Molecular Characterization of Chikungunya Virus, Philippines, 2011–2013

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During 2011–2013, a nationwide outbreak of chikungunya virus infection occurred in the Philippines. The Asian genotype was identified as the predominant genotype; sporadic cases of the East/Central/South African genotype were detected in Mindanao. Further monitoring is needed to define the transmission pattern of this virus in the Philippines.

Chikungunya fever is a mosquitoborne infection that causes large outbreaks mainly in tropical and subtropical countries. The causative agent is chikungunya virus (CHIKV), an enveloped, single-stranded positive-sense RNA virus (family *Togaviridae*, genus *Alphavirus*). Phylogenetic analysis of the E1 gene of CHIKV revealed 3 genotypes: West African, East/Central/South African (ECSA), and Asian (*1*). In 2005, large outbreaks occurred in the islands in the Indian Ocean and India that were caused by the Indian Ocean lineage (IOL) virus, which newly emerged from the ECSA genotype (*2*). More recently, the emergence and potential spread of ECSA and Asian genotypes in the Americas have been major public health concerns in the region. (*3*).

In the Philippines, CHIKV was first isolated in 1965 (*4*). Since then, sporadic cases have been reported, including those among US Peace Corps volunteers stationed in the islands of Mindanao, Cebu, and Masbate in 1986 (*5*), and a local community outbreak in Cavite, Luzon Island, was reported in 1996 (*6*). However, the first nationwide CHIKV outbreak was identified starting in 2011 (Philippines Department of Health, unpub. data). Previous studies of chikungunya fever in the Philippines focused on clinical and serologic analyses (*7*), and recent reports on the molecular surveillance of CHIKV in the country were limited and analyzed only samples collected in 2012 (*8*,*9*). We conducted genetic analysis to characterize recent CHIKV infections that caused a large nationwide outbreak in the Philippines during 2011–2013.

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The Study

Serum samples were collected through the chikungunya fever surveillance under the Philippine Integrated Disease Surveillance and Response of the Department of Health Epidemiology Bureau from different provinces in the Philippines during 2011–2013. Samples were collected from patients suspected to have chikungunya fever manifesting with symptoms such as fever, rash, and arthralgia. Samples were sent to the Research Institute for Tropical Medicine, which serves as the National Reference Laboratory for Dengue and Other Arboviruses. We screened serum samples by using CHIKV IgM-capture ELISA (NovaLisa, NovaTec Immundiagnostica GmbH, Dietzenbach, Germany). We extracted viral RNA using the QIAamp Viral RNA kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and amplified the partial E1 gene using 1-step reverse transcription PCR followed by direct Sanger sequencing with primers as previously described (*10*,*11*). We conducted phylogenetic analysis using the maximum-likelihood method as implemented in MEGA 6 software (http://www.megasoftware.net/). Molecular clock analysis and Bayes factor calculation were performed using BEAST software 1.8.0 (http://beast.bio.ed.ac.uk/) to select the best migration event model of CHIKV among countries. Bayes factor analysis was used to test phylogeographic hypothesis whether posterior migration rate between locations in whole evolutionary history was significantly higher than the expected prior migration rate, assuming truncated Poisson probability (*12*).

A total of 5,729 serum samples were collected from persons suspected to have chikungunya within 5 days after symptom onset. Fever, rash, and arthralgia were the most common symptoms (53%, 47%, and 34% of patients, respectively). Of the 5,729 serum samples, 2,891 were IgM positive by ELISA. We conducted reverse transcription PCR on 382 representative samples among IgM-negative patients in accordance with the CHIKV outbreak surveillance strategy of the Philippines, of which 131 samples tested positive. Partial E1 gene sequence (733 nt) was obtained from 31 samples. Sequences were submitted to GenBank (accession nos. LC064714–LC064744).

Phylogenetic analysis identified 28 Asian genotype viruses and 3 ECSA genotype viruses (Figure 1). Sequence

[1]These authors contributed equally to this article.

