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# A new "American" subgroup of African-lineage Chikungunya virus detected in and isolated from mosquitoes collected in Haiti, 2016

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## Abstract

As part of on-going arboviral surveillance activity in a semi-rural region in Haiti, *Chikungunya virus* (CHIKV)-positive mosquito pools were identified in 2014 (the peak of the Caribbean Asian-clade epidemic), and again in 2016 by RT-PCR. In 2014, CHIKV was only identified in *Aedes aegypti* (11 positive pools/124 screened). In contrast, in sampling in 2016, CHIKV was not identified in *Ae. aegypti*, but, rather, in (a) a female *Aedes albopictus* pool, and (b) a female *Culex quinquefasciatus* pool. Genomic sequence analyses indicated that the CHIKV viruses in the 2016 mosquito pools were from the East-Central-South African (ECSA) lineage, rather than the Asian lineage. In phylogenetic studies, these ECSA lineage strains form a new ECSA subgroup (subgroup IIa) together with Brazilian ECSA lineage strains from an isolated human outbreak in 2014, and a mosquito pool in 2016. Additional analyses date the most recent common ancestor of the ECSA IIa subgroup around May 2007, and the 2016 Haitian CHIKV genomes around December 2015. Known CHIKV mutations associated with improved *Ae. albopictus* vector competence were not identified. Isolation of this newly identified lineage from *Ae. albopictus* is of concern, as this vector has a broader geographic range than *Ae. aegypti*, especially in temperate areas of North America, and stresses the importance for continued vector surveillance.

## Introduction

From May through July 2014, a severe outbreak of Chikungunya fever (CF) occurred in Haiti, with almost 65,000 suspected cases reported to the Pan American Health Organization (PAHO) [1]. During this time, our group began a surveillance study in the Gressier region of Haiti within a population of schoolchildren diagnosed with undifferentiated febrile illness [2,3]. In this cohort, *Chikungunya virus* (CHIKV) was detected in 90 plasma specimens

2DReadiness_Armed-2DForces-2DHealth-2DSurveillance-2DBranch_Global-2DEmerging-2DInfections-2DSurveillance-2Dand-2DResponse&d=DwIGaQ&c=pZJPUDQ3SB9JplYbifm4nt2lEVG5pWx2KikqINpWlZM&r=Ve6EldqWejwMbm2ADbtjgM-3vIGcddjo18T0wf_wJys&m=40dKywmWfBLR5r_AQAgJxJdFwsVRiQ3OtIbV5DHxuaE&s=BR-UiD7JZ5QbOa8Ve2-ql_2jTeTWEHZDup9cj6UkuEs&e=), a grant from the National Center for Emerging and Zoonotic Infectious Diseases (CDC) (CDC/HHS U01 CK000510 to JAL), and a grant from the National Institutes of Health to JGM (R01 AI26357-01S1 to JGM, https://urldefense.proofpoint.com/v2/url?u=https-3A__www.niaid.nih.gov_&d=DwIGaQ&c=pZJPUDQ3SB9JplYbifm4nt2lEVG5pWx2KikqINpWlZM&r=Ve6EldqWejwMbm2ADbtjgM-3vIGcddjo18T0wf_wJys&m=40dKywmWfBLR5r_AQAgJxJdFwsVRiQ3OtIbV5DHxuaE&s=mslyvsCDd2Tz_SqkbfnhbHhNsDMOemaw_6piYwkdM1c&e=). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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between May 29 and August 13, 2014. As previously reported, we obtained the complete genomic sequences of 10 CHIKV isolates from these human cases; their viral genomes belonged to the Asian lineage, and remained essentially unchanged during the three-month outbreak [4]. Only rare CF cases have occurred in our study cohort after the 2014 outbreak and the National Public Health Laboratory in Port-au-Prince has reported only two suspected CF cases to PAHO in the intervening time period [5], consistent with cessation of the initial human epidemic.

