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Chikungunya viruses containing the A226V mutation detected retrospectively in Cameroon form a new geographical subclade

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ABSTRACT

Background: Chikungunya virus (CHIKV) is a re-emerging arbovirus associated with sporadic outbreaks in Cameroon since 2006. Viral whole genomes were generated to analyze the origins of evolutionary lineages, the potential of emergence/re-emergence, and to infer transmission dynamics of recent Cameroon CHIKV outbreak strains.

Methods: Samples collected between 2016 and 2019 during CHIKV outbreaks in Cameroon were screened for CHIKV using reverse transcription PCR (RT-PCR), followed by whole genome sequencing of positive samples.

Results: Three coding-complete CHIKV genomes were obtained from samples, which belong to an emerging sub-lineage of the East/Central/South African genotype and formed a monophyletic taxon with previous Central African strains. This clade, which we have named the new Central African clade, appears to be evolving at 3.0×10^{-4} nucleotide substitutions per site per year (95% highest posterior density (HPD) interval of 1.94×10^{-4} to 4.1×10^{-4}). Notably, mutations in the envelope proteins (E1-A226V, E2-L210Q, and E2-I211T), which are known to enhance CHIKV adaptability and infectious potential in *Aedes albopictus*, were present in all strains and mapped to established high-density *Ae. albopictus* populations.

Conclusions: These new CHIKV strains constitute a conserved genomic pool of an emerging sub-lineage, reflecting a putative vector host adaptation to *Ae. albopictus*, which has practically displaced *Aedes aegypti* from select regions of Cameroon.

1. Introduction

Arthropod-borne viral infections are a major public health threat and continue to cause outbreaks worldwide (Mayer et al., 2017; Wilder-Smith et al., 2017). These infections are caused by viruses mainly from the *Peribunyaviridae, Flaviviridae*, and *Togaviridae* families (Gubler, 2001; Hughes et al., 2020). Hematophagous arthropods, primarily mosquitoes, are the principal vectors responsible for the transmission of medically important viruses such as Zika, West Nile, chikungunya, yellow fever, and dengue (Gould et al., 2017; Gubler, 2001). The transmission dynamics and epidemiology of these viruses are poorly understood in less resourced and endemic regions like sub-Saharan Africa. Consequently, critical data are lacking to inform public health decisions

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to curb arboviral spread in Africa (Braack et al., 2018; Gould et al., 2017).

Chikungunya virus (CHIKV) is a positive-sense, single-stranded RNA *Alphavirus* belonging to the family *Togaviridae*. CHIKV has a genome size of approximately 11.8 kb and has been evolutionarily classified into three genotypes: West African (WA), East/Central/South African (ECSA), and Asian (Rahman et al., 2019; Schuffenecker et al., 2006). However, between 2005 and 2006, CHIKV outbreaks in the Indian Ocean region caused by CHIKV with an alanine to valine mutation in the envelope protein gene at position 226 (E1-A226V) gave rise to the Indian Ocean Lineage (IOL), a sub-lineage of ECSA (Weaver, 2014; Schuffenecker et al., 2006). This virus has caused several outbreaks in Africa, Asia, Europe, Indian/Pacific Oceans, and the Americas (Petersen and Powers, 2016; Staples et al., 2009).

Transmitted primarily by the mosquito vectors Aedes aegypti and Aedes albopictus, CHIKV infection often presents with only mild fever and a rash in humans and is usually not fatal. However, CHIKV infection can be associated with chronic morbidity due to debilitating polyarthralgia (Gubler, 1998). Additionally, it has recently been linked to numerous other complications including cardiomyopathies, as well as autoimmune and neurological diseases (Alvarez et al., 2017; Bonifay et al., 2018; Pinheiro et al., 2016; Tanay, 2017). Currently, there are no licensed antiviral therapies to treat CHIKV infection (Chang et al., 2014; Erasmus et al., 2016) and there is no licensed vaccine. CHIKV first posed a problem to US military members in Thailand from 1962 to 1964, then in Vietnam, and more recently in Puerto Rico and other Western countries from 2010 to 2016 (Frickmann and Herchenröder, 2019). Troops deployed to austere environments for extended periods are susceptible to CHIKV, highlighting the need to better understand regional genomes of the pathogen to inform mitigation strategies. The debilitating impact of CHIKV on deployed forces degrades readiness and force health protection in tropical environments.

Cameroon, a region endemic for arboviruses, has a history of yellow fever, dengue, and chikungunya outbreaks (Demanou et al., 2014; Fokam et al., 2010; Nemg Simo et al., 2019; Peyrefitte et al., 2007; Vicens et al., 1993; Yousseu et al., 2018). The southern Guinean subequatorial region of the country provides an ideal wet-dense tropical ecology for sylvatic and urban populations of both *Ae. aegypti* and *Ae. albopictus*. Several seroprevalence and molecular characterization studies have been conducted on CHIKV outbreaks in Cameroon since 2006 (Demanou et al., 2010; Demanou et al., 2015; Kuniholm et al., 2006; Peyrefitte et al., 2007). However, these approaches are limited in their ability to completely characterize the viruses, resolve the evolutionary history of circulating strains, and inform public health policy. Recent viral whole genome sequencing (WGS) efforts have revealed the emergence of evolutionarily distinct sub-lineages within the ECSA genotype, prompting the need for constant genomic surveillance beyond traditional molecular or serological diagnostics. In this study, viral whole genomes were generated to analyze the origins of evolutionary lineages, the potential of emergence/re-emergence, and to infer transmission dynamics of recent CHIKV outbreak strains in Cameroon.