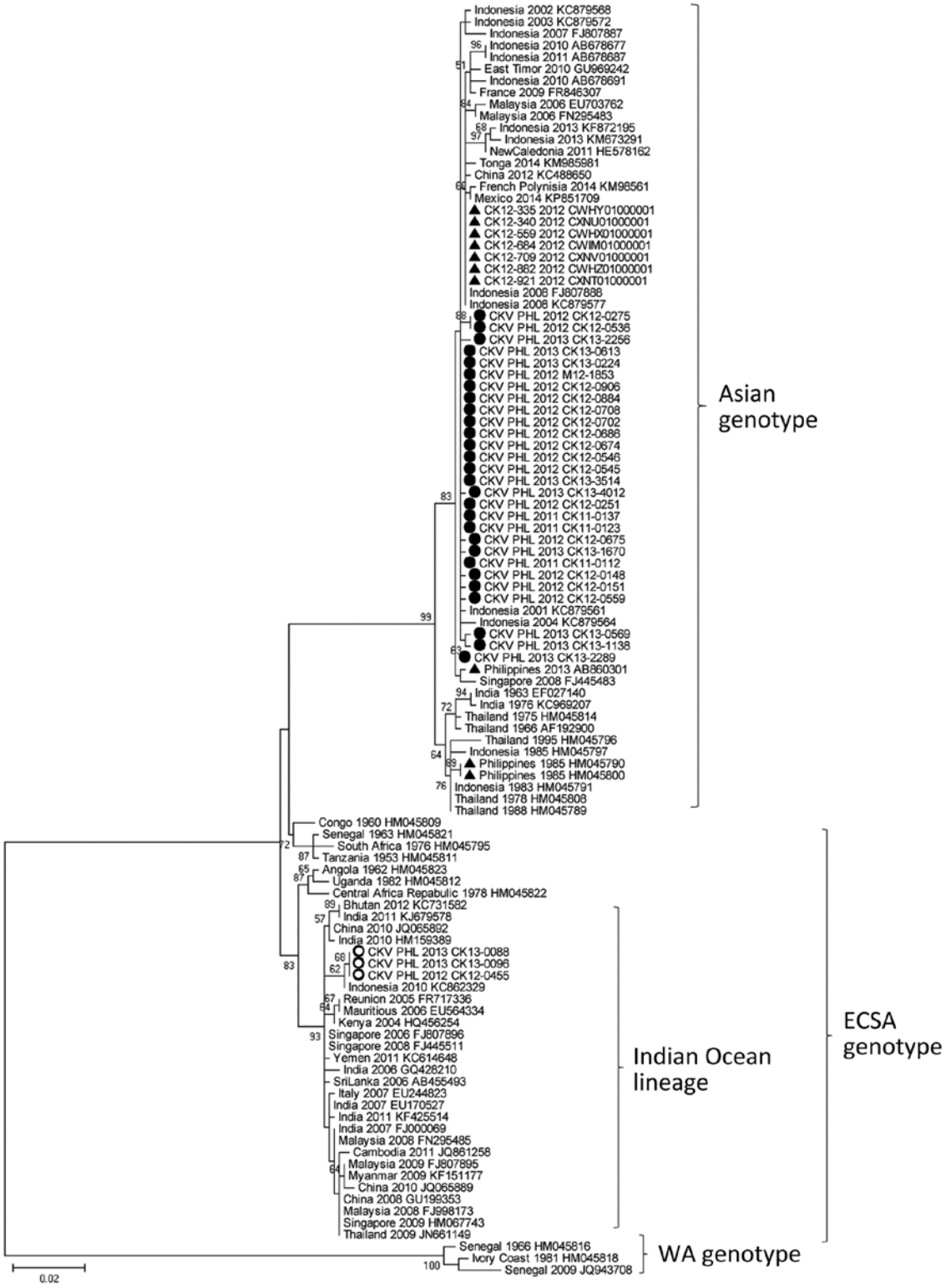

Figure 1. Phylogenetic analysis of partial (733 nt) E1 gene of 31 CHIKVs detected in the Philippines in this study during 2011–2013 compared with 77 global strains. The tree was constructed using maximum-likelihood method with the Kimura 2-parameter model and 1,000 bootstrap replications. Bootstrap values >50% are indicated on the branches of the tree. Black circles indicate Asian genotypes; open circles indicate ECSA genotypes analyzed in this study; triangles indicate reference strains collected in the Philippines. CHIKV, chikungunya virus; ECSA, East/Central/South African, WA, West African. Scale bar indicates nucleotide substitutions per site.

