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Isolation and genetic analysis of the chikungunya virus from *Aedes aegypti* and *Aedes albopictus* mosquitoes captured in Central America

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Abstract
Introduction. The habitat of mosquitoes belonging to the genera *Aedes* spp., *Culex* spp., *Culiseta* spp. is in South and Central America, including Nicaragua. Monitoring of the spread of mosquito vectors and assessment of the infection with arboviruses can provide information on possible occurrence of new diseases or an increase in the reported cases, changes in the infectivity of viruses for humans due to changes in pathogen transmitters.
The purpose of this study was isolation and identification of arboviruses belonging to the *Flavivirus* and *Alphavirus* genera from *A. albopictus, A. aegypti, Culiseta* spp., *Culex* spp. mosquitoes captured in forests of Nicaragua.
Materials and methods. *A. albopictus, A. aegypti, Culiseta* spp., *Culex* spp. mosquitoes were captured during the dry season in 2021 in forested areas of Nicaragua in four different locations. Mosquitoes were sorted into pools, each containing 5-8 mosquitoes (236 pools in total). Using the reverse transcription polymerase chain reaction, the pools were tested for the presence of chikungunya (CHIKV), dengue, Zika, and yellow fever viruses. Positive pools were inoculated into the C6/36 cell culture to obtain isolates and for their further sequencing.
Results. The dengue virus was detected only in *Aedes* spp. mosquitoes: in 7 pools — *A. aegypti*, in 1 — *A. albopictus.* CHIKV was also detected only in *Aedes* spp. mosquitoes: in 3 pools — *A. aegypti*, in 1 — *A. albopictus.* The sequencing of nucleotide sequences of *6K*, *E1*, *E2,* and *NS1* genes of CHIKV isolated from *A. albopictus* mosquitoes showed that compared to the similar gene sequences from CHIKV isolates recovered from *A. aegypti* mosquitoes, the 6K gene region contained 4 nucleotide and 4 amino acid substitutions, while the E1 region contained 16 nucleotide substitutions, 10 of them led to amino acid substitutions; the E2 region contained 14 nucleotide and 11 amino acid substitutions; the NS1 region contained 33 nucleotide and 19 amino acid substitutions.

Keywords: *chikungunya, dengue, arboviruses, Aedes albopictus, Aedes aegypti, Culiseta spp., Culex spp., polymerase chain reaction, sequencing, isolate, nucleotide substitution, amino acid substitution*

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Изоляция и генетический анализ вируса Чикунгунья из комаров *Aedes aegypti* и *Aedes albopictus,* отловленных в Центральной Америке

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Аннотация

Введение. Ареал обитания комаров родов *Aedes* spp., *Culex* spp., *Culiseta* spp. распространяется на Южную и Центральную Америку, включая Никарагуа. Мониторинг за распространением комаров-переносчиков и оценка их инфицированности арбовирусами могут предоставить информацию о возможности появления новых или увеличении случаев уже регистрируемых заболеваний, изменении инфекционности вирусов для человека при смене переносчика возбудителя.

Целью настоящей работы были выделение и идентификация арбовирусов, принадлежащих к родам *Flavivirus* и *Alphavirus*, из комаров видов *A. albopictus, A. aegypti, Culiseta* spp., *Culex* spp., отловленных в лесах Никарагуа.

Материалы и методы. Комары *A. albopictus, A. aegypti, Culiseta* spp., *Culex* spp. были отловлены в 2021 г. в сухой сезон в лесной зоне в Никарагуа в четырех разных локациях. Комаров объединяли в пулы по 5–8 особей (всего 236 пулов). Методом полимеразной цепной реакции с обратной транскрипцией пулы анализировали на наличие вирусов Чикунгунья (ВЧ), денге, Зика и жёлтой лихорадки. Положительные пулы инокулировали в культуру клеток C6/36 с целью получения изолятов и их дальнейшего секвенирования.