2. Methodology

2.1. Ethical review

This non-human subject study was reviewed and approved by the Naval Medical Research Center (NMRC) Institutional Review Board (IRB), approval number NAMRU3.PJT.19.01, in compliance with all applicable Federal regulations governing the protection of human subjects.

2.2. Samples

This retrospective study used de-identified human plasma samples originally collected as part of dengue and chikungunya outbreak responses in Cameroon. The samples were stored at −80°C at the Centre Pasteur Cameroon in Yaoundé prior to WGS at the Noguchi Memorial Institute for Medical Research (NMIMR) located in Accra, Ghana. Samples sent to NMIMR lacked personally identifiable information, and instead samples were identified using laboratory identification codes.

Samples were collected from individuals in all age categories who met the World Health Organization definition for CHIKV infection. Samples were collected as part of sentinel site surveillance, clinical case evaluation, and outbreak investigations occurring at medical facilities throughout Cameroon between 2016 and 2019. The specific facilities were Medico-Social Health Center of Yaoundé and Mfou District Hospital in the Centre region, community-based facilities and Londji Health Center in the South region, and New Bell District Hospital in the Littoral region.

2.3. PCR detection of chikungunya virus

Viral RNA extraction was done using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. CHIKV detection and data interpretation/validation were per the United States Centers for Disease Control and Prevention (US CDC) Trioplex real-time RT-PCR protocol (CDC, 2017) on an ABI 7500 system using the SuperScript III Platinum One-step qRT-PCR Enzyme Kit (Invitrogen, Waltham, MA, USA). The PCR-positive samples were selected for whole genome library preparation and sequencing.

2.4. Whole genome sequencing (WGS)

Sequencing libraries were prepared using the KAPA Hyper Prep Kit (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's instructions. Viral enrichment was done using custom target capture probes (Twist Bioscience, San Francisco, CA, USA). In brief, extracted RNA was fragmented, spiked with HELA RNA to increase ligation efficiency, and reverse-transcribed to cDNA. Dual indexing of cDNA libraries was achieved using IDT unique dual indexes (IDT, Coralville, IA, USA). Libraries were enriched using the 1-plex pooling strategy described previously by quartering the amount of reagents for the enrichment step (Blackley et al., 2016). Barcoded pooled libraries were sequenced on an Illumina MiSeq with v3 reagent kits (Illumina, San Diego, CA, USA).

2.5. Whole genome sequencing analysis

Illumina sequence adaptors were removed from the sequencing reads using Cutadapt v1.9 (dev1), and low-quality reads or bases were filtered using Prinseq-lite v0.20.3. Reads were aligned to the non-redundant National Center for Biotechnology Information (NCBI) database using BLAST. Viral hits were screened for appropriate references and viral reads were aligned to the reference genome using Bowtie2 v2.0.6. Duplicates were removed with Picard (http://broadinstitute.github.io/picard), and a new consensus sequence was generated using a combination of Samtools v0.1.18 and custom scripts (https://github.com/jtladner/Scripts/blob/master/reference_based_assembly/consensus_fasta.py). Only bases with Phred quality scores ≥20 were used in consensus calling, and a minimum of three times read-depth coverage, in support of the consensus, was required to make a call; positions lacking this depth of coverage were treated as missing (that is, called as

'N'). The final consensus sequence was submitted for annotation using the VIGOR4 Genome Annotation tool at the Virus Pathogen Database and Analysis Resource (ViPR) (https://www.viprbrc.org/).

2.6. Phylogenetic analysis

The sequences from the current study were submitted to the online Genome Detective virus tool (https://www.genomedetective.com/) for genotyping. The strains were aligned to complete CHIKV genomic sequences retrieved from NCBI. The sequences selected for phylogenetic analysis covered the three major lineages of CHIKV. Genome alignments and phylogenetic construction were conducted using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) and Mega X software (Kumar et al., 2018), respectively. To correct for the effects of ambiguous alignments due to polymorphisms in the 5′ and 3′ untranslated regions, the sequences were trimmed to the open reading frames (ORFs), and all subsequent phylogenetic analyses were conducted on the ORFs. A maximum likelihood phylogenetic analysis was conducted on the sequences using the GTR+G4 nucleotide substitution model predicted by ModelFinder (Kalyaanamoorthy et al., 2017) as the best fit model and implemented in IQ-TREE 1.6.11 (http://www.iqtree.org). The robustness of each node of the phylogenetic tree was ascertained using the bootstrap method with 1000 replicates.