Table. Bayes factor of migration events of Asian genotype of chikungunya virus based on 65 cases, Philippines, 2011–2013*

Bayes factor	Locations
68.8	Philippines, Indonesia
68.0	India, Thailand
54.2	Indonesia, New Caledonia
25.6	French Polynesia, Mexico
22.0	France, Indonesia
19.5	Indonesia, Malaysia
11.3	East Timor, Indonesia
7.6	Indonesia, Singapore
6.8	Indonesia, Thailand
3.4	China, Tonga
3.3	China, Indonesia
2.3	East Timor, France
2.3	Philippines, Thailand
2.2	Mexico, Tonga
2.1	French Polynesia, Tonga
2.1	China, French Polynesia
2.0	Philippines, Tonga
1.9	China, Mexico
1.7	China, Philippines
1.3	Malaysia, Singapore

*The migrations of Asian genotype viruses between countries were determined by using Bayes factor analysis. Bayes factor value >5 is considered a significant migration.

analysis of E1 gene showed >99% nt similarity among 28 Asian genotype viruses (data not shown). Bayes factor analysis for the migration events also showed that migration might have occurred between Indonesia and Philippines with high probability (Table). CHIKV-positive patients were found only in Mindanao Island in 2011 but were detected in other parts of the country in 2012 and 2013. All CHIKV detected in parts of the country other than Mindanao were identified as Asian genotype.

The 3 ECSA genotype viruses were detected in the Davao Del Sur and Davao Oriental in Mindanao Island in 2012 and 2013, and all were clustered into IOL (Figures 1, 2). This finding indicates that the 2 genotypes co-circulated in the island during the outbreak. ECSA genotype viruses in the Philippines were closely related to the Indonesian viruses (GenBank accession no. KC862329) detected in 2010. Sequencing analysis showed that all Philippine ECSA viruses possess the alanine to valine substitution (A226V) in the E1 gene (online Technical Appendix Tables 1, 2, http://wwwnc.cdc.gov/EID/article/22/5/15-1268-Techapp1.pdf).

Conclusions

The recent large CHIKV outbreaks in other Asian countries were caused mainly by the IOL of ECSA genotype (*2*). However, the 2011–2013 outbreak in the Philippines was caused mainly by the Asian genotype; the reason that this large outbreak was caused by this genotype is unknown. Previous reports have confirmed the reemergence of Asian genotype viruses in the Philippines (*8,9*). In this study, phylogenetic analysis and Bayes factor calculation showed that the Philippines viruses were closely related to Indonesian viruses, which might explain why the outbreak started in southern Mindanao, near Indonesia. Although sequence data of CHIKV in the database are not enough to identify the exact origin of the virus, we tried to estimate when CHIKV was introduced into the Philippines with molecular clock analysis using the dataset of Asian genotype viruses (online Technical Appendix Figures 1, 2). The results suggested that circulating Asian genotype viruses in the Philippines were introduced from Indonesia before 2010.

We also detected IOL of ECSA genotype, which possesses the E1-A226V substitution, and its co-circulation with the Asian genotype in the Philippines. However, the geographic distribution of ECSA genotype in 2012 and 2013 was limited to southern provinces in Mindanao. After the large outbreak in the Indian Ocean region in 2005, IOL of ECSA genotype rapidly spread to Asian countries and then co-circulated with the endemic Asian viruses, then eventually became the predominant genotype (*13*). However, this circulation pattern differs from what we observed in the Philippines. Until the early 2010s, most of the viruses