Результаты. Вирус денге был выявлен только в комарах *Aedes* spp.: в 7 пулах — *A. aegypti*, в 1 — *A. albopictus.* ВЧ также был выявлен только в комарах *Aedes* spp.: в 3 пулах — *A. aegypti*, в 1 — *A. albopictus.* Секвенирование нуклеотидных последовательностей генов *6K*, *E1*, *E2* и *NS1* ВЧ, выделенного из комаров *A. albopictus*, показало, что по сравнению с аналогичными последовательностями генов из изолятов ВЧ, выделенных из комаров *A. aegypti*, в области гена белка 6K обнаружено 4 нуклеотидных и столько же аминокислотных замен, в области E1 — 16 нуклеотидных замен, 10 из которых приводили к аминокислотным заменам, в области E2 — 14 нуклеотидных и 11 аминокислотных замен, в области NS1 — 33 нуклеотидные и 19 аминокислотных замен.

Ключевые слова: *Чикунгунья, денге, арбовирусы, Aedes albopictus, Aedes aegypti, Culiseta spp., Culex spp., полимеразная цепная реакция, секвенирование, изолят, нуклеотидная замена, аминокислотная замена*

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Introduction

Mosquitoes of the genera *Aedes* spp., *Culex* spp., *Culiseta* spp. are vectors for numerous pathogens causing viral diseases such as dengue fever, Zika fever, yellow fever, chikungunya fever, Venezuelian equine encephalomyelitis, Sindbis fever and belong to families *Flaviviridae* (genus Flavivirus) and *Togaviridae* (genus Alphavirus) [1–6]. Habitats of mosquitoes belonging to the genera *Aedes* spp., *Culex* spp., *Culiseta* spp. are located in South and Central America, including Nicaragua [2, 3, 7–10]. The monitoring of the spread of mosquito vectors and the assessment of their infectivity can provide information on possible occurrence of new diseases or an increase in the reported cases, while the change of a pathogen's vector can cause changes in the infectivity of viruses for humans [5]. Isolation of viruses directly from vectors captured in their natural habitats and exploration of isolated strains are essential for development of diagnostic tools, prevention and treatment of diseases caused by alphaviruses and flaviviruses.

The purpose of this study was isolation and identification of arboviruses belonging to the genera Flavivirus and Alphavirus from *A. albopictus, A. aegypti, Culiseta* spp., *Culex* spp. mosquitoes captured in forests of Nicaragua.

Materials and methods

Mosquitoes

Mosquitoes were captured during the dry season in 2021 in a forested area of Nicaragua in four different locations having the following coordinates:

- location 1 — 12.325527N 85.974662W;
- location 2 — 12.323326N 85.974275W;
- location 3 — 11.908210N 85.932490W;
- location 4 — 11.903555N 85.938758W.

Representatives of the genera *Aedes* spp. (*A. albopictus, A. aegypti*), *Culiseta* spp., and *Culex* spp. were among the captured mosquitoes. After their species had been identified, mosquitoes were sorted into pools of 5–8 insects of the same species captured in the same location. A total of 236 pools were formed. Each pool was homogenized in 300 µl of the Leibovitz L-15 medium (Gibco, Thermo Fisher Scientific), pH 7.4, using ceramic beads and the SpeedMill Plus homogenizer (Analytik Jena) to obtain the suspension. 140 µl of homogenate from each pool was used for RNA extraction, which was performed using a QIAamp Viral RNA reagent kit (Qiagen). The reverse transcription polymerase chain reaction (RT-PCR) was performed using protocols for each pathogen. The pools tested positive for any of the viruses under study were used for subsequent isolation of the virus on cell cultures.

