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Molecular genome tracking of East, Central and South African genotype of Chikungunya virus in South-east Asia between 2006 and 2009

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ABSTRACT

Objective: To understand the epidemiology of the East, Central and South African (ECSA) genotype of Chikungunya virus (CHIKV) in terms of emerging and re-emerging infections, this study has been aimed at investigating the evolutionary parameters, genomic signatures and molecular tracking of the CHIKV ECSA genotype in South-east Asia and coastal areas of the Indian Ocean between 2006 and 2009 by using phylogenetic analysis and the Bayesian Markov Chain Monte Carlo (BMCMC) evolutionary estimation. **Methods:** Nearly complete genome sequences of 53 CHIKV isolates from all genotypes were subjected to phylogenetic analysis and evolutionary parameter estimation. The amino acids of 67 of ECSA genotype during 2006 to 2009 were compared for finding molecular signature tracking. The ECSA genotype signatures were visualized to find the possible transmission root was projected onto a geographic map. **Results:** Phylogenetic analysis showed the ECSA genotype was divided into 2 groups. The first group comprises viruses from India and Southeast Asian countries. The second group consists of strains typically circulating in Sri Lanka in 2008. The evolutionary parameters of these groups depicted the time of the most recent common ancestor at approximately 7.5 years ago. The genomic signatures revealed the positions of amino acid variation in each group. **Conclusions:** The molecular evolution projected onto a geographical map showed the routes of CHIKV transmission from 2006 to 2009. Molecular tracking will assist in understanding transmission routes, epidemiology and molecular evolution of CHIKV.

1. Introduction

Chikungunya virus (CHIKV), a positive stranded RNA virus, is a mosquito-transmitted member of the *Alphavirus* genus of the family Togaviridae. The main mosquito vectors of this virus are the members of the *Aedes* family, *Ae. aegypti* and *Ae. albopictus*. Recent outbreaks of CHIKV have exhibited unusual severity with suspected mortality among the affected people in several areas of the world[1,2]. The virus causes an acute infection of abrupt onset, characterized by high fever, arthralgia, myalgia, headache, and rash[3]. The typical clinical sign of the disease is poly-arthralgia which may persist for months or years. Arthralgia in CHIKV is more severe and more common than in dengue virus infection.

CHIKV was first isolated during an outbreak in Tanzania in 1952[4]. Two complete genome prototypes showed that the genome structure was similar to other alpha viruses and most closely related to O'nyong-nyong virus (ONN)[5]. The 1.18 kb genome of CHIKV is organized as follows: 5'cap-nsP1-nsP2-nsP3-nsP4-junction region-C-E3-E2-6k-E1-poly (A)-3'. The genome comprises nonstructural and structural polyproteins. Phylogenetic analyses based on partial E1 sequences from African and Asian isolates revealed the existence of three lineages of CHIKV; West African, Asian and East, Central and South African (ECSA)[6].

CHIKV in Asia is an urban disease, usually found in dengue-endemic areas and transmitted largely by *Aedes aegypti* (*Ae. aegypti*) mosquitoes. However, the predominant *Aedes* sp. in locations such as Réunion Island, where CHIKV emerged in 2005, was *Ae. albopictus*[7]. The potential of *Ae. albopictus* mosquitoes to transmit CHIKV was confirmed by the spread of CHIKV into rural areas during the later stages of outbreaks in India in 2007[8]. The emerging strains have an alanine to valine substitution at codon 226 (A226V) of the envelope 1 (E1) gene in Réunion Island[9] and India[1]. The mutation in this position might increase the potential of virus transmission by *Ae. albopictus* mosquitoes[10].

CHIKV appears to have spread from Africa to Asian tropical countries[6]. The first case reported in Asia was in Bangkok, Thailand in 1958. Subsequently, it spread and

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affected other Asian and southeast Asian countries, such as Myanmar, Philippines, Malaysia, Pakistan, and the Pacific islands[3]. Furthermore, the first outbreak in India was recorded in 1963[11]. Some sporadic cases were found later in many parts of India. After 20 years, the re-emergence was documented in Malaysia between 2001 and 2003[12]. In Africa and Asia, the re-emergence was unpredictable, with intervals of 7-8 years up to 20 years between consecutive epidemics. Since the end of 2004 and during 2005 and 2006, CHIKV has emerged in the islands of the south-western Indian Ocean. The most affected island is the French island of La Réunion[13].