While our group, and others, have identified the CHIKV strain responsible for the 2014 Caribbean and South American epidemic as being in the Asian clade, there have been isolated reports of identification of the East-Central-South African (ECSA) lineage in Brazil, including identification in association with a localized outbreak in 2014 [6,7], and from patients and a mosquito pool in 2016 [8–10]. The Asian lineage appears to have emerged originally from the ECSA lineage, but quickly adapted to transmission in urban settings, unlike the ECSA lineage that is maintained in a sylvatic cycle and spills-over into the human population causing small localized outbreaks [11,12]. Considering that *Ae. aegypti* primiarly feed on humans, and are commonly found in urban settings, it is not surprising that *Ae. aegypti* is a successful vector of both the Asian and ECSA CHIKV lineages, whereas *Ae. alpopictus*, a forest dweller, is more successful in transmitting the ECSA lineage (11). Adaptive mutations of Asian lineage CHIKV (in the *envelope protein* gene segments 1 and 2, E1 and E2, including E1 T98A and A226V, and E2 L210Q) have also been identified that result in greater infectivity to mosquitoes (*Ae. aegypti* and *Ae. albopictus*) and increased vector competency of *Ae. albopictus* [13,14]. Another mutation, of the opal stop codon at the end of the nsP3 gene, is associated with reduction of arthralgia signs in an animal model, effecting virus pathology [15].

We report here results of screening for CHIKV in mosquito pools collected in Haiti during the 2014 CHIKV epidemic, and again in 2016. Our data document the apparent recent introduction of the "American" ECSA CHIKV lineage IIa into Haiti, and its carriage by *Ae. albopictus*.

## Methods

In 2014, adult *Aedes* mosquitoes were collected using Bio-Gents (BG) Sentinel traps (Bioquip, Rancho Dominguez, CA) within households and courtyards in Gressier/Leogane where children suspected of CHIKV infection resided. The traps were set from 7:00am to 6:00pm for four consecutive days. This work was approved by the University of Florida (UF) and Haitian National IRBs, and residents provided informed consents. In 2016, mosquitoes were also collected using BG Sentinel traps which were set for one day per week for twelve consecutive weeks at eight static locations within a ten mile radius in the commune of Gressier, a semi-rural setting in the Ouest department of Haiti. Trap locations were selected based on environmental considerations, security of traps, and in areas with known human arbovirus-caused illnesses. During both 2014 and 2016 trapping events, trap bags were transported to our UF BSL2-plus field laboratory in Haiti where mosquitoes were frozen at -20˚C, after which they were identified by species (targeting *Ae. aegypti*, *Ae. albopictus*, *or Cx. quinquefasciatus*) and sexed by trained technicians using morphological keys by Leopold Rueda and Walter Reed Biosystematics Unit identification guides [16,17]. Thereafter, the mosquitoes were sorted according to location, collection date, species (*Ae. aegypti*, *Ae. albopictus*, and 'other' with inclusion of *Cx. quinquefasciatus* for 2016 collections), and sex.

The mosquitoes caught in 2014 were pooled for homogenization at the UF field laboratory in Haiti. Each pool contained 1–10 mosquitoes and was tested for CHIKV by molecular methods [18]. Mosquitoes collected in 2016 were stored at -70˚C and shipped on dry ice to the

Lednicky BSL3 laboratory at the Emerging Pathogens Institute at UF for further processing and virus detection and isolation. As these mosquitoes were collected in an area with previous active CHIKV transmission, and due to the possibility that viruses such as *Yellow fever virus* or other BSL3 agents may have been present in the mosquitoes, homogenization and RNA extraction at UF were performed in our BSL3 laboratory.

Mosquitoes from the 2016 collections were homogenized in refrigerated phosphate buffered saline (PBS) with two sizes of very high-density zirconium oxide beads (2mm and 0.1mm, Glen Mills, Clifton, NJ, USA) [19]. The homogenates were centrifuged, and the resulting supernatant halved: (a) one aliquot was placed in lysis buffer to initiate extraction of viral RNA (vRNA) using a Qiagen QIAamp viral RNA mini kit (Qiagen, Germantown, MD, USA), and (b) the remaining supernatant placed in PBS containing trehalose (15% (w/v) final trehalose concentration upon mixing with supernatant) for storage at -80˚C for cryopreservation of virus particles for future isolation attempts in cell cultures. Each pool contained no more than 25 mosquitoes of the same species and sex, from at the same trap location. Extracted nucleic acids were subsequently screened by real-time (rt) RT-PCR for CHIKV, DENV, and ZIKV vRNAs using published protocols [18,20,21].

