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Chikungunya Virus Sequences across the First Epidemic in Nicaragua, 2014–2015

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Abstract. Chikungunya is caused by the mosquito-borne arthrogenic alphavirus, chikungunya virus (CHIKV). Chikungunya was introduced into the Americas in late 2013 and Nicaragua in mid-2014. Here, we sequenced five imported and 30 autochthonous Nicaraguan CHIKV from cases identified in the first epidemic in the country between August 2014 and April 2015. One full-length and two partial genomic sequences were obtained by deep sequencing; Sanger methodology yielded 33 *E1* sequences from five imported and 28 autochthonous cases. Phylogenetic analysis indicates that Nicaraguan CHIKV all belonged to the Asian genotype, Caribbean clade. Moreover, *E1* gene sequences revealed accumulation of mutations in later months of the epidemic, including four silent mutations in 11 autochthonous cases and three non-synonymous mutations in three autochthonous cases. No mutations contributing to increased transmissibility by *Aedes albopictus* were identified in the *E1* gene. This represents the most comprehensive set of CHIKV sequences available from the Americas to date.

Chikungunya is a reemerging infectious disease caused by a mosquito-borne arthrogenic alphavirus, chikungunya virus (CHIKV). CHIKV is an enveloped virus with a 12-kb positive-sense RNA genome that contains a 5' untranslated region (UTR) followed by nonstructural protein genes (*NS1–NS4*), structural protein genes (*C*, *E3*, *E2*, *6K*, and *E1*) under the control of a subgenomic promoter, and a 3' UTR including a terminal poly-A tail. Often initially mistaken for dengue, chikungunya manifests clinically as fever and arthralgia involving intense pain and inflammation in joints typically lasting weeks or months but sometimes years.¹ Other symptoms may include headache, muscle pain, or rash. Currently there is no licensed vaccine to prevent or medicine to treat CHIKV infection. Areas endemic for chikungunya include Africa and southeast Asia.² Since 2004, CHIKV has expanded into the Indian and Pacific Oceans and Europe, (<http://www.who.int/mediacentre/factsheets/fs327/en/>), and since the end of 2013, it has caused explosive epidemics in the Caribbean and Latin America, with imported cases and some autochthonous transmission reported in the United States.^{3,4} However, limited numbers of CHIKV genomes are available from the Americas, despite the recent epidemic.^{4,5}

CHIKV can be classified into three distinct genotypes: Asian, East/Central/South African (ECSA), and western African.^{2,4} Viral sequences from St. Martin Island, the point of introduction in the Americas, belonged to the CHIKV Asian genotype.⁴ Chikungunya is generally spread through the bite of *Aedes aegypti* mosquitoes, but transmission by *A. albopictus* has been reported.^{6–8} During the Reunion Island outbreak in 2005–2006, a single mutation, A226V, in the structural *E1* gene of certain ECSA strains enhanced CHIKV transmissibility by *Ae. albopictus*, potentially aiding in the spread of CHIKV in unprecedented regions.^{9,10} In July 2014, CHIKV was introduced into Nicaragua. Here, we sequenced CHIKV from imported and autochthonous Nicaraguan cases to determine the

origin of CHIKV strains circulating in Nicaragua, and we analyzed the viral envelope *E1* gene for any variation that could provide insight into the introduction and dissemination of CHIKV across time in Nicaragua.

In Nicaragua, the first imported case was identified on July 9, 2014, and the first autochthonous case on September 23, 2014. Chikungunya cases were identified through a national surveillance program implemented by the Ministry of Health and two ongoing pediatric studies conducted in Managua, the capital city of Nicaragua: the Pediatric Dengue Cohort Study,¹¹ a community-based cohort ongoing since 2004, and the Hospital-based Dengue Study, based at the National Pediatric Reference Hospital, ongoing since 1998.¹² CHIKV testing was included in both studies in 2014. The studies were approved by the University of California, Berkeley, and Nicaraguan Ministry of Health institutional review boards. All samples were tested using CHIKV-specific real-time reverse-transcription polymerase chain reaction (RT-PCR) (Supplemental Methods). A total of 35 CHIKV-positive samples from five imported and 30 autochthonous cases, spanning the period from August 2014 to April 2015, were sequenced (Table 1). Four samples were sequenced directly from serum and for the rest, CHIKV was isolated in Vero cells and then sequenced (Table 1). Supplemental Table 1 lists detailed information about each strain sequenced, including the sample type (isolate or serum), case type (imported or autochthonous), source (Nicaraguan national surveillance, cohort study, or hospital study), date of collection, and mutations identified.