To investigate the origins and better understand the evolutionary dynamics of the sequenced strains, a Bayesian inference phylogenetic approach based on the Bayesian Markov chain Monte Carlo (MCMC) method was implemented in BEAST v1.10.4 (Suchard et al., 2018) to determine the time of the most recent common ancestor (tMRCA) of study strains and the evolutionary rates of genotypes/clades. A total of 70 CHIKV genomes were used in this analysis, comprising 38 ECSA (18 African, 20 IOL), 17 Asian, 12 West African, and three strains from the current study. The presence of temporal signal in the complete and subdivided datasets was determined by performing a root-to-tip genetic divergence and sampling date correlation analysis using maximum-likelihood trees generated as described above and implemented in TempEst v1.5.3 (Rambaut et al., 2016). For each dataset, the strict molecular clock model and the coalescence constant tree prior were implemented (as convergence was not achieved for many parameters using the relaxed clock model, even after more than 100 million generations). The analysis was computed for at least 30 million MCMC steps for each dataset, sampling trees at every 1000 generations. Parameter convergence was inspected in TRACER v1.7.1 (http://tree.bio.ed.ac.uk/software/tracer/) to achieve an effective sample size (ESS >200). A maximum clade credibility (MCC) phylogeny was computed from the posterior distributions of the complete dataset, excluding 10% as burn-in using TreeAnnotator (via BEAST). Tree visualization and annotation was done with FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

To visualize how the sequenced strains fit into the African CHIKV landscape, a maximum likelihood phylogenetic tree was created with all available CHIKV partial E1 sequences of African origin from NCBI, using a partial E1 sequence from an o'nyong'nyong virus (NCBI accession **AF079456.1**) as an external outgroup. Akaike Information Criterion (AIC)-based SMS (smart model selection) (Lefort and Longueville, 2017) was used to determine the model of best fit. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A maximum likelihood tree using a general time reversible model was then created in Mega X with a discrete gamma distribution and invariable sites (GTR+G+I). Tree visualization and annotation was done with FigTree v1.4.4.

2.7. Mosquito prevalence and insecticide resistance

To gain a better perspective on the vector environment where these sequenced strains were collected, CHIKV vector prevalence for *Ae. aegypti* and *Ae. albopictus* alongside insecticide resistance data were mined from Tedjou et al. (Tedjou et al., 2020; Tedjou and Armel, 2019) and Kamgang et al. (Kamgang et al., 2011; Kamgang et al., 2017), respectively. These data were presented alongside population density data from the 2005 Cameroon census (https://microdata.worldbank.org/) and visualized on region and district-specific maps created in ArcGIS (v10.7.1).

3. Results

Three complete CHIKV genomes were obtained after sequencing. All three strains belong to the ECSA genotype (Table 1). Details of genome structure and organization, as well molecular fingerprints in non-structural proteins (NSPs) are presented in **Supplementary Material** File 1. The opal stop codon between nsP3 and nsP4 was intact for all three genomes.

3.1. Structural protein molecular fingerprints

In the structural polyproteins, the E1 envelope protein segment (439 amino acids (aa)) demonstrated 10 aa substitutions that were shared among all of the three strains from this study. One substitution, K157R, was present in only CHIKV/CAM/2016/Yaoundé. Among the substitutions was the highly characterized A226V, which has been shown to enhance *Ae. albopictus* competence for CHIKV and result in increased viral transmission potential (Tsetsarkin et al., 2007). Also present were the less characterized mutations M269V, I317V, and V322A, which have been reported previously in India (Harsha et al., 2020; Singh et al., 2012). Five novel aa substitutions were also present. The E2 protein segment (423 aa) had 21 aa substitutions, and 20 of these mutations were shared by all three strains, while the Q282K mutation was present in only the CHIKV/CAM/2018/Mfou strain. Notably, aa substitutions L210Q and I211T, which have been shown to enhance CHIKV infectivity in *Ae. albopictus*, were present (Sahu et al., 2013; Tsetsarkin et al., 2009). The remaining 19 mutations in the E2 protein have not been reported previously and could be novel. The 64 aa long E3 protein showed a single substitution, I23T, which was present in all strains. The 6K protein (61 aa) showed 2 aa substitutions, I54V and S60N, which were shared between all of the strains in the study. In the capsid protein (261 aa), 2 aa substitutions (K63R and K122R) were observed and were present in all three stains. A summary of all of the aa substitutions is presented in Table 2, and those that are unique to the three Cameroon strains are detailed in Table 3.

3.2. Phylogenetic relationships

The strains recovered from this study belong to the ECSA genotype and formed a monophyletic taxon, the new Central African Clade (nCAC), with strains collected in Congo, Gabon, and Cameroon (Fig. 1). The time scaled phylogeny indicates that the common recent ancestor of the Cameroon CHIKV strains existed around 2012, with a 95% highest posterior density (HPD) interval of 2010 to 2013. However, the nCAC was estimated to have originated around January 1998, with a 95% HPD interval of April 1995 to March 2000. The nCAC was evolving at 3.0×10^{-4} nucleotide substitutions per site per year, with a 95% HPD interval of 1.94×10^{-4} to 4.1×10^{-4} substitutions/site/year. The substitution rates of the datasets and the root-to-tip genetic divergence results are presented in Table 4. Partial CHIKV E1 phylogenetic analyses

Table 1
CHIKV sequencing and assembly results

Strain	Number of reads	G+C content (%)	Mean coverage (X)	Assembled sequence length	GenBank accession number
CHIKV/CAM/2018/Yaoundé	734 581	50.5	8526	11 561	**MT666071**
CHIKV/CAM/2016/Yaoundé	1 889 829	50.4	20 932	11 562	**MT666072**
CHIKV/CAM/2018/Mfou	2 727 185	50.4	31 695	11 562	**MT666073**