There are few reports on molecular evolution and evolutionary parameters of CHIKV. Power *et al*[6] have reported the evolution rate of CHIKV based on partial E1 gene sequences and determined the time of emergence of each genotype of CHIKV. Another study on evolutionary parameter estimation based on the BMCMC framework was performed by Cherian *et al*[14]. Their report comprises an estimation of substitution rates and ancestral time for all CHIKV genotypes based on whole genome and partial E1 gene datasets of re-emerged CHIKV on the Indian subcontinent between 2005 and 2007.

Recently, the ECSA genotype was the cause of outbreaks in several countries such as India, Sri Lanka, Thailand, Malaysia, Singapore and China[1,15-17]. To understand the epidemiology of the ECSA genotype in terms of emerging and re-emerging infections, this study has been aimed at investigating the evolutionary parameters, genomic signature and molecular tracking of the ECSA genotype of CHIKV in countries of Southeast Asia and at the Indian Ocean between 2006 and 2009.

2. Materials and methods

2.1. Viral sequence samples

To estimate the evolution parameter of overall CHIKV, the 53 nearly complete genome (total length of 11 235 nt) from all genotypes of CHIKV were estimated. To understand the genomic signature and molecular tracking of ECSA genotype, sixty-three nearly complete genome sequences of the ECSA genotype of CHIKV isolated between 2006 and 2009 were analyzed in this study. All the virus sequences are available at GenBank. Details of the sequences are shown in Table 1.

2.2. Estimating the rate of evolution and evolutionary parameters

All genotypes of CHIKV, 53 nearly complete genome sequences were subjected to phylogenetic analysis and subsequent estimation of evolutionary parameters. Multiple alignments were carried out using Muscle version 3.8[18]. The best fitting nucleotide substitution model was selected by Bayesian information criterion (BIC) as implemented in Model Test 3.7[19]. GTR (General Time Reversible) + I (proportion of Invariant sites) was found to be the best-fit model for nucleotide substitution. The Bayesian Markov Chain Monte Carlo (BMCMC) was executed by using BEAST 1.5.4 package[20]. The evolutionary parameters, the rates of substitution, the divergence time and the most recent common ancestor (tMRCA) were estimated by MCMC framework. The best-fit molecular clock model was chosen by Bayes Factor (BF). The Bayesian skyline plot[21] was used to estimate population dynamics. Demographic models with less complex parameters (constant size, exponential growth and logistic growth) were applied independently and the best-fit models selected by BF tests were used to quantitatively estimate the growth rate and other demographic parameters. Uncorrelated exponential molecular clock was chosen with the assumption of constant population growth. The BMCMC analysis contained 8×10^7 states, with every 5 000 states sampled, and 10% of the chain was discarded as burn-in. Convergences and effective sample sizes of the estimates were checked using Tracer version 1.5[available from http://tree.bio.ed.ac.uk/]. The maximum clade credibility tree was generated using Tree Annotator and projected by using FigTree[available from http://tree.bio.ed.ac.uk/].

2.3. Genomic signature

To determine the molecular tracking of ECSA genotype, the 63 sequences encoding amino acid sequences of the ECSA genotype of CHIKV isolated between 2006 and 2009 were compared among all countries in relation to time period. To visualize the molecular genomic signature tracking of the ECSA genotype of CHIKV in this study, the possible transmission root was projected onto a geographic map.