To obtain complete genomic sequence information and determine the origin of CHIKV strains circulating in Nicaragua, whole-genome amplification of viral RNA from a subset of samples (three serum samples from autochthonous cases in October 2014), combined with Nextera technology, was used to generate libraries for deep sequencing (Supplemental Methods). In brief, complementary DNA for each sample was synthesized using random hexamers and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and then amplified using multiple displacement amplification with phi29 DNA Polymerase (New England Biolabs, Ipswich, MA). Amplified DNA was purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA) and prepared for high-throughput

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TABLE 1
Nicaraguan CHIKV samples used in this study

Total	Case type*	Types of sample	Source	August 2014	October 2014	November 2014	December 2014	January 2015	February 2015	March 2015	April 2015
Nicaraguan CHIKV samples (35)	Imported (5)	Virus isolates (5)	National surveillance (5)	5	–	–	–	–	–	–	–
			Pediatric cohort study (13)	–	3	3	2	1	2	1	1
	Autochthonous (30)	Virus isolates (26)	Pediatric hospital study (13)	–	–	2	5	2	4	–	–
			Serum samples (4)	National surveillance (2)	–	2	–	–	–	–	–
			Pediatric cohort study (2)	–	2	–	–	–	–	–	–

CHIKV = chikungunya virus.

*Autochthonous case: if the individual had not traveled; imported case: if the patient returned ill from a country with a current CHIKV epidemic.

sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA). The libraries were pooled in equimolar ratios and sequenced on the HiSeq2000 sequencer (Illumina) to generate 100-bp reads. Reads for each sample were mapped to full-length CHIKV sequences from the National Center for Biotechnology Information (NCBI), using the “Bowtie2” software.¹³ “Samtools”¹⁴ and in-house Python (Python Software Foundation, Beaverton, OR; <http://www.python.org>) scripts were used to generate pileups and consensus nucleotide sequences.

Complete full-length sequence from one individual (Nicaragua/11540/KT192707/2014) (Figure 1A) was obtained, along with partial genome sequences from two other samples (Nicaragua/4916/NA/2014 and Nicaragua/11519/NA/2014; data not shown) because of lower-than-expected coverage across the CHIKV genome. Two of the sequences cover the NSP3 region (Nicaragua/11540/KT192707/2014 and Nicaragua/4916/NA/2014), and both share the same four amino acid deletion in NSP3 previously reported in Indonesia/0706aTw/FJ807897/2007 (Figure 1A) and related Asian genotype strains.¹⁶

Phylogenetic analysis was performed to identify the origin of the Nicaraguan CHIKV. Full-length genomic nucleotide sequences of Nicaragua/11540/KT192707/2014 and 36 reference strains were available from GenBank and the European Virus Archive. Nicaragua/11540 is a sample from October 2014 (first month of autochthonous cases used in the study); thus, it is representative of CHIKV strains circulating early in the epidemic in Nicaragua. FASTA format alignment was performed by MAFFT FFT-NS-2 (v7.221). The evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model.¹⁷ The tree with the highest log likelihood is shown in Figure 1A. Initial trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach.

Our phylogenetic analysis identified that Nicaraguan strains belong to the Caribbean clade⁵ of the Asian genotype, most similar to BritishVirginIslands/99659/KJ451624/2014, St.Martin/H20235/NA/2013, and St.Martin/H20236/NA/2013 (Figure 1A).

To investigate the molecular evolution of CHIKV through its emergence and spread in Nicaragua, primers for Sanger sequencing were designed according to BritishVirginIslands/99659/KJ451624/2014 to amplify and sequence the *E1* gene (Supplemental Table 2). The PCR product of ~2.4 kb was sequenced using Sanger methodology (Supplemental Methods).