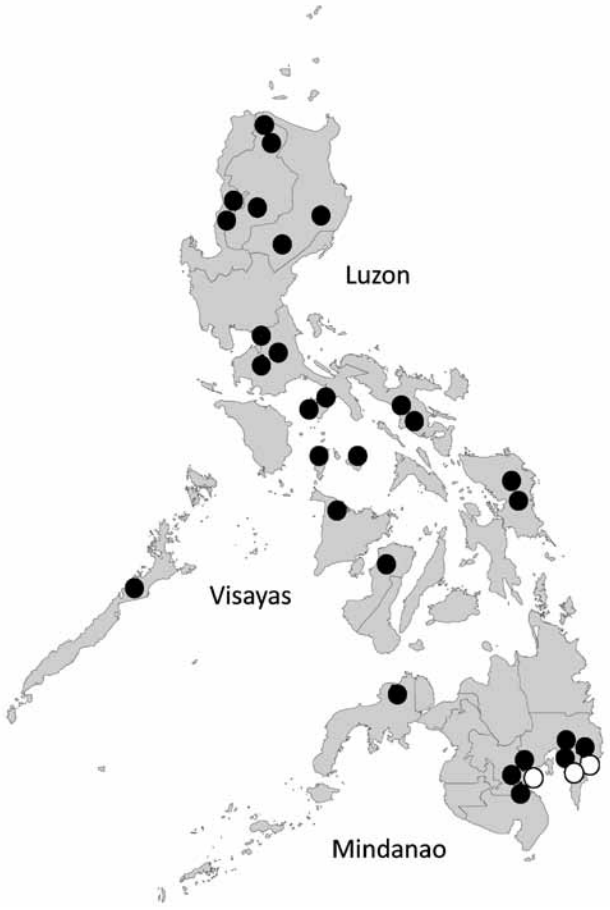

Figure 2. Geographic distribution of CHIKV genotypes in the Philippines. The location of samples collected in this study are indicated by circles; 1 circle represents 1 sample. Black circles indicate Asian genotype; white (open) circles indicate East/Central/South African genotype. CHIKV, chikungunya virus.

circulating in the Philippines and Indonesia were still Asian genotype, and ECSA genotype viruses had been reported in West Kalimantan, Indonesia, in 2011 (*14*) and in the Philippines in 2012 several years after the large outbreak in the Indian Ocean region (*2*). The movement of persons near the border of these countries might play a key role in CHIKV transmission.

As part of the national vector control program, surveillance of *Aedes aegypti* and *Ae. albopictus* mosquitoes has been conducted in several areas in the Philippines. A previous report showed that the proportion of these 2 mosquito species was almost the same in Metro Manila (*15*). If the proportion of *Ae. albopictus* mosquitoes increases, the ESCA genotype virus with A226V mutation could spread more rapidly in the country. Thus, monitoring the spread of ECSA genotype viruses and the proportion of the *Aedes* mosquitoes in the Philippines is important.

We have demonstrated that the Asian genotype CHIKV, which is closely related to the Indonesian viruses, was identified in Mindanao in 2011 and spread to other regions in 2012 and 2013. Like the Asian genotype, ECSA genotype virus was first detected in Mindanao in 2012. Mindanao might play a key role for the introduction of the CHIKV into the Philippines. Further monitoring is necessary to define the transmission pattern of CHIKV, including cross-border transmission.

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Molecular Characterization of Chikungunya Virus, Philippines, 2011–2013

Technical Appendix

Technical Appendix Table 1. Chikungunya viruses newly sequenced in this study, Philippines, 2011–2013, n = 31

Strain name	GenBank accession no.	Genotype	Amino acid at position 226	Year collected	Month collected	Country	Region	Province
CKV_PHL_2013_CK13–0613	LC064714	Asian	Alanine	2013	July	Philippines	CAR	Ifugao
CKV_PHL_2013_CK13–2256	LC064715	Asian	Alanine	2013	Apr	Philippines	CAR	Apayao
CKV_PHL_2012_CK12–0151	LC064716	Asian	Alanine	2012	Nov	Philippines	NCR	NCR
CKV_PHL_2013_CK13–1138	LC064717	Asian	Alanine	2013	Nov	Philippines	1	LaUnion
CKV_PHL_2013_CK13–2289	LC064718	Asian	Alanine	2013	Aug	Philippines	1	Ilocos Sur
CKV_PHL_2012_CK12–0559	LC064728	Asian	Alanine	2012	Oct	Philippines	2	Isabela
CKV_PHL_2013_CK13–1670	LC064729	Asian	Alanine	2013	July	Philippines	2	Quirino
CKV_PHL_2013_CK13–3514	LC064730	Asian	Alanine	2013	Aug	Philippines	2	CagayanValley
CKV_PHL_2012_CK12–0148	LC064731	Asian	Alanine	2012	Aug	Philippines	4A	Laguna
CKV_PHL_2012_CK12–0251	LC064732	Asian	Alanine	2012	Aug	Philippines	4A	Laguna
CKV_PHL_2012_CK12–0545	LC064733	Asian	Alanine	2012	Oct	Philippines	4B	Romblon
CKV_PHL_2012_CK12–0546	LC064734	Asian	Alanine	2012	Dec	Philippines	4B	Romblon
CKV_PHL_2012_CK12–0702	LC064735	Asian	Alanine	2012	Nov	Philippines	4B	Marinduque
CKV_.PHL_2012_CK12–0708	LC064736	Asian	Alanine	2012	Nov	Philippines	4B	Marinduque