Virus isolation

The remaining mosquito suspension (200 µl) was filtered through the 0.45 µm PES filter. The obtained filtrate was used to infect the monolayer of C6/36 cells grown in 24-well plates. The inoculation was performed in 100 µl using the stock and dilution 1:10. Seven days after the infection, 100 µl of the supernatant were used for the next passage. A total of 5 successive passages were conducted. After the infection, the cells were examined daily for the presence of any signs of a cytopathic effect.

Molecular and genetic study

The RT-PCR-based molecular and genetic study was performed using the following procedure. The 140 µl mosquito suspension was used to extract RNA using a QIAamp Viral RNA reagent kit (Qiagen) in accordance with the instructions of the manufacturer. Then using the reverse primer pNS1CHVrev2-3 for the chikungunya virus (CHIKV; the *NS1* gene region); the mixture of reverse primers for the dengue virus panDVrev1 and panDVrev2 (the 3'-UTR region), which were common for all 4 types of the virus (**Table 1**); the reverse primer for the Zika virus pZVrev [9]; the reverse primer for yellow fever virus pYFVrev (**Table 2**)

Table 1. Nucleotide sequences of primers and probes for detection of CHIKV and dengue virus RNA in collected samples using real-time RT-PCR

Oligonucleotide	Nucleotide sequence, 5'–3'	PCR product size
	CHIKV (the *NS1* gene region)	
pNS1CHVfor	GTGTGCTGTTCTCAGTAGGGTCAACG	218 bp
pNS1CHVrev	GTCTGCGTGGTGGGTTACCGC	
zNS1CHVfor	**FAM**-GGCTACGTCGTTAAGAGAATAACGATGAGCCC-**BHQ1**	
	Dengue virus (the 3'-UTR region)	
panDVfor	GACTAG**Y**GGTTAGAGGAGACCC	190 bp
panDVrev1	CGTTCTGTGCCTGGAATGATG	
panDVrev2	CGCTCTGTGCCTGGATTGATG	
zDVfor	**FAM-**GCATATTGACGCTGGGA**R**AGACCAGAG**-BHQ1**	

Note. Y — either C or T; **R** — either A or G.

Table 2. Nucleotide sequences of primers for amplification of Zika and yellow fever virus RNA using RT-PCR and electrophoresis detection

Oligonucleotide	Nucleotide sequence, 5'–3'	PCR product size
	Zika (the *NS5* gene region)	
pZVfor	CCGCGCCATCTGGTATATGT	450 bp
pZVrev	CTCCACTGACTGCCATTCGT	
	Yellow fever (the *E* gene region)	
pYFVfor	TACCCTGGAGCAAGACAAGT	465 bp
pYFVrev	GCTTTTCCATACCCAATGAA	

[11] and a reagent kit for RT (Syntol), the cDNA was generated from the viral RNA template using an RT reaction. During the first stage, 2 μl of reverse primers (10 pmol/μl) were mixed with 6 μl of the extracted RNA; then, the mixture was heated at 95°C for 5 min. Then, the tubes were cooled down at the room temperature for 2 min and 22 μl of the mixture were added for RT (9 μl of deionized water, 12 μl of the 2.5-fold buffer for RT (Syntol), 1 μl of MMLV-reverse transcriptase (Syntol)) and were incubated at 42°C for 30 min. The mixture was heated at 95°C for 5 min to inactivate the reverse transcriptase.

For the Zika virus and yellow fever virus, agarose gel electrophoresis detection of PCR amplicons was performed using non-original primers from other researchers [9, 11], which are listed in Table 2. The amplification was performed in accordance with the following program: 95°C — 1 min 30 sec; 30 cycles: 95°C — 20 sec, 55°C — 15 sec, 72°C — 30 sec; 72°C — 10 min.

The real-time PCR was performed for CHIKV and dengue viruses using original primers and probes. The DT*prime* instrument (DNA-Technology) was used for amplification following the program: 95°C — 1 min 30 sec; 40 cycles: 95°C – 15 sec, 55°C – 40 sec.