3. Results

3.1. Estimation of evolutionary parameters from all genotype

Nearly complete genome sequences (total length of 11 235 nt) of 53 CHIKV isolates from all genotypes were subjected to phylogenetic analysis and evolutionary parameter estimation. The best fitting nucleotide substitution model (GTR+I) and molecular clock model (uncorrelated exponential, constant population growth) were applied for Bayesian MCMC analysis. Phylogenetic analysis showed that viruses isolated in 2006-2009 clustered in the ECSA genotype, except for some strains from Malaysia from 2006 and Indonesia which clustered in the Asian genotype (Figure 1). The ECSA genotype isolated between 2006 and 2009 clustered in 2 groups. The first group consisted of Sri Lanka strains from 2006 and 2008, India and Sri Lanka strains from 2006-2007 (INDSRI06-07), Sri Lanka strains from 2008 (SRI08(i)) and Thailand, Malaysia, Singapore and China strains from 2008-2009. SRI08(i) was shown to be separate from other countries in this cluster (Figure 1). Another group was the variation of Sri Lanka isolates in 2008 (SRI08(ii)). One isolate from China also clustered in this group. The genetic distance was determined at 0.25% and 0.28% for nucleic acid and amino acid sequences, respectively.

The evolutionary parameters of 53 nearly complete genome sequences were estimated based on the model of best fit. The mean substitution rate is 5.72×10^{-4} subs/site/year with a 95% HPD limit of $3.58-8.08\times10^{-4}$. According to phylogenetic analysis of ECSA, the estimated time to the most recent common ancestor (tMRCA) for ECSA which divided into two groups is approximately 7.5 years (4.7-11.2 years). tMRCA of the first group has been estimated at approximately 6 years (6.00-6.51 years).

Table 1
The detail of genomic of CHIKV in this study.

Accession	Strain	Countries	Group	Isolation date
GU199351	SD08Pan	China	–	12-Mar-2008
GU199350	FD080008	China	–	04-Mar-2008
GU199353	FD080231	China	China08	27-Dec-2008
GU199352	FD080178	China	China08	03-Oct-2008
FJ000068	KA51	India	India06	Aug-2006
GQ428211	RGCB05/KL06	India	India06	07-Oct-2006
FJ000062	IND-GJ52	India	India06	Sep-2006
FJ000067	IND-MH51	India	India06	Aug-2006
EF210157	DRDE-06	India	India06	2006
FJ000066	IND-KR51	India	India06	2006
EF027137	IND-06-RJ1	India	India06	2006
EF027135	IND-06-KA15	India	India06	2006
EF027138	IND-06-TN1	India	India06	2006
FJ000065	IND-GJ53	India	India06	Sep-2006
EF027136	IND-06-MH2	India	India06	2006
EU564335	CHIK31	India	India06	31-Oct-2006
EF027134	IND-06-AP3	India	India06	2006
GQ428210	RGCB03/KL06	India	India06	07-Oct-2006
FJ000063	IND-KA52	India	India06	Oct-2006
FJ000064	IND-GJ51	India	India06	Sep-2006
GQ428213	RGCB120/KL07	India	India07	13-Jul-2007
GQ428212	RGCB80/KL07	India	India07	12-Jul-2007
FJ000069	IND-KR52	India	India07	Jun-2007
GQ428215	RGCB356/KL08	India	India08	29-May-2008
GQ428214	RGCB355/KL08	India	India08	29-Jun-2008
FJ807899	0810bTw	Malaysia	Malay08	2008
FJ807892	0812aTw	Malaysia	Malay08	2008
FJ445511	SGEHICHD13508	Singapore	Singapore(i)	Jan-2008
FJ445510	SGEHICHS277108	Singapore	Singapore(i)	Jan-2008
FJ445445	SGEHICHS425208	Singapore	Singapore(ii)	Aug-2008
FJ445463	SGEHICHD96808	Singapore	Singapore(ii)	Jul-2008
FJ445432	SGEHICHS422308	Singapore	Singapore(ii)	Jul-2008
FJ445430	SGEHICHD93508	Singapore	Singapore(ii)	Jul-2008
FJ445433	SGEHICHS422808	Singapore	Singapore(ii)	Aug-2008
FJ445431	SGEHICHS421708	Singapore	Singapore(ii)	Jul-2008
FJ445484	SGEHICHT077808	Singapore	Singapore(ii)	May-2008
FJ445443	SGEHICHS424108	Singapore	Singapore(ii)	Aug-2008
FJ445502	SGEHICHD122508	Singapore	Singapore(ii)	Aug-2008
GU189061	SL15649	Sri Lanka	SriLanka2006	2006
AB455493	SL11131	Sri Lanka	SriLanka2006	Dec-2006
AB455494	SL10571	Sri Lanka	SriLanka2006	Dec-2006
FJ445428	LKRGCH1507	Sri Lanka	SriLanka2007	May-2007
FJ445427	LKMTCH2707	Sri Lanka	SriLanka2007	Jul-2007
FJ513632	LK(PB)CH3008	Sri Lanka	SriLanka2008(ii)	Mar-2008
FJ445426	LKEHCH13908	Sri Lanka	SriLanka2008(ii)	Apr-2008
FJ513673	LK(EH)CH17708	Sri Lanka	SriLanka2008(ii)	Apr-2008
FJ513654	LK(EH)CH6708	Sri Lanka	SriLanka2008(ii)	Apr-2008
FJ513645	LK(EH)CH4408	Sri Lanka	SriLanka2008(ii)	Apr-2008
FJ513679	LK(EH)CH20108	Sri Lanka	SriLanka2008(i)	Apr-2008
FJ513657	LK(EH)CH7708	Sri Lanka	SriLanka2008(ii)	Apr-2008
GU013530	LK(EH)chik19708	Sri Lanka	SriLanka2008(i)	Apr-2008
GU013528	LK(PB)chik3408	Sri Lanka	SriLanka2008(i)	Mar-2008
FJ513628	LK(PB)CH1008	Sri Lanka	SriLanka2008(i)	Mar-2008
FJ513629	LK(PB)CH1608	Sri Lanka	SriLanka2008(i)	Mar-2008
GU013529	LK(PB)chik6008	Sri Lanka	SriLanka2008(i)	Mar-2008
FJ513637	LK(PB)CH5808	Sri Lanka	SriLanka2008(i)	Mar-2008
FJ513635	LK(PB)CH5308	Sri Lanka	SriLanka2008(i)	Mar-2008
FJ513675	LK(EH)CH18608	Sri Lanka	SriLanka2008(ii)	Apr-2008
GU301780	CU-Chik10	Thailand	Thailand08-09	21-Oct-2008
GU908223	CU-Chik_OBF	Thailand	Thailand08-09	14-Aug-2009
GQ905863	CU-Chik661	Thailand	Thailand08-09	25-May-2009
GU301779	CU-Chik009	Thailand	Thailand08-09	04-Sep-2009
GU301781	CU-Chik683	Thailand	Thailand08-09	27-Jul-2009