Table 2
Amino acid substitutions in ORFs. AA (amino acid), *n* (number of strains); substitutions in bold are those that have been reported previously

Non-structural polyprotein										Structural polyprotein									
nsP1		nsP2		nsP3		nsP4		E1		E2		E3		6K		Capsid	
AA change	*n*	AA change	*n*	AA change	*n*	AA change	*n*	AA change	*n*	AA change	*n*	AA change	*n*	AA change	*n*	AA change	*n*
29PS	3	R34C	3	T122I	3	Q46H	3	N9S	3	G57K	3	I23T	3	I54V	3	K63R	3
D75E	3	H374Y	3	V175I	3	T101I	3	T37I	3	I74T	3			S60N	3	K122R	3
V156I	3	A604V	3	A196V	3	E119K	2	K157R	1	G79E	3						
L172V	3	C642Y	3	P326S	3	K230N	3	**A226V**	3	V85A	3						
K224N	1			Q328P	3	Q235R	1	S250P	3	S118G	3						
E234K	3			V331A	3	V497A	3	**M269V**	3	K149R	3						
M383L	3			T337A	3	Q500L	3	**I317V**	3	N160T	3						
I384L	3			T338M	3	I514T	3	**V322A**	3	A164T	3						
T481I	3			Q344R	3	V604I	3	K324R	3	L181M	3						
P482L	1			K352E	3			G348E	3	S194G	3						
A487T	3			I376T	3			V399I	3	**L210Q**	3						
L507R	3			T378M	3					**I211T**	3						
				A382T	3					V222I	3						
				V399I	3					A227V	3						
				M449T	3					A246D	3						
				S462N	3					M267R	3						
				N483S	3					Q282K	1						
										S299N	3						
										A344T	3						
										I415L	3						

ORF, open reading frame.

Table 3
Unique amino acid substitutions in strains of the new Central African clade (nCAC)

Strain	Non-structural polyprotein				Structural polyprotein				
	nsP1	nsP2	nsP3	nsP4	E1	E2	E3	6K	Capsid
CHIKV/CAM/2018/Yaoundé[a]	K224N	R34C, A604V	T122I, A196V, Q328P, S462N	Q46H, T101I, E119K, Q235R, V497A	K157R, S250P, I317V, K324R, G348E, V339I	W64R, V85A, L210Q, A227V, A246D	-	-	-
CHIKV/CAM/2016/Yaoundé[a]	P482L	R34C, A604V	T122I, A196V, Q328P, S462N	Q46H, T101I, E119K, V497A	S250P, I317V, K324R, G348E, V339I	V85A, L210Q, A227V, A246D	-	-	-
CHIKV/CAM/2018/Mfou[a]	-	R34C, A604V	T122I, A196V, Q328P, S462N	Q46H, T101V, V497A	S250P, I317V, K324R, G348E, V339I	V85A, L210Q, A227V, A246D, Q282K	-	-	-
CHIKV/*H.sapiens*/CMR/667/2006[a]	-	-	Q328P, V349A, S462I	-	-	A246D	H60R	-	Q37R
GABOPY1[b]	-	I457S	T122I, S153T, S462N	T101I, E119K	S250P	-	-	-	R13G
BRAZZA_MRS1[c]	E508V	A604V, T17I, M557T	T122I, A196V, Q328L, S462N	T101I, E119K, V497A	S250P, K324R	V85A, L210Q, A246D, T265I	-	-	-

[a] Cameroonian partial E1 sequences (**KJ508821.1**,**KJ508819.1** (Maurice et al., 2015) and **EF051584** (Peyrefitte et al., 2007)) were derived from sporadic CHIKV surveillance, stemming from febrile dengue-like symptom outbreaks.
[b] GABOPY1 (**KP003812.1**) from a 2007 CHIKV outbreak in Gabon.
[c] BRAZZA_MRS1 (**KP003813.2**) from a 2011 CHIKV outbreak in Congo.

Table 4
Nucleotide substitution rates of datasets used in the molecular clock analysis and the root-to-tip genetic divergence results

Dataset	Substitution rate (BEAST analysis) Mean ($\times 10^{-4}$)	95% HPD ($\times 10^{-4}$)	Root-to-tip analysis (TempEst) Correlation coefficient	R^2
Complete	2.15	1.98–2.32	0.57	0.33
ECSA	1.65	1.46–1.84	0.81	0.67
Old ECSA	1.20	0.99–1.41	0.90	0.81
IOL	4.49	3.54–5.50	0.70	0.48
Asian	4.20	3.66–4.74	0.99	0.99
West African	2.02	1.64–2.40	0.93	0.87
nCAC	3.00	1.94–4.10	0.99	0.99

ECSA, East/Central/South African; HPD, highest posterior density; IOL, Indian Ocean Lineage; nCAC, new Central African Clade.