The Sanger methodology yielded sequences from five imported CHIKV cases from August 2014 and 28 autochthonous

cases from October 2014 to April 2015, including one already sequenced by deep sequencing (Nicaragua/11540/KT192707/2014) (Supplemental Table 1). For sample Nicaragua/11540, *E1* sequences obtained from full-genome deep sequencing and Sanger methodology were 100% identical. *E1* sequences from all five imported cases (August 2014) and five autochthonous cases (October 2014) from the early months of the epidemic shared 100% identity with the BritishVirginIslands/99659/KJ451624/2014 reference genome (Supplemental Table 1). However, later in the epidemic season (November 2014 to April 2015), 14 of the 23 autochthonous cases showed silent mutations at four sites (T10080C/E1-L33, G10665A/E1-T228, T11076C/E1-F365, or G11130T/E1-P383) or non-synonymous mutations at three sites (C10415T/E1-A145V, A10465G/E1-I162V, or G10852A/E1-V291I), with nucleotide numbering according to BritishVirginIslands/99659/KJ451624/2014 (Figure 1B, Supplemental Table 1). Accumulation of substitutions over the course of an epidemic is not surprising. Of the mutations in *E1* gene that appeared in later months of the epidemic, five mutations (T10080C/E1-L33 silent, C10415T/E1-A145V, A10465G/E1-I162V, G10665A/E1-T228 silent, and G10852A/E1-V291I) appeared only once in certain samples, whereas the other two mutations (G11130T/E1-P383 and T11076C/E1-F365) were identified in several cases. G11130T/E1-P383 appeared five times, spanning sequences from cases from November 2014 to February 2015, two of which also contained a second mutation (either C10415T/E1-A145V or G10852A/E1-V291I). T11076C/E1-F365 appeared in four samples spanning cases from November 2014 to April 2015, as can be seen in the small branch in Figure 1B. Interestingly, this mutation appears in numerous distinct *E1* sequences, including several from Brazil in 2014.¹⁸ It has not yet been determined whether these genetic variations play a role in viral infectivity or transmission, and further studies are needed to investigate their implications. Furthermore, consistent with other Asian genotype viruses, all 35 Nicaraguan samples sequenced thus far contain E1-A226 and E1-T98¹⁹ and therefore do not display the mutations that have been associated with adaptation to *Ae. albopictus* (data not shown). It should be noted that Figure 1B is intended to demonstrate the Caribbean origin of the Nicaraguan samples and the extent of mutations accumulated, but not the phylogenetic relationship among these Nicaraguan strains, given the limited number of mutations identified.

In summary, Nicaraguan CHIKV samples (five imported and 30 autochthonous cases) from national surveillance and two ongoing pediatric studies from the first epidemic in the country were sequenced. One full-length sequence obtained