For the samples collected in 2016, pools that yielded a positive result for CHIKV vRNA were inoculated onto subconfluent (40%) Vero E6 cells in a 75cm$^2$ flask with reduced-serum media and incubated at 37˚C in 5% $CO_2$ for up to 30 days for virus isolation attempts. The inoculated Vero E6 cells were refed every three days. Upon observation of virus-specific cytopathic effects (CPE) throughout 50% of the monolayer, spent media and scraped cells in spent media were collected and again tested by molecular methods for CHIKV, DENV, and ZIKV vRNAs. Additionally, the mosquito species (*Ae*. *aegypti*, *Ae*. *albopictus*, and *Cx*. *quinquefasciatus*) was confirmed in virus-positive pools by published PCR protocols [22–25]. Mosquito pools of 'other' species were not assessed. The homogenate and/or the spent media were used for whole genome sequencing by Sanger sequencing methods as previously reported [26] to obtain complete CHIKV genome sequences.

Pan-genomic alignment comprising of all CHIKV genomes publicly available in GenBank and the two 2016 genomes sequenced in this study were obtained using the MUSCLE algorithm implemented in MEGA7 (http://www.megasoftware.net/) [27–29]. Evidence of recombination was assessed using the set of algorithms implemented in the RDP4 software (http://web.cbio.uct.ac.za/~darren/rdp.html) [30]. Recombinant genomes were excluded from subsequent analyses. Presence of nucleotide substitution saturation was assessed using DAMBE6 (http://dambe.bio.uottawa.ca/DAMBE/) [31] and phylogenetic signal was evaluated using Tree-Puzzle (http://www.tree-puzzle.de/) [32].

Maximum likelihood (ML) phylogenetic inference was performed using the software IQ-TREE package and was based on the best-fit model chosen according to Bayesian Information Criterion [33,34]. UFBoot—Ultrafast Bootstrap (BB) Approximation (2,000 replicates) was chosen to assess statistical robustness for internal branching order in the phylogeny, and strong statistical support along the branches was defined as BB>90% [35].

The presence of temporal signal was assessed using TempEst v1.5 (http://tree.bio.ed.ac.uk/software/tempest) [36]. Time-scaled tree phylogenies were obtained performing Bayesian coalescent inference using BEAST v1.8.4 software package (http://beast.bio.ed.ac.uk), [37,38] testing the constant size demographic model against Bayesian Skyline Plot, [39] and assessing the fit of the strict or uncorrelated lognormal relaxed molecular clock model. Markov chain Monte Carlo samplers were run for 500 million generations and runs with ESS >200 (after 10% burn-in) were considered of proper mixing. The HKY substitution model [40] was used with empirical base frequencies and gamma distribution of site-specific rate of heterogeneity. Best model to fit the data was estimated by marginal likelihood estimates (MLE) obtained

**Table 1. Characteristics of CHIKV-positive mosquito pools, Haiti, 2016.**

| Pool ID | Trap location | Date collected | Mosquito sex | Mosquito species | GenBank accession number |
|---|---|---|---|---|---|
| 16-5-1701 | 5 | May 17 | Female | *Aedes albopictus* | MG000876 |
| 16-5-1931 | 4 | June 27 | Female | *Culex quinquefasciatus* | MG000875 |

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using path sampling and stepping-stone sampling methods [37,41]. The strength of evidence against the null hypothesis ($H_0$) was evaluated via MLE comparison with the more complex model ($H_A$), referred to as they Bayes Factor (BF), wherein *ln*BF<2 indicates no evidence against $H_0$.

## Results

Between May and November 2014, a total of 350 mosquitoes were caught within and around 61 households in the Gressier/Leogane area, and between May and August 2016, 1756 mosquitoes were captured from eight locations in Gressier, Haiti. In rtRT-PCR screens on the year 2014 samples for CHIKV vRNA, 11 (8.9%) of 125 *Ae. aegypti* pools were positive, and none of 24 *Ae. albopictus* pools (p = 0.2. Fishers exact test, two tail). For the year 2016 samples, CHIKV vRNA was identified in 2 (1%) of 171 mosquito pools tested: (a) two female *Ae. albopictus* mosquitoes caught on May 17, 2016, and (b) twenty-three female *Cx. quinquefasciatus* caught on June 27, 2016. No CHIKV were identified in any of the 82 *Ae. aegypti* pools (n = 805 mosquitoes).