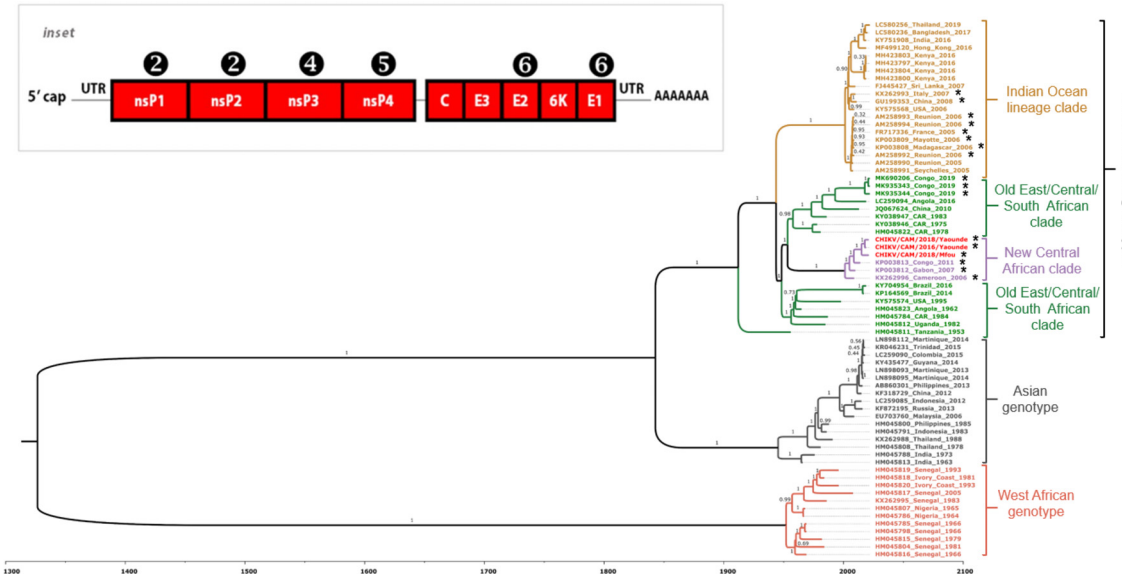

Figure 1. Maximum clade credibility (MCC) phylogeny based on the complete ORFs of 70 CHIKV strains used for molecular clock analysis. Black asterisks indicate sequences containing the A226V mutation. The MCC tree was based on the strict molecular clock model and the coalescence constant tree prior. Taxon labels include accession number/country and year of isolation for sequences extracted from GenBank, while new sequences are labeled with strain names. Posterior probabilities are present on all clades. The inset shows a summary of the maximum number of unique amino acid substitutions between each strain in each gene for the three new CHIKV strains from Cameroon described in Table 3.

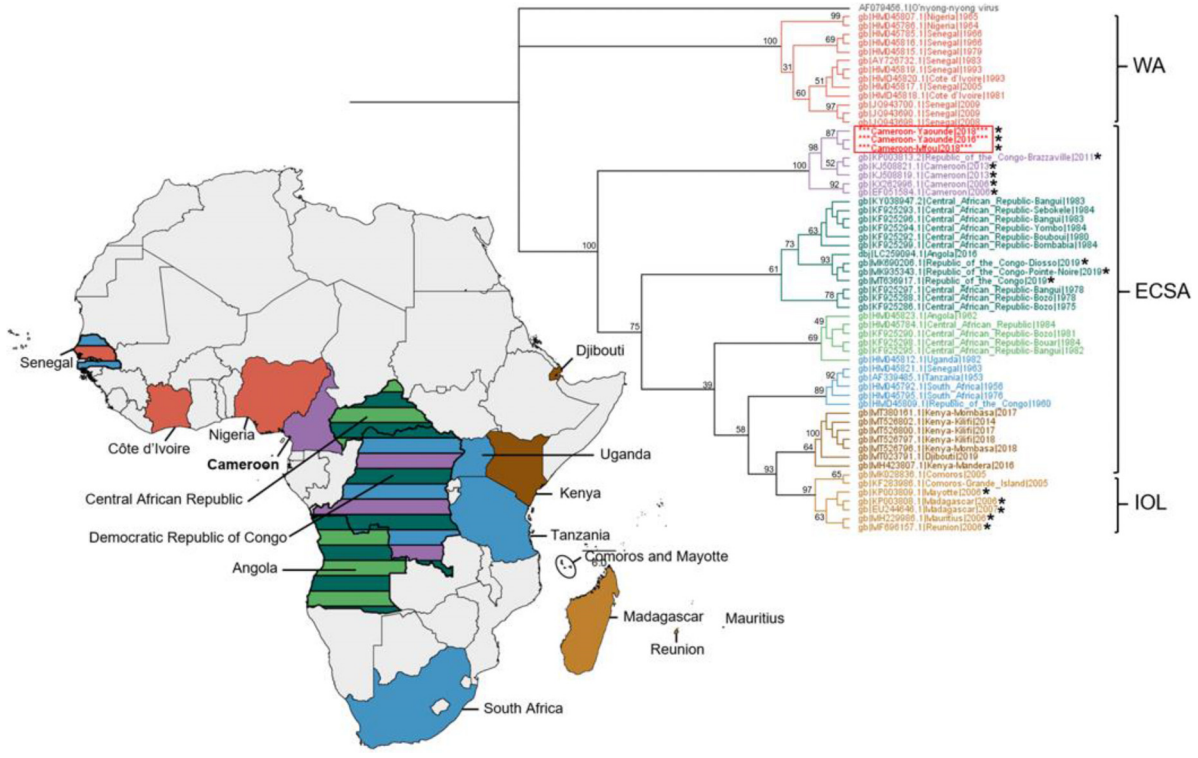

Figure 2. Molecular phylogenetic analysis of 60 CHIKV partial E1 sequences. Sequences obtained in this study are denoted in bright red with three red asterisks. Taxa are represented by NCBI accession number, county(-city), and year. Each clade is separated by color and bracketed by strain (WA = West African, ECSA = East/Central/South African, IOL = Indian Ocean Lineage). Black asterisks indicate sequences containing the A226V mutation. Evolutionary history was inferred by the maximum likelihood method based on the general time reversible model with discrete gamma distribution rates among invariant sites (GTR+G+I). Bootstrap values are present on major clades. The tree is drawn to scale, with branch lengths measured by the number of substitutions per site. The accompanying map of Africa is colored by clade.