SriLanka06 = Sri Lanka isolates in 2006, SriLanka07 = Sri Lanka isolates in 2007, SriLanka08(i) = Sri Lanka isolates in 2008 group 1, SriLanka08(ii) = Sri Lanka isolates in 2008 group 2, India06 = India isolates in 2006, India07 = India isolates in 2007, India08 = India isolates in 2008, China08 = China isolates in 2008, Malay08 = Malaysia isolates in 2008, Singapore08(i) = Singapore isolates in first period of 2008 (January), Singapore08(ii) = Singapore isolates in second period of 2008 (May-Aug), Thailand08-09 = Thailand isolates between 2008 and 2009.

Figure 1. BMCMC tree analysis of whole CHIKV genome applying best-fit model.
The nodes show the estimated age with 95% HPD in square brackets.

3.2. Genomic signature of ECSA genotype tracking

Sequences encoding amino acids of the ECSA genotype were compared among 63 CHIKV strains isolated from India, Sri Lanka, Malaysia, Singapore Thailand and China (Table 2). A tendency for amino acid alterations was ascribed to altogether 10 positions with 5 located in non-structural proteins and 5 in structural proteins.

Although the comparison of virus from Sri Lanka and India isolated in 2006 showed close relatedness some strains displayed different genomic signatures at various positions (position 394 of nsP3, position 27 of the capsid protein and position 211 of E1). For 2007, two genomic signature positions in structural proteins were found in isolates from Sri Lanka and India. The A226V mutation in particular was typical for India strains, while Sri Lanka strains still showed alanine at this position. Another signature was found in E2 at position 252.