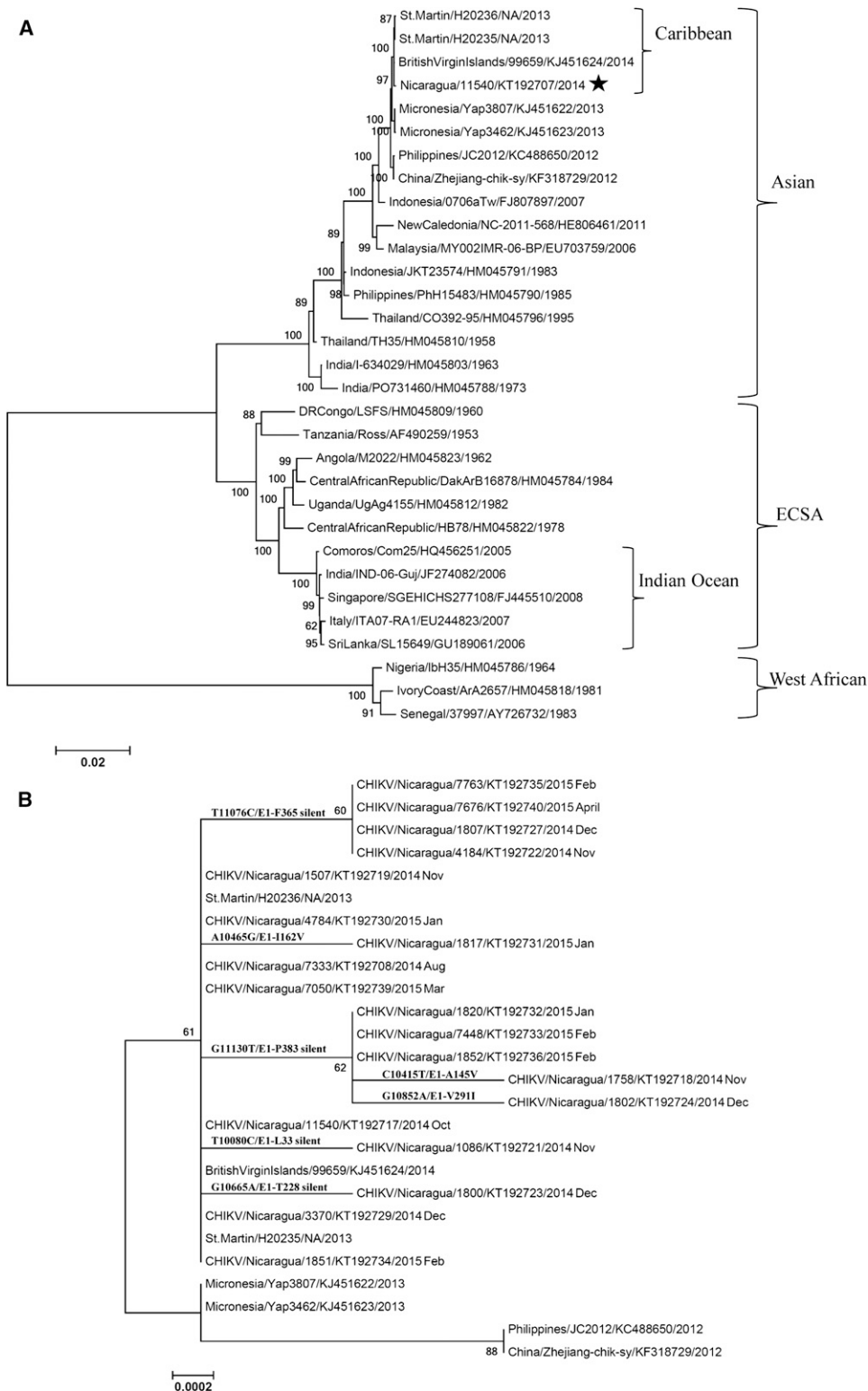


FIGURE 1. Phylogenetic analysis of Nicaraguan chikungunya viruses (CHIKV). (A) Nicaraguan CHIKV belongs to the Caribbean clade of the Asian genotype. Phylogenetic analysis included 37 full-length genomic nucleotide sequences (one Nicaraguan strain and 36 reference strains), with the format of country/strain/GenBank no./year. The three major genotypes and two clades are labeled. Bootstrap values are shown on major branches. (B) Mutations in the *E1* gene of Nicaraguan CHIKV. This analysis included *E1* gene sequences of 19 representative monthly Nicaraguan CHIKV strains, with the format of country/strain/GenBank no./year month, and *E1* sequences of seven reference strains in the format of country/strain/GenBank no./year. For simplicity, each month only one sample identical to and all the samples different from the BritishVirginIslands/99659/KJ451624/2014 strain were included in the subtree, resulting in elimination of 14 duplicate identical Nicaraguan CHIKV sequences from the subtree. The relevant mutations are indicated at each branch point. Both trees are drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are shown on major branches. All positions containing gaps and missing data were eliminated. Phylogenetic analyses were conducted in MEGA6.¹⁵

by deep sequencing demonstrated the origin of the Nicaraguan strains to be the Caribbean clade⁵ of the Asian genotype, containing the four amino acid deletion in NSP3.¹⁶ Sanger methodology yielded 33 *E1* sequences from five imported cases from August 2014 and 28 autochthonous cases from October 2014 to April 2015. The imported cases were observed in August 2014 (the first month of epidemic included in this study), with *E1* sequences 100% homologous to the British Virgin Islands/99659/KJ451624/2014 strain. Beginning in October 2014, all cases used in this study were autochthonous, with either 100% homology to the British Virgin Islands/99659/KJ451624/2014 strain or with accumulation of mutations (starting November 2014). The accumulated mutations included four silent mutations in 11 autochthonous cases and three non-synonymous mutations (E1-A145V, E1-I162V, or E1-V291I) in three autochthonous cases. All 35 sequences contained E1-A226 and E1-T98 mutations, and thus do not display the mutations suggestive of enhanced CHIKV transmissibility in *Ae. albopictus*.¹⁹ It should be noted that E1-A226V alone only affects increased infectivity and transmissibility in *Ae. albopictus* when it occurs in the ECSA lineage but not in the Asian lineage strains unless accompanied by additional mutations.²⁰ Altogether, this study represents the most comprehensive set of CHIKV sequences available from the Americas to date.

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