they exhibited some genetic diversity, including single nucleotide substitutions and insertions/deletions in the genes, reflecting ongoing evolution. Continued surveillance will be crucial for determining the mutation rate in this viral population.

The genome of Kudep virus exhibits the size and structural features typical of alphacoronaviruses. Its closest genomic similarity is to the *Cardioderma bat*

a/*a*





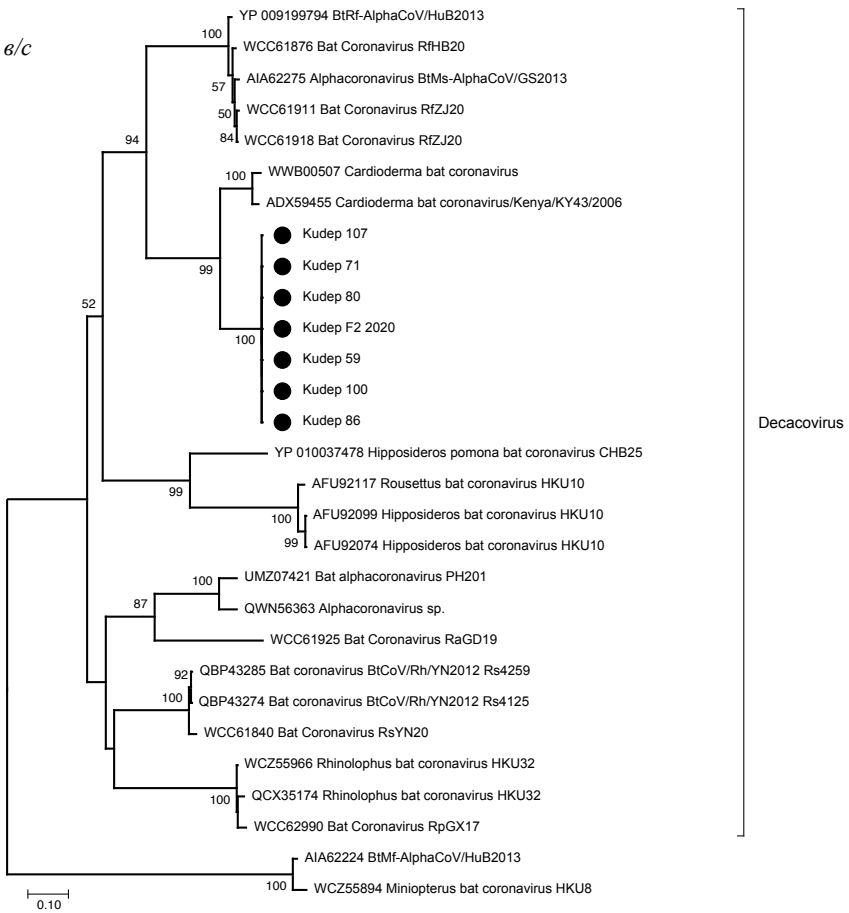


Figure. Phylogeny of Kudep virus based on analysis of RdRp (*a*), spike protein S (*b*), and nucleocapsid protein N (*c*) of certan representatives of the genus *Alphacoronavirus*.

The Kudep virus is marked with a black circle. Human coronaviruses are marked with a transparent triangle.

Рисунок. Филогения вируса Кудеп, построенная на основе анализа аминокислотной последовательности RdRp (*а*), спайкового белка S (*б*), белка нуклеокапсида N (*в*) отдельных представителей рода *Alphacoronavirus*.

Вирус Кудеп отмечен черным кружком; вирусы человека – прозрачным треугольником.

coronavirus (strains BtKY43, 2A/Kenya/BAT2621/2015, and 2B/Kenya/BAT2618/2015), identified in the heart-nosed bat (*Cardioderma cor*) in Kenya [20]. Both Kudep virus and BtKY43 lack genes for non-structural accessory proteins, such as ORF4, ORF7, ORF8, and ORF9, which are typically found in Asian bat alphacoronaviruses [25]. Whole-genome comparisons show similar levels of identity (~ 67–72%) between Kudep virus and BtKY43, as well as several alphacoronaviruses found in Chinese horseshoe bats (*Rhinolophus* spp.). Amino acid sequence analysis of structural and non-structural proteins places Kudep virus almost equidistant from both African and Asian viruses, with RdRp identities ranging from 87 to 92%.

Phylogenetic analysis places Kudep virus within the *Decacovirus* subgenus, which includes viral lineages associated with the various *Rhinolophus* species (e.g., *Rhinolophus bat coronavirus HKU32* and YN2012), as well as HKU10 viruses found in *Hipposideros* bats and *Rousettus* fruit bats in China. Additionally, this subgenus includes viruses from false vampire bats (e.g., BtKY43 from Kenya and *Megaderma* bat coronavirus from Bangladesh). These findings suggest considerable ecological plasticity within the *Decacovirus* subgenus, facilitating cross-species transmission among diverse bat families and even across chiropteran suborders [26]. Kudep virus consistently forms a distinct lineage within *Decacovirus* in phylogenetic trees based on RdRp and nucleocapsid (N) proteins, clustering closely with BtKY43. Phylogenetic analysis based on the spike (S) protein places Kudep virus closer to the Chinese horseshoe bat lineage YN2012 [11].

To determine the taxonomic position of the Kudep virus, we analyzed the similarity of the concatomer of its conserved domains within the *ORF1ab* gene (nsp5 (3CLpro), NiRAN, nsp12 (RdRp), ZBD, nsp13 (Hel1)) to those of other known alphacoronaviruses. The highest observed similarity was 91% amino acid identity (with BtKY43), which meets the ICTV demarcation criteria (92% amino acid identity) for defining distinct coronavirus species (https://ictv.global/report/chapter/coronaviridae/coronaviridae) [19]. Based on these findings, we propose that the Kudep virus represents a novel species within the *Decacovirus* subgenus of the genus *Alphacoronavirus*.

RT-PCR screening results demonstrate the active circulation of both the previously described sarbecovirus Khosta-1 and the newly identified Kudep alphacoronavirus in the surveyed region. In the greater horseshoe bat colony at Kolokolnaya Cave, infection rates reached 70.5% for Khosta-1 and 59.2% for Kudep virus during the autumn of 2021. These rates are consistent with reported bat coronavirus prevalence, which can range from 0% to 60–70%, depending on the location and season [27–30]. Notably, spring 2024 sampling revealed lower infection rates (13%), but the presence of both viruses suggests persistent infections exist within the host population.

Co-infections of individual bats with Khosta-1 and Kudep viruses were identified in both surveyed colonies (Khostinskaya-1 and Kolokolnaya). In 2021, such cases accounted for over half of the positive samples. The detection of these coronaviruses in both autumn and spring over a four-year period (2020–2024) supports the presence of a stable natural foci for both betacoronavirus Khosta-1 and alphacoronavirus Kudep, associated with the greater horseshoe bat.

Evidence of human and animal activity in the caves, including spelunkers, domestic goats seeking cool shelter in summer, jackals, and wild cats, highlights potential exposure and transmission risks for these bat coronaviruses to humans or other animals. These findings underscore the need for continuous surveillance and ecological studies to monitor zoonotic transmission risks. The identification of viral diversity in natural biomes and the study of the evolutionary processes that lead to the emergence of new viral infections is a key task in modern virology. These studies have significant practical implications for controlling the emergence of new and re-emerging infections. Epidemic outbreaks will continue to occur in the future, underscoring the need for international collaboration to conduct continuous monitoring of the gene pool of potentially zoonotic viruses [31, 32].

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