The 2008 isolates from Sri Lanka were divided into two groups. Comparison among these groups revealed differences in seven genomic signature positions comprising nsP3 (positions 38, 394 and 444), capsid (position 27), E3 (position 15) and E1 (positions 211 and 268). The phylogenetic tree confirmed these clusters. Interestingly, the Sri Lanka groups were separate from CHIKV of other countries. However, all viruses from Sri Lanka collected in 2008 exhibited the same A226V mutation as viruses from other countries.

The first local transmission of CHIKV in Singapore occurred in January 2008[16]. The genomic signature of the first episode (SGEICHD13508, SGEHICHS277108; denoted Singapore(i)) outbreak was similar to that of the Sri Lanka 2007 strains. These strains showed alanine in the E1 protein. After the first episode, the genomic signature of CHIKV in Singapore (denoted Singapore(ii)) was similar to that of strains isolated in India between 2007 and 2008. Leucine at position 539 of nsP2 was substituted by serine and a substitution of lysine by glutamine was found in the E2 protein at position. Furthermore, the A226V mutation in the E1 protein was dominant in these strains.

The outbreak in Thailand was reported between 2008 and 2009[15]. The genomic signatures were similar to those of the 2008 (second episode) strains from India, Malaysia and Singapore.

Sporadic cases were found in China in 2008 as imported cases[20]. The genomic signature in the first cases in March 2008 (FD080008 and SD08pan) was similar to that of the Sri Lanka 2007 strains. The nsP2 had leucine at position 539 and the E1 protein had alanine at position 226. Viruses isolated in late 2008 (FD080178 and FD080231) have genomic signatures resembling 2008-2009 isolates from India, Malaysia, Thailand and Singapore.

3.3. Geographic mapping of genomic signatures.

The epidemiology routes were projected by geographic mapping and time periods (Figure 2). Epidemiology mapping was carried out by phylogenetic, parameter estimation and genomic signature results. Since 2006, viruses have been circulating in India and Sri Lanka. In 2008, Sri Lanka viruses split into two groups and remained limited to Sri Lanka. During early 2008, CHIKV was imported from India (2007 strain) to Singapore as Singapore(i) group viruses. The second episode of CHIKV in Singapore originated from India and the virus circulated in India, Malaysia and Thailand.
The strains imported to China had originated from Sri Lanka and Malaysia. The first strains were imported from Sri Lanka. During late 2008, viruses were imported from Malaysia.

Table 2
Genomic signature of ESCA genotype of CHIKV during 2006–2009.

Protein	Position		Isolates													
	Polypeptide	Protein	SriLanka06	SriLanka07	SriLanka08 (i)	SriLanka08 (ii)	India06	India07	India08	Singapore08 (i)	Singapore08 (ii)	Malay08	Thailand 08–09	China SD08Pan	China FD080008	China08
nsP2	1074	539	L	L	L	L	L	S	S	L	S	S	S	L	L	S
nsP3	1371	38	Y	Y	H	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	1727	394	I	I/M	I	M	M	M	M	M	M	M	M	M	I	M
	1777	444	T	T	T	M	T	T	T	T	T	T	T	T	T	T
Capsid	27	27	I	I	I	V	I/V	I	I	I	I	I	I	V	I	I
E3	279	15	S	S	F	S	S	S	S	S	S	S	S	S	S	S
E2	577	252	K	K	K	K	K	Q	Q	K	Q	Q	Q	K	K	Q
E1	1020	211	K	K	K	N	K/N	K	K	K	K	K	K	K	K	K
	1035	226	A	A	V	V	A	V	V	A	V	V	V	A	A	V
	1078	269	V	V	V	M	V	V	V	V	V	V	V	M	V	V

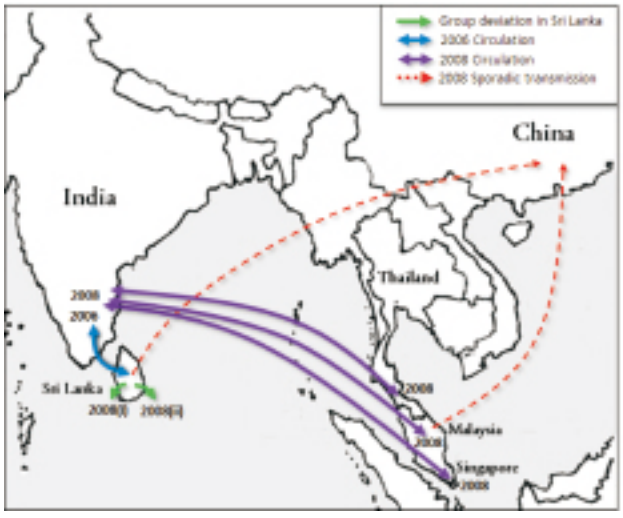

Figure 2. The geographical tracking of CHIKV between 2006 and 2009.
The epidemic spread was tracked based on molecular genomic signatures and period time.