Upon culturing the two CHIKV-positive pools from 2016, CHIKV-induced CPE were observed 14 days post-infection of Vero cells inoculated with aliquots of the *Ae. albopictus* and *Cx. quinquefasciatus* homogenates, but not in non-inoculated controls maintained in parallel. The supernatant from both tested positive for CHIKV vRNA by rtRT-PCR, and vRNA purified from each were subsequently used for sequencing, in addition to the vRNA purified directly from mosquito homogenate from the May 17 pool (Table 1). As the mosquitoes had been identified to species by manual inspection, confirmatory testing of mosquito species in the pooled samples was accomplished using the PCR methods devised by Das *et al* [22] and by Smith *et al* [23]. The PCR tests indicated that only *Ae. albopictus* were present in the May 17 pool and only *Cx. quinquefasciatus* in the June 27 pool.

Sequencing analyses revealed that the two isolates did not contain any of the expected mutations in the E1 and E2 regions that contribute to changes in vector competency, nor changes to the opal stop codon. These sequences were highly similar to one another (99%); however, compared to previous CHIKV isolates from Haiti in 2014, the sequences were different, sharing only 93% identity.

All sequenced CHIKV strains cluster into three main lineages: West African, East-Central-South African (ECSA), and the Asian lineage [12]. Based on our tests, no recombination (data not shown) or substitution saturation (S1A Fig) were detected, and likelihood mapping displayed relatively low phylogenetic noise (9.8%) (S1B Fig), indicating that the dataset was optimal for phylogenetic analysis. Our pan-genomic ML phylogenetic analysis of all CHIKV genomes available indicated that the two novel CHIKV genomes obtained from *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes in Haiti in 2016 belong to the ECSA lineage (Fig 1).

Presence of temporal signal that allows for reconstruction of the evolutionary history of the ECSA lineage was assessed (S2 Fig) before performing Bayesian coalescent phylogenetic inference. The time-scaled Maximum Clade Credibility (MCC) phylogeny of the ECSA lineage (Fig 2 and S3 Fig) was inferred using the Bayesian Skyline demographic enforcing an uncorrelated lognormal relaxed clock as determined by model testing (S1 Table). While the 2013–2014