(Fig. 2) further support this finding and suggest that there may be other smaller clades akin to the nCAC within the ECSA group.

3.3. Mosquito prevalence and insecticide resistance

Merged mosquito prevalence data from Tedjou et al. (Tedjou et al., 2019; Tedjou et al., 2020) show that *Ae. albopictus* vectors predominate in downtown and suburban areas in Yaoundé, while rural areas seem to have roughly equal *Ae. aegypti* and *Ae. albopictus* populations. Although no published data exist for Mfou, a similar prevalence trend towards *Ae. albopictus* can be seen in the urban and suburban surrounding areas of Yaoundé, Mbalmayo, and Akonolinga (Fig. 3). Within Yaoundé, the 2018 sequenced sample was collected from Bastos in Yaoundé II, while the 2016 sample

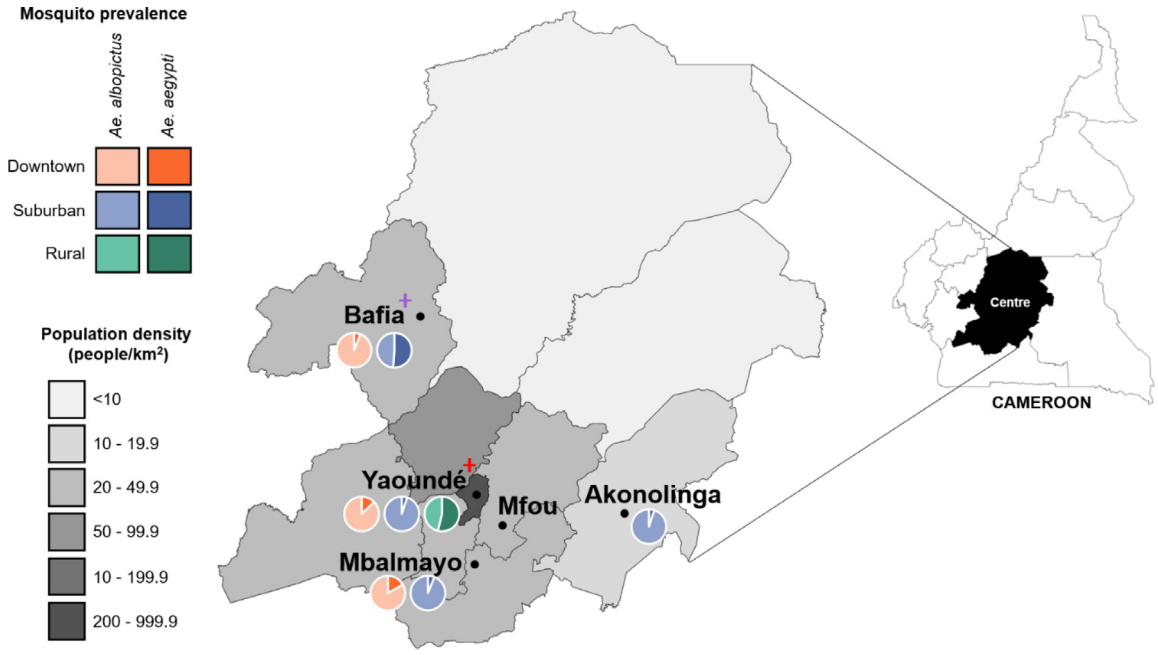

Figure 3. Geographic distribution of *Aedes albopictus* and *Aedes aegypti* in Centre, Cameroon. Black borders denote administrative districts within Centre, Cameroon and are shaded based on population density gathered from the 2005 census. '+' denotes cities with mosquito insecticide resistance data; a purple cross indicates susceptible populations, while a red cross indicates deltamethrin-, bendiocarb-, and DDT-resistant populations that were susceptible to permethrin and malathion. Data were merged from Tedjou et al., 2019 and 2020, and Kamgang et al., 2017 and 2011.