The in-house designed oligonucleotides and the procedure described previously were used for generating CHIKV *6K*, *E1*, *E2,* and *NS1* gene fragments and their sequencing [12].

Prior to sequencing, all the PCR products were gel-purified using a Cleanup Standard reagent kit (#BC022, Evrogen) and cloned the pGEM-T Easy vector (Promega) in accordance with the manufacturer's instructions. Then, the clones were selected, plasmids were extracted, and the Sanger sequencing was performed using standard primers for sequencing T7 and SP6. The obtained nucleotide sequences were aligned using the MEGA11 software[1].

Phylogenetic analysis

The phylogenetic analysis was conducted using the molecular dating method, the BEAST v. 1.10.4 software package and the associated software suite BEAUti v. 1.10.4[2]. When constructing trees, we used the HKY[3] nucleotide substitution algorithm with a strict molecular clock. The analysis included 10 million trees, every 1,000th tree was selected. The MEGA11 software was used for tree construction. The multiple alignment was performed using genetic sequences of genome fragments (E2, 6K, E1) of CHIKVs isolated from mosquitoes (30 sequences) in different regions of the world. The position in the genome fragment, which was used for the phylogenetic analysis, was 8574–11 303 bp (the positions are shown for the prototype strain isolated in 1959, GenBank KX262990). The phylogenetic tree was extended with 8 nucleotide sequences of CHIKV isolated from humans, 3 of which were obtained from patients in Nicaragua in 2014 and 2015, and 4 of them were reference sequences of the virus.

The infectivity of mosquitoes was measured following the previously described procedure [10, 13].

Results and discussion

The mosquitoes captured in four locations represented three genera *Aedes* spp., *Culex* spp., *Culiseta* spp. As can be seen from **Table 3**, the best represented genus was *Aedes* spp. Accounting for 954 mosquitoes: 604 (67%) mosquitoes belonged to *A. aegypti*, 314 (33%) mosquitoes belonged to *A. albopictus*. Out of 105 pools of *A. aegypti* mosquitoes, 7 pools were tested positive for the dengue virus RNA, while 3 pools were tested positive for CHIKV RNA. Zika virus and yellow fever virus RNAs were not detected. No simultaneous detection of CHIKV RNA and dengue virus RNA was recorded. Out of 54 pools of *A. albopictus* mosquitoes, the dengue virus RNA was detected in 1 pool and the CHIKV RNA was also detected in 1 pool. Zika virus and yellow fever virus RNAs were also not detected. The simultaneous detection of CHIKV and dengue virus RNA was not reported. No RNA of the tested viru-

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Table 3. Genus and number of captured mosquitoes

Mosquito species	Number of mosquitoes	Number of pools	PCR results (positive/negative)			
			dengue virus	CHIKV	yellow fever virus	Zika virus
Aedes spp.	954	159	8/151	4/155	0/159	0/159
A. aegypti	640	105	7/98	3/102	0/105	0/105
A. albopictus	314	54	1/53	1/53	0/54	0/54
Culex spp.	278	46	0/46	0/46	0/46	0/46
Culiseta spp.	188	31	0/31	0/31	0/31	0/31

ses was detected in 46 pools of mosquitoes belonging to the genus *Culex* spp. and in 31 pools of mosquitoes representing the genus *Culiseta* spp.

A. albopictus mosquitoes from the CHIKV positive pool were captured in location 1 and *A. aegypti* mosquitoes from the positive pools were captured in location 4. The dengue virus RNA was detected in pools of *A. aegypti* mosquitoes captured in location 2, and *Aedes albopictus* mosquitoes captured in location 2.

The minimum infection rate for *A. aegypti* mosquitoes for the dengue virus was 10.0 and for CHIKV – 4.6. The minimum infection rate for *A. albopictus* mosquitoes for the dengue virus and CHIKV was 3.0. The obtained results lead to the assumption that mosquitoes of the genus *Aedes* spp. were infected with the dengue virus and CHIKV.