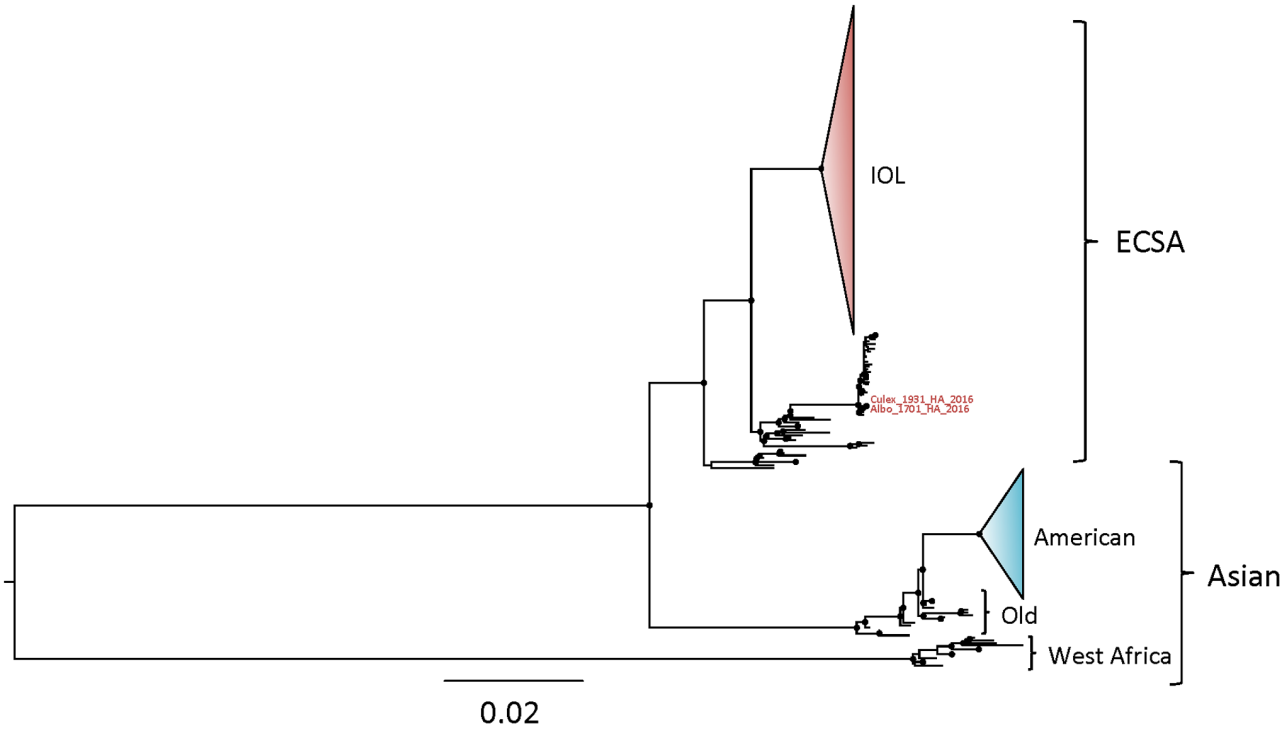


**Fig 1. Pangenomic maximum likelihood phylogenetic inference of CHIKV.** Phylogeny was inferred based on maximum likelihood method using the software IQ-TREE for the complete dataset of publicly available CHIKV genomes. Indian Ocean lineage (IOL), East/Central/South African (ECSA) lineage and Asian lineage are indicated. Branch lengths reflect genetic distances, and diamonds at each node shows strong statistical support based on ultrafast-bootstrap (BB>90%).

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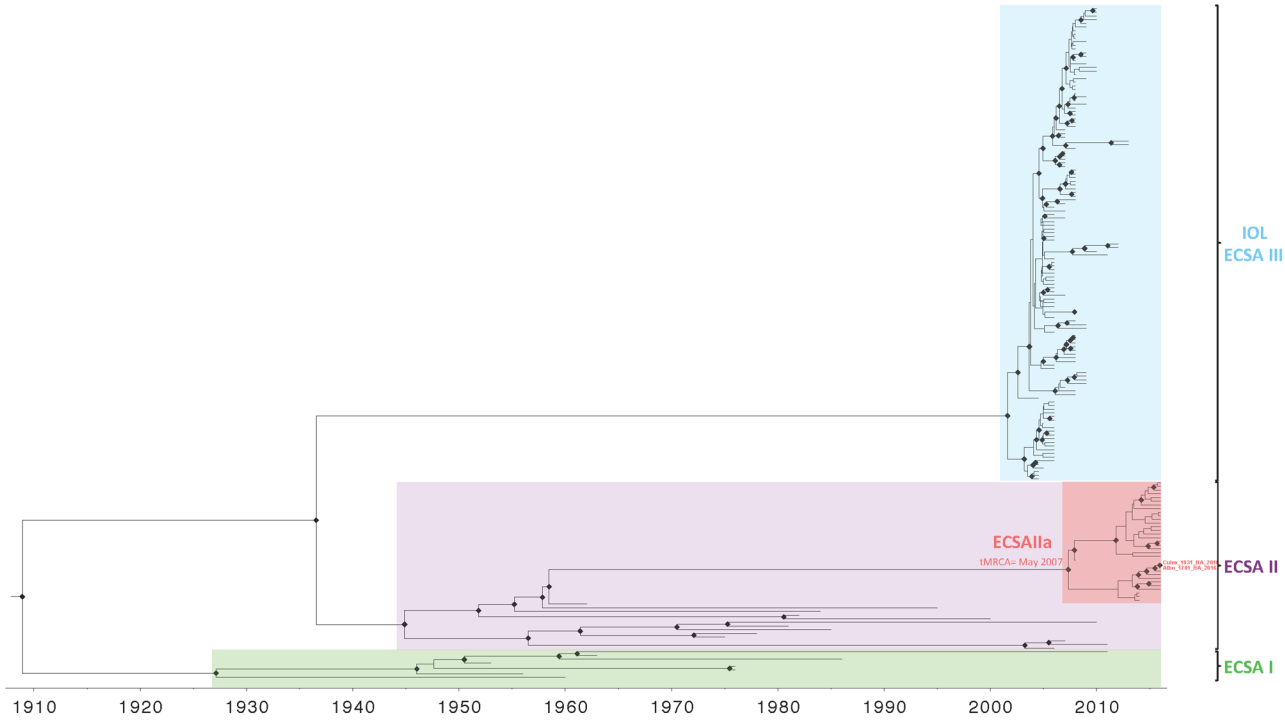