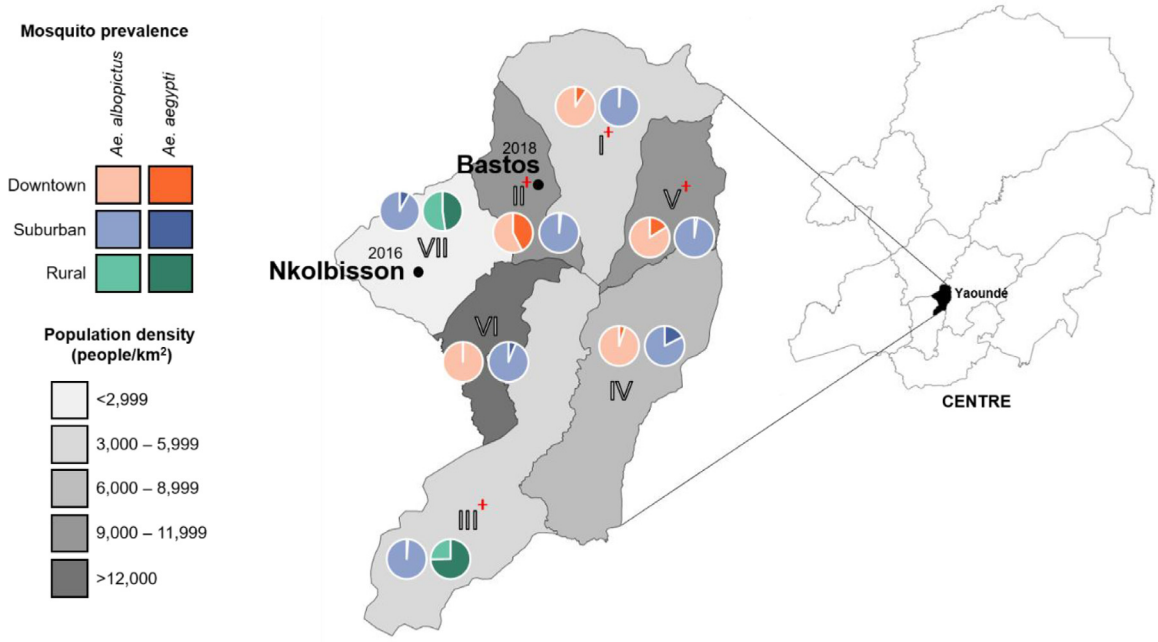

Figure 4. Geographical distribution of *Aedes albopictus* and *Aedes aegypti* in Yaoundé (Centre, Cameroon). Black borders denote municipalities within Yaoundé (I through VII) and are shaded based on population density gathered from the 2005 census. '+' denotes municipalities with mosquito insecticide resistance; a red cross indicates deltamethrin-, bendiocarb-, and DDT-resistant populations that were susceptible to permethrin and malathion. Data were merged from Tedjou et al., 2020 and Kamgang et al., 2017.

was from Nkolbisson in Yaoundé VII. Vector prevalence in these municipalities is largely municipality-specific, with an equal proportion of both vectors in urban Yaoundé II and rural Yaoundé VII; in both areas, suburban environments primarily comprise *Ae. albopictus* (Fig. 4). *Ae. aegypti* and *Ae. albopictus* from Yaoundé appear to be deltamethrin-, bendiocarb-, and DDT-resistant, while still susceptible to permethrin and malathion (Kamgang et al., 2011; Kamgang et al., 2017).

4. Discussion

The persistent re-emergence of CHIKV highlights the evolutionary fitness and adaptability of the virus. The strains recovered in this study share the same basic genomic architecture as others isolated in Congo, Gabon, and Cameroon (Moyen et al., 2014; Peyrefitte et al., 2007).

Phylogenetic analyses were based on the complete ORFs and partial E1 segments. The former has proven to be more accurate in comparison to the latter (Volk et al., 2010), but sequence availability is largely skewed towards the latter, and therefore both approaches were incorporated. The analyses revealed that the strains from the current study are of the ECSA genotype.

Molecular clock analysis estimates that the new sequences have existed since around 2012. However, their mother clade (nCAC) had a common ancestor around 1998, the same period as the emergence of the IOL outbreak strains. Although the nCAC and IOL might have emerged around the same period, the phylogeny suggests that nCAC is an emerging sub-lineage of the Central African ECSA harboring the E1-A226V and E2-I211T substitutions, the signature genomic fingerprints of the IOL. Although, the nCAC and IOL diverged long ago, the selection mechanism resulting in the evolutionary convergence of these same independent mutations is likely to be the same since the vector that manifests this selective advantage has been identified in some of the Central African outbreaks (Chen et al., 2016). The endemicity of the nCAC to Central Africa suggests that it constitutes a conserved genomic pool with a limited geographical distribution. With 99.0% nucleotide identity between the strains recovered from this study and their sister taxa, it could be suggested that this sub-clade might have originated from CHIKVs that caused epidemics in Central Africa during the mid-2000s (Zeller et al., 2016). The silent circulation of the nCAC containing the *Ae. albopictus* adaptive mutation for nearly two decades indicates the need for constant surveillance in these regions and underscores the idea (Chen et al., 2016) that Central Africa contains rich, deeply divergent, co-circulating strains.