The minimum infection rate for mosquitoes, undoubtedly, is an important variable; however, it depends on the number of captured mosquitoes (the size of the studied subset) and the number of mosquitoes in the pool, thus affecting the sensitivity of the employed method. In some studies of the minimum infection rate among mosquitoes infected with alphaviruses and flaviviruses, the rate ranged from 0 to 12, thus implying a potential risk of infection with this virus transmitted by the particular mosquitoes [10, 13]. The possible coinfection with dengue and chikungunya viruses was described earlier; the examination of serological markers of flaviviruses and alphaviruses demonstrated the presence of antibodies against the dengue virus and CHIKV [9, 14]. The RT-PCR positive mosquito pools – 8 pools with the dengue virus and 4 pools with CHIKV were used to isolate viruses.

C6/36 cells were used to isolate viruses from the mosquito pools. Each pool was put through 5 successive passages. In the cells infected with samples containing CHIKV (based on the RT-PCR results), the disturbance of the cell monolayer – the development of a cytopathic effect – was observed in the 2nd – 3rd passages. In the 5th passage, the 100% cytopathic effect was observed after 72 hours. In the samples containing dengue viruses (based on the PCR results), the cytopathic effect was not as pronounced. In each passage, the authenticity of the isolates and the absence of cross-contamination were verified using RT-PCR. After the isolation had been completed, the isolates were sequenced. The resulting isolates contained CHIKV and dengue viruses types 1 and 2. In isolates containing dengue viruses, only the type of the virus was identified due to the short length of PCR product fragments.

As can be seen from the electropherogram of amplicons (**Fig. 1**) obtained using universal primers for all the 4 types of the dengue virus, the amplification produces 2 amplicons 190 and 106 bp long. The sequencing of fragments showed that both of them were specific [15, 16].

The sequencing of nucleotide sequences of CHIKV *6K*, *E1*, *E2,* and *NS1* genes from *A. albopictus* mosquitoes (1 pool, location 1) showed that it was the same strain, which we had isolated in the same location in 2018 [12]. Compared to the similar gene sequences from the CHIKV isolates obtained from *A. aegypti* mosquitoes (3 pools, location 4), the 6K gene region had 4 nucleotide and 4 amino acid substitutions; the E1 region had 16 nucleotide substitutions, 10 of which resulted in amino acid substitutions; the E2 region had 14 nucleotide and 11 amino acid substitutions and the NS1 region had 33 nucleotide and 19 amino acid substitutions (**Table 4**). Three isolates from *A. aegypti* mosquitoes did not have any amino acid substitutions relative

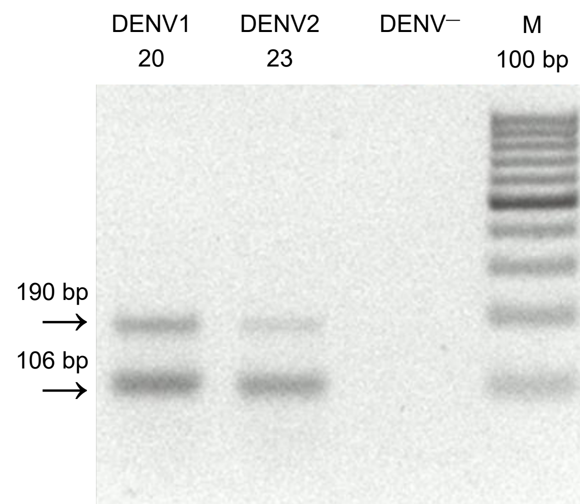

Fig. 1. Agarose gel electrophoresis (2%) of PCR products.

DENV1 20 and DENV2 23 — amplicons of dengue viruses types 1 and 2 from 20 and 23 pools, respectively; DENV⁻ — negative PCR controls for the dengue virus; M 100 bp – DNA molecular weight marker.

to each other. The isolated strain was deposited in the genetic sequence database of GenBank (NCBI) with accession number OQ320495.