**Fig 2. Maximum clade credibility tree of the ECSA lineages.** Time-scaled phylogenetic maximum clade credibility tree inferred using the Bayesian Skyline demographic enforcing a uncorrelated lognormal relaxed clock implemented in BEAST v1.8.4. Black diamonds represent branches supported by posterior probability >0.90.

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CHIKV outbreak in the Americas aligned with the Asian lineage, our MCC phylogeny shows, in accordance with the ML phylogeny, that the novel CHIKV genomes obtained in Haiti cluster together with strains isolated in Brazil in 2014 [7] that belong to the ECSA lineage (Fig 2 and S3 Fig). The MCC tree portrayed the clear distinction between the subgroups ECSA isolates from Africa (ECSA I and II), and from the Indian Ocean region (IOL) (ECSA III) [42,43], and a new distinct ECSA subgroup IIa (ECSA IIa) arising from the ECSA II lineage (Fig 2 and S3 Fig).

The estimated time of the most recent common ancestor (tMRCA) for the new ECSA IIa subgroup was found to be May 2007 with a 95% highest posterior density (HPD) interval of April 2006 –January 2008. The MCC tree shows presence of two clades within the ECSA IIa lineage, one of which contained both Brazilian and Haitian sequences. The tMRCA for this clade was December 2013 (HPD 95% interval of April 2012 –February 2015). The Haitian strains obtained in this study share a common ancestor that was dated around December 2015 with a HPD 95% interval of October 2015 –January 2016, suggesting recent introduction to the country.

## Discussion

Here we report the first detection of the CHIKV ECSA lineage in Haiti, with our Haitian strains forming a new ECSA subgroup IIa together with CHIKV strains previously reported from Brazil [8,9]. Our molecular clock analysis suggests that this "American" ECSA lineage diverged from the African ECSA lineages sometime in the range of mid-2012-early 2015, within the range of the time period when the major Asian-clade CHIKV epidemic started in the Americas. Our analyses further suggest that the Haiti ECSA lineage IIa strain diverged from the earlier Brazilian strains sometime between October, 2015 and January, 2016, suggesting that it was introduced into Haiti after passage of the main CHIKV Asian clade epidemic. We have previously noted what appears to have been transfer of arbovirus strains between Brazil and Haiti [44,45]; under these circumstances, movement of the ECSA CHIKV strain from Brazil to Haiti in the time period noted would clearly be plausible.

Not unexpectedly, given the massive size of the initial CHIKV epidemic in Haiti and the Caribbean, our 2014 studies documented CHIKV in close to 9% of the *Ae. aegypti* pools sampled. In contrast, we were not able to identify CHIKV in any of the 24 *Ae. albopictus* pools collected in 2014. While numbers are small and differences between rates of *Ae. aegypti* and *Ae. albopictus* identification are not statistically significant, these findings lend credence to the idea that *Ae. aegypti* was the primary vector for the Asian clade epidemic strain. In contrast, in 2016, the two CHIKV ECSA lineage IIa strains identified were from *Ae. albopictus* and *Culex quinquefasciatus*–with no identification in any *Ae. aegypti* pools. Again, numbers are small; however, our findings raise the possibility that *Ae. albopictus*, even in the absence of genetic changes that have been associated with increased *Ae. albopictus* transmission, plays a more important role in transmission of this new clade than does *Ae. aegypti*. This is of potential public health concern, given that *Ae. albopictus* is highly prevalent in the Caribbean and the Americas, with a range that reaches further into temperate regions of the United States than is seen with *Ae. aegypti* [11,46]. While *Cx. quinquefasciatus* was defined as a poor CHIKV vector in one study [47], this concept should also be re-examined in contemporary terms with the viruses in circulation and relevant mosquito subspecies. It is plausible that the CHIKV genome could adapt for enhanced vector to human transmission by *Cx. quinquefasciatus*, particularly as others have detected CHIKV in wild-caught *Cx. quinquefasciatus* [48].