Strain name	GenBank accession no.	Genotype	Amino acid at position 226	Year collected	Month collected	Country	Region	Province
CKV_PHL_2012_M12–1853	LC064737	Asian	Alanine	2012	July	Philippines	4B	Palawan
CKV_PHL_2012_CK12–0674	LC064738	Asian	Alanine	2012	Oct	Philippines	5	Albay
CKV_PHL_2012_CK12–0675	LC064739	Asian	Alanine	2012	Jun	Philippines	5	Albay
CKV_PHL_2013_CK13–0569	LC064740	Asian	Alanine	2013	July	Philippines	6	Negros Occidental
CKV_PHL_2013_CK13–4012	LC064741	Asian	Alanine	2013	Nov	Philippines	6	Aklan
CKV_PHL_2012_CK12–0884	LC064742	Asian	Alanine	2012	Dec	Philippines	8	Samar
CKV_PHL_2012_CK12–0906	LC064743	Asian	Alanine	2012	Feb	Philippines	8	Samar
CKV_PHL_2013_CK13–0224	LC064744	Asian	Alanine	2013	July	Philippines	9	Zamboanga Del Norte
CKV_PHL_2011_CK11–0112	LC064719	Asian	Alanine	2011	Oct	Philippines	11	Davao Del Sur
CKV_PHL_2011_CK11–0123	LC064720	Asian	Alanine	2011	Oct	Philippines	11	Davao Del Sur
CKV_PHL_2011_CK11–0137	LC064721	Asian	Alanine	2011	Sept	Philippines	11	Davao Del Sur
CKV_PHL_2012_CK12–0275	LC064722	Asian	Alanine	2012	Oct	Philippines	11	CompostellaValley
CKV_PHL_2012_CK12–0536	LC064724	Asian	Alanine	2012	Nov	Philippines	11	CompostellaValley
CKV_PHL_2012_CK12–0686	LC064725	Asian	Alanine	2012	Oct	Philippines	11	CompostellaValley
CKV_PHL_2012_CK12–0455	LC064723	ECSA	Valine	2012	Jan	Philippines	11	Davao Del Sur
CKV_PHL_2013_CK13–0088	LC064726	ECSA	Valine	2013	Jan	Philippines	11	Davao Oriental
CKV_PHL_2013_CK13–0096	LC064727	ECSA	Valine	2013	May	Philippines	11	Davao Oriental

*CAR, Cordillera Administrative Region; ECSA, East/Central/South African; NCR, National Capital Region.

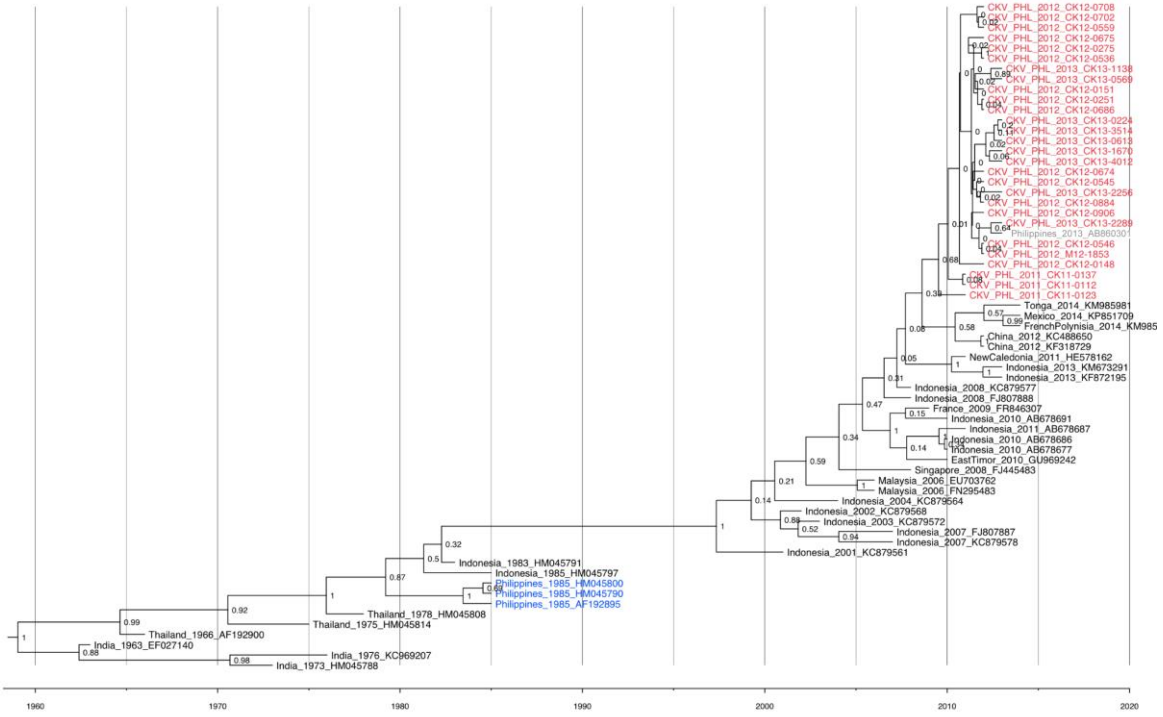
Technical Appendix Table 2. Reference strains obtained from GenBank used in this study,* n = 77