The occurrence of 42 aa substitutions in virus NSPs, of which 40 were shared among the three strains (Table 2), suggests these are stable adaptive mutations essential for viral survival and pathogenicity. Unfortunately, the lack of information on CHIKV NSPs means no meaningful inferences can be made about these mutations. Mutations in CHIKV E1 and E2 envelope structural proteins have been noted to play key roles in viral evolution and host adaptation (Tsetsarkin et al., 2007; Tsetsarkin et al., 2009). A major shift in the dynamics of CHIKV transmission has been the increase in epidemic potential and transmissibility by *Ae. albopictus*, as conferred adaptive mutations enhance vector competence (Schuffenecker et al., 2006; Tsetsarkin et al., 2007). Analysis of the E1 protein segment revealed that the positively selected E1-A226V mutation, which has increased CHIKV fitness in *Ae. albopictus*, was present in all strains recovered from the study. This finding corroborates the findings of earlier studies, i.e., *Ae. albopictus* invasion is a rising problem of public health concern in Cameroon (Simard et al., 2005), which was shortly followed by the first CHIKV isolation in Cameroon (Peyrefitte et al., 2007). Although the genomic data of the virus were not available at the time, sequencing was done later (strain: CHIKV/*Homo sapiens*/CMR/667/2006, GenBank accession number **KX262996**). Analysis of the E1 segment of CHIKV/*Homo sapiens*/CMR/667/2006 shows the presence of the E1-A226V mutation, suggesting that *Ae. albopictus* CHIKV transmission might have happened earlier than previously thought. Subsequently, the analysis of partial E1 protein segments of CHIKV strains collected from Cameroon in 2013 identified the E1-A226V mutation (Demanou et al., 2015). In addition to the E1-A226V mutation, three other aa substitutions, which had previously been reported in isolates of Indian origin, were present in all of the strains of the current study. Seven remaining substitutions were unique to strains in this study.

The E2 protein segment had the highest proportion of aa substitutions across the genomic segments. Many of these mutations have not yet been reported, but the aa substitutions E2-L210Q and E2-I211T have been characterized previously (Tsetsarkin et al., 2009; Tsetsarkin and Weaver, 2011). Tsetsarkin et al., 2007 showed that a synergistic effect of E1-A226V with E2-I211T is needed to see a significant increase in *Ae. albopictus* midgut infectivity. This change, however, did not have any effect on the primary vector *Ae. aegypti* (Tsetsarkin et al., 2009). All strains recovered from this study harbored the E2-I211T substitution, suggesting that these strains would be transmitted well by *Ae. albopictus*. The E2-L210Q substitution has also been shown to increase CHIKV fitness in *Ae. albopictus*; the presence of E2-L210Q enhanced infectivity of *Ae. albopictus* midgut cells but not Ae. aegypti or human cells lines (Tsetsarkin and Weaver, 2011). Nineteen other uncharacterized mutations were observed. The abundance of polymorphic sites on the antigenic E2 protein presents a great challenge to vaccine development due to the likelihood of high antigenic variation. The E3 and 6K protein segments were somewhat conserved, showing few uncharacterized substitutions. The capsid protein, however, showed a high number of substitutions, many of which were uncharacterized.

Previous concerns regarding the rapid geographic expansion of *Ae. albopictus* in Central Africa have clearly manifested, as there is an overwhelming presence of *Ae. albopictus* in urban and suburban Yaoundé and surrounding areas, displacing native *Ae. aegypti* populations (Kamgang et al., 2011; Kamgang et al., 2017). This suggests that there is an increased epidemic potential for CHIKV in Cameroon, as Cameroonian strains (historically and herein) contain the E1-A226V *Ae. albopictus*-adaptive substitution. Amplifying this risk are previous findings that there is widespread *Ae. aegypti* and *Ae. albopictus* insecticide resistance in Yaoundé, which has appeared despite no known vector control efforts in these areas (Kamgang et al., 2017). The evolutionary rate of the nCAC of 3.0×10^{-4} nucleotide substitutions per site per year, which is two times that of the main ECSA, presents the nCAC with the opportunity for increased infectivity and pathogenicity (Sanjuán and Domingo-Calap, 2016); previous literature (Caron, 2012) has also suggested that an increase in CHIKV co-infections could also result in increases in viral fitness.

In conclusion, CHIKV strains recovered from this study belong to an emerging sub-lineage of the East/Central/South African genotype and had a common recent ancestor around 2012. The identification of E1-A226V, E2-L210Q, and E2-I211T mutations, which confer an adaptive advantage in the mosquito vector *Ae. albopictus*, means there is likely a selective shift towards this vector for effective dissemination of the virus in both urban and peri-urban settings. The high evolutionary rate of the emerging clade from the ancestral taxa highlights the possibility of increased CHIKV fitness and pathogenicity. Furthermore, the identification of many novel amino acid substitutions is indicative that there is an imminent need to characterize these mutations to assess their effects on the evolutionary fitness of the pathogen. These functional studies combined with known mosquito prevalence data shed light on pathogen adaptation and evolution and highlight the concern for further CHIKV outbreaks in Cameroon, framing future public health decisions.

Declarations

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by a Military Service member or employee of the US Government as part of that person's official duties.

Ethics statement: The study protocol NAMRU3.PJT.19.01 was approved by the Naval Medical Research Center Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects.

Author contributions

Conceptualization: MRW, MD, AGL. Writing – original draft: BA. Sampling: FBSY, FBNS, MD. Molecular assays: BA, FBSY, FBNS, SK, CY, M-TM, REB. Whole genome sequencing: BA, FBSY, SK, CY, M-TM, REB, KP. Bioinformatics analysis and data interpretation: BA, HGC, RRD, MRW. Writing – review and editing: BA, FBSY, FBNS, SK, CY, M-TM, REB, SMC, NA, SN-P, ATF, JHKB, HGC, RRD, MRW, MD, AGL. Coordination: NA, SN-P, ATF, JHKB, WA, DW, MD, AGL. All authors have read and agreed to the published version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.09.058.

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