There are some limitations when conducting mosquito surveillance efforts and utilizing wild-caught mosquitoes for virus detection. It is possible that during tests of our mosquito

pools additional CHIKV-positive pools were missed due to the limits of detection by rtRT-PCR. We have found on numerous occasions that virus isolation in cell cultures enhances the ability to identify virus-positive samples when the viral loads are too low for rtRT-PCR, but virus isolation is resource-intensive and impractical for every mosquito pool. To further improve our chances of virus detection, excess PBS is not used during our mosquito homogenization protocol so as not to dilute the concentration of virus in the homogenates, as that negatively impacts downstream applications such as detection by RT-PCR and virus isolation in cultured cells. CHIKV was detected in approximately 1% of all wild-caught mosquito pools identified by species and tested for CHIKV vRNA (2/171 x 100) in the 2016 portion of the study. This detection rate is ten-fold greater than a previous estimate for the natural infection rate for CHIKV in *Ae. aegypti* and *Ae. albopictus* mosquitoes [49], underscoring the potential utility of our approach.

Given the high levels of infection seen with the 2014 CHIKV Asian clade epidemic in the Caribbean and South America, it is unlikely that we will see another major Asian clade epidemic in the near future. However, we are seeing a very different pattern with the CHIKV ECSA lineage IIa strains, with only small numbers of reported cases and localized outbreaks [9,10]. This would be consistent with some level of endemicity in either the vector population or a natural reservoir, possibly within a sylvatic cycle similar to what has been reported in Africa. While there are no nonhuman primates to serve as a CHIKV reservoir in Haiti, other mammals and some birds have been noted as potential reservoirs [50]. Of additional concern, studies of wild-caught mosquitoes have generated evidence of vertical transmission in both *Ae. aegypti* and *Ae. albopictus* [51–53], which indicates that CHIKV can be maintained within the mosquito population until human immunity wanes over time and another outbreak can occur. This, in turn, underscores the importance of continuing vector surveillance and screening for clinical CHIKV infections, to detect possible ongoing endemic infection, outside of epidemic settings.

## Supporting information

**S1 Fig. Substitution saturation and phylogenetic signal for pan-genomic CHIKV dataset.** (A) Scatter plot of nucleotide transition (s) and transversion (v) substitutions over genetic distance measured by TN93 nucleotide substitution model. (B) Likelihood triangle showing supports for each of three alternative topologies (tips), unresolved quartets (center) and partly resolved quartets (edges).
(TIF)

**S2 Fig. Assessment of temporal signal.** The plot represents regression analysis of root-to-tip genetic distance for the ECSA lineage assessed using TempEst v1.5. The positive slope and the correlation coefficient "r" indicate presence of temporal signal for the dataset.
(TIF)

**S3 Fig. ECSA maximum clade credibility tree of the ECSA lineages with tips.** Time-scaled phylogenetic maximum clade credibility tree inferred using the Bayesian Skyline demographic enforcing a uncorrelated lognormal relaxed clock implemented in BEAST v1.8.4. Black diamonds represent branches supported by posterior probability >0.90.
(TIFF)

**S1 Table. Molecular clock and demographic tree prior model comparison.**
(DOCX)

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## Author Contributions

**Conceptualization:** Sarah Keller White, John Glenn Morris, Jr., Bernard A. Okech, John A. Lednicky, James C. Dunford.

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**Writing – review & editing:** Sarah Keller White, Carla Mavian, Marco Salemi, John Glenn Morris, Jr., Maha A. Elbadry, Bernard A. Okech, John A. Lednicky, James C. Dunford.

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