Country	Year collected	GenBank accession no.	Genotype
Angola	1962	HM045823	ECSA
Bhutan	2012	KC731582	ECSA
Cambodia	2011	JQ861258	ECSA
Central African Republic	1978	HM045822	ECSA
China	2008	GU199353	ECSA
China	2010	JQ065889	ECSA
China	2010	JQ065892	ECSA
China	2012	KC488650	Asian
Congo	1960	HM045809	ECSA
East Timor	2010	GU969242	Asian
France	2009	FR846307	Asian
French Polynesia	2014	KM98561	Asian
India	1963	EF027140	Asian
India	1976	KC969207	Asian
India	2006	GQ428210	ECSA
India	2007	EU170527	ECSA
India	2007	FJ000069	ECSA
India	2010	HM159389	ECSA
India	2011	KF425514	ECSA
India	2011	KJ679578	ECSA
Indonesia	1983	HM045791	Asian
Indonesia	1985	HM045797	Asian
Indonesia	2001	KC879561	Asian
Indonesia	2002	KC879568	Asian
Indonesia	2003	KC879572	Asian
Indonesia	2004	KC879564	Asian
Indonesia	2007	FJ807887	Asian
Indonesia	2008	FJ807888	Asian
Indonesia	2008	KC879577	Asian
Indonesia	2010	AB678677	Asian
Indonesia	2010	AB678691	Asian
Indonesia	2010	KC862329	ECSA
Indonesia	2011	AB678687	Asian
Indonesia	2013	KF872195	Asian
Indonesia	2013	KM673291	Asian
Italy	2007	EU244823	ECSA