The analysis of the nucleotide and amino acid substitutions showed that they, to a greater degree, differed from the known substitutions typical of the studied region [7]. Such significant differences can be explained by the fact that most of the CHIKV isolates were obtained by other researchers from sera of infected individuals and only rarely from mosquitoes. In addition, most of the isolates were obtained from mosquitoes captured in urban areas and rarely – in remote rural areas like mosquitoes in this study. The differences in nucleotide sequences of CHIKV isolates in all the 4 genes can also be explained by differences in locations of captured mosquitoes and by different mosquito species that carried the detected virus.

Thus, CHIKV and dengue type 1 and type 2 viruses were detected when isolating viruses from *Aedes* spp., *Culex* spp. and *Culiseta* spp. mosquitoes captured in forests in Nicaragua. The analysis of some CHIKV genes demonstrated high genetic diversity among viruses isolated from *A. albopictus* and *A. aegypti* mosquitoes.

The phylogenetic analysis of the nucleotide sequences (**Fig. 2**) showed that the closest relative of the isolated CHIKV is the virus isolated from a human in Africa in 2011 (GenBank KJ679577). The emergence of

Table 4. Nucleotide and amino acid substitutions in *6K*, *E1*, *E2,* and *NS1* gene regions in CHIKV isolates recovered from *A. albopictus* and *A. aegypti* mosquitoes captured in different locations in Nicaragua

Substitutions *A. albopictus* > *A. aegypti*							
6K protein gene		E1 protein gene		E2 protein gene		NS1 protein gene	
N	A	N	A	N	A	N	A
A37G	E13K	G28A	T10A	C145T	H49Y	C7T	P3S
A54T	F18L	T79A	M27L	A406G	K136E	T21C	–
C107T	L36P	G150A	–	T541G	C181G	C253T	R85C
T176C	A59V	C441T	–	T543C	C181G	A293G	K98R
		T443C	A148V	A545G	Q182R	G307A	A103T
		C579G	–	C558T	–	T389C	M130T
		G633A	–	G662A	R221K	T489C	–
		T683C	T228M	A760G	M254V	T498G	–
		C852T	–	G775A	G259R	A540G	–
		G955A	K319E	C914T	A305V	T581C	L194S
		G968T	I323S	C944T	A315V	A699G	–
		G979A	K327E	G1019A	R340H	A890G	Y297C
		A1081G	A361T	T1082C	V361A	C1014T	–
		C1106T	V369A	G1146A	–	A1047G	–
		C1217T	V406A			A1067G	Q356R
		T1308C	–			T1085C	L362P
						G1114A	A372T
						T1176C	–
						C1249T	R417C
						T1366A	W456R
						G1489A	A497T
						G1491A	A497T
						A1493T	E498V
						G1512A	–
						A1580T	E527V
						C1587T	–
						A1619C	N540T
						G1622C	R541P
						A1623G	R541P
						T1625G	I542R
						T1626A	I542R
						C1627G	P543G
						C1628G	P543G
Σ H: 4	Σ A: 4	Σ H: 16	Σ A: 10	Σ H: 14	Σ A: 11	Σ H: 33	Σ A: 19

Note. N – nucleotide substitution; A – amino acid substitution. The position of a nucleotide or amino acid substitution is shown from the beginning of a gene.