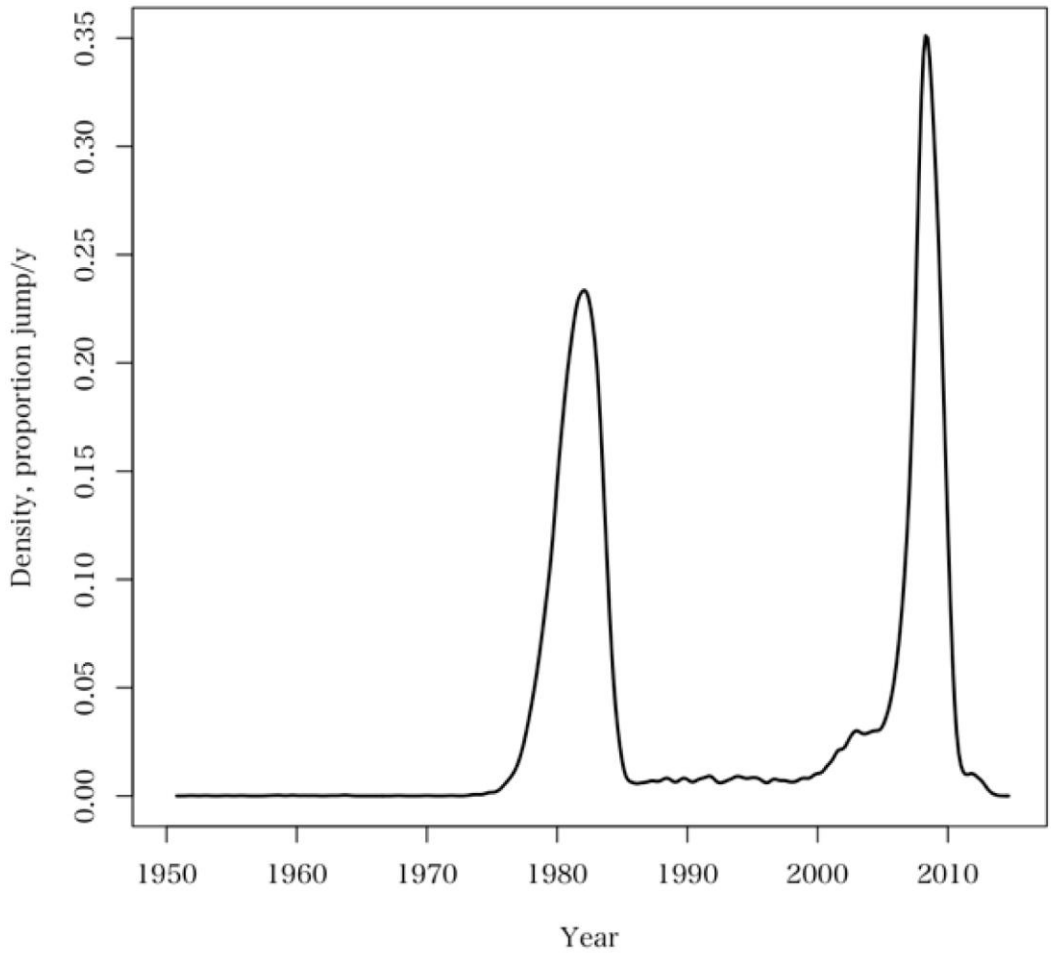
Country	Year collected	GenBank accession no.	Genotype
IvoryCoast	1981	HM045818	WA
Kenya	2004	HQ456254	ECSA
Reunion	2005	FR717336	ECSA
Malaysia	2006	EU703762	Asian
Malaysia	2006	FN295483	Asian
Malaysia	2008	FJ998173	ECSA
Malaysia	2008	FN295485	ECSA
Malaysia	2009	FJ807895	ECSA
Mauritious	2006	EU564334	ECSA
Mexico	2014	KP851709	Asian
Myanmar	2009	KF151177	ECSA
New Caledonia	2011	HE578162	Asian
Philippines	1985	HM045790	Asian
Philippines	1985	HM045800	Asian
Philippines	2012	CWHY01000001	Asian
Philippines	2012	CXNU01000001	Asian
Philippines	2012	CWHX01000001	Asian
Philippines	2012	CWIM01000001	Asian
Philippines	2012	CXNV01000001	Asian
Philippines	2012	CWHZ01000001	Asian
Philippines	2012	CXNT01000001	Asian
Philippines	2013	AB860301	Asian
Senegal	1963	HM045821	ECSA
Senegal	1966	HM045816	WA
Senegal	2009	JQ943708	WA
Singapore	2006	FJ807896	ECSA
Singapore	2008	FJ445483	Asian
Singapore	2008	FJ445511	ECSA
Singapore	2009	HM067743	ECSA
South Africa	1976	HM045795	ECSA
Sri Lanka	2006	AB455493	ECSA
Tanzania	1953	HM045811	ECSA
Thailand	1966	AF192900	Asian
Thailand	1975	HM045814	Asian
Thailand	1978	HM045808	Asian
Thailand	1988	HM045789	ECSA
Thailand	1995	HM045796	ECSA

Country	Year collected	GenBank accession no.	Genotype
Thailand	2009	JN661149	ECSA
Tonga	2014	KM985981	Asian
Uganda	1982	HM045812	ECSA
Yemen	2011	KC614648	ECSA

*ECSA, East/Central/South African; WA, West African.



Technical Appendix Figure 1. Maximum clade credibility (MCC) tree of Asian genotype of chikungunya virus. MCC tree of Asian genotype was inferred by molecular clock analysis. Branch lengths are temporally scaled, and the x-axis shows the time unit (year). Red indicates Philippine viruses analyzed in this study; blue indicates Philippine strains in the 1980s obtained from GenBank. The posterior probability values are indicated at nodes.



Technical Appendix Figure 2. Markov jump density of viral migrations of chikungunya virus over time between Philippines and Indonesia. The Markov jump along the branches of the posterior trees of Asian genotype were estimated, and the history of the migrations between the Philippines and Indonesia was inferred by summarizing the Markov jump density using kernel density estimation.