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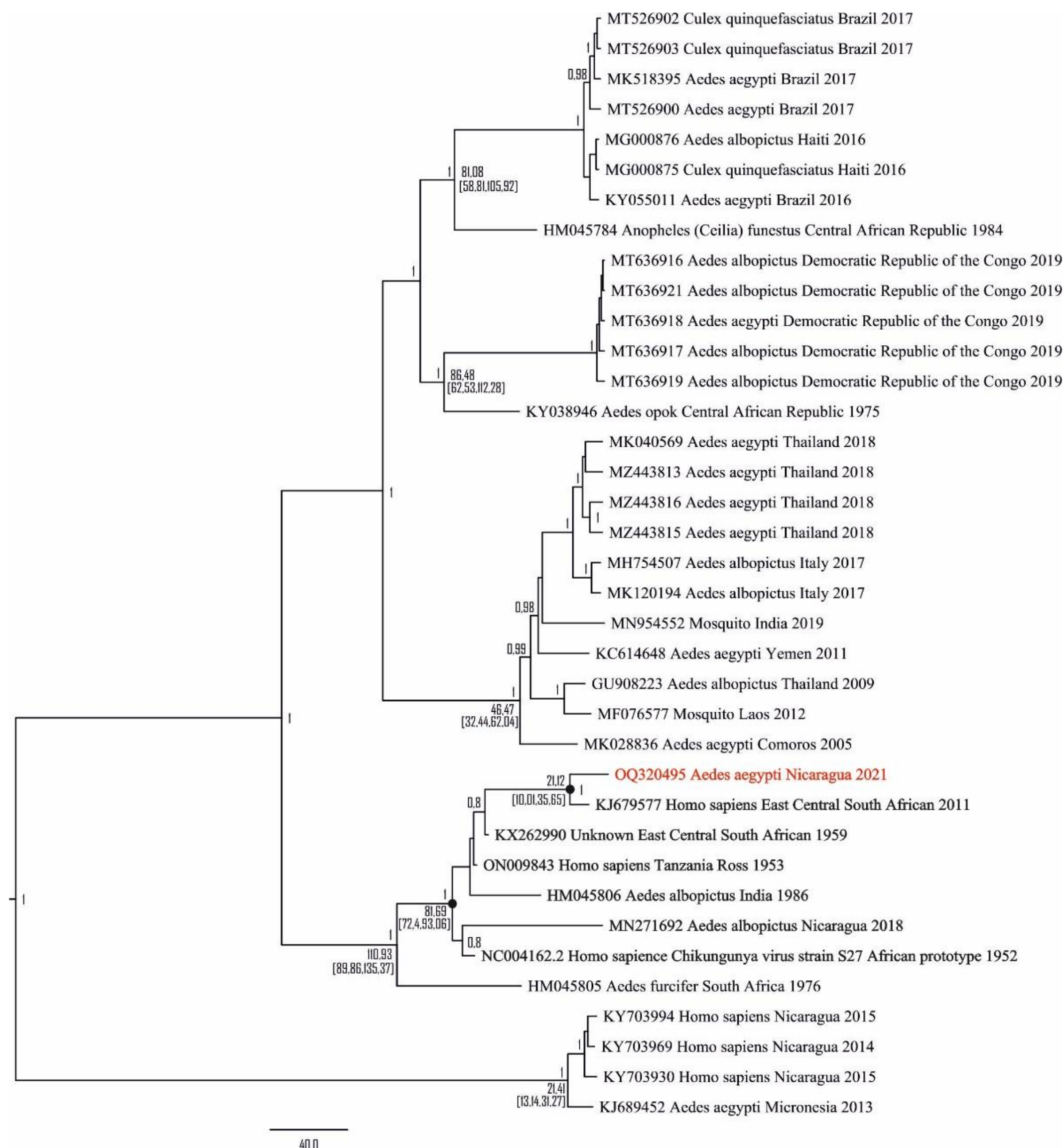

Fig. 2. Phylogenetic tree of CHIKV E2, 6K, E1 genome fragments.

The nodes of the tree indicate the posterior probability > 0.75 and the age of the group. The virus under study is highlighted. The scale shows the branch length representing years.

the common ancestor of these strains is estimated to have occurred 21 years ago (95% HPD 10–35). The isolate under study is also included in the cluster with prototype strains isolated in 1952–1986 in Tanzania and Africa.

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