4. Discussion

Over the past several years, CHIKV has been causing health care problems. Its emergence and re-emergence has been widely reported from several countries. Between 2005 and 2008, the ECSA genotype has been observed in several countries[22–25]. This study has provided the evolutionary parameters of the ECSA genotype and its genomic signature to facilitate epidemiological tracking of CHIKV.

A few studies have estimated the evolutionary rate of CHIKV[6, 17]. Cherian *et al*[17] have estimated the substitution rate of 27 CHIKV complete genomic sequences at 8.8×10^{-4} subs/site/year with a 95% HPD limit of $3.3-14.8\times10^{-4}$. Furthermore, the rate of evolution was determined at 6×10^{-4} subs/site/year (with standard deviation of 4×10^{-4}) by Power *et al*[6]. The evolutionary parameters in this study were approached by MCMC analysis. The best fit evolution models are similar to the approach by Cherian *et al* and were tested by likelihood analysis[14]. The mean substitution rate in this study was 5.72×10^{-4} subs/site/year with a 95% HPD limit of $3.58-8.08\times10^{-4}$. The evolutionary parameter estimation in this study did not significantly differ from that previously arrived at by Cherian *et al*[14] and Power *et al*[6]. In conclusion, the evolutionary rate of CHIKV genomic sequences was around $5-8\times10^{-4}$ subs/site/year.

ECSA genotype of CHIKV was widely spread into many countries. For the ECSA genotype in the period between 2006 and 2009, phylogenetic analysis showed divergence into 2 groups. The estimated tMRCA for these two groups was approximately 5–12 years. Phylogenetic analysis and evolutionary results suggest that these viruses have the same common ancestors and might have originated from the India strains or Sri Lanka strains. Group 2 can only be found in Sri Lanka, except for the sporadic case from China (SD08pan), while the other group was wide spread in many countries.

The important signature, the A226V mutation in the E1 protein, was found with increased frequency in coastal areas of the Indian Ocean. This mutation has been proven by laboratory-based evidence to have caused vector alteration from *Ae. Aegypti* to *Ae. Albopictus* mosquitoes[26]. *Ae. albopictus* was the main vector in the coastal areas of the Indian Ocean. This mutation may be responsible for the increased transmissibility of the virus and higher epidemic potential[1].

Leucine at nsP2 position 539 was the early signature. This position was mostly found in the 2006–2007 strains from India and Sri Lanka. Serine substitution was found in 2008 with the altered strains circulating in Southeast Asian and India. In the 2008 Sri Lanka strain, leucine is still preserved at this position. The genomic signatures in nsP3 have varied without a specific trend for alteration except for the Sri Lanka strain (ii). The K252Q substitution in the E2 protein has been observed in many Kerala isolates. This substitution was predominant recently in 2008 isolates and might be responsible for immunogenicity of the E2 protein[27].

The genomic signatures of the ECSA genotype of CHIKV since 2006 have been tracked by geographic mapping and time periods. In 2006, viruses were circulating in India and Sri Lanka. According to phylogenetic analysis and genomic signature, in 2008, Sri Lanka viruses split into two groups. Early isolates from Singapore (first episode) showed genomic signatures closely related to the 2007 Sri Lanka and 2007 India isolates. Subsequently, by mid to late 2008, the genomic signatures were altered to those of the 2008 strains from India and neighboring countries. Strains from Malaysia and Thailand displayed the same genomic signature as the 2008 late episode strain from Singapore. Furthermore, this genomic signature pattern was still found in India suggesting that this particular CHIKV virus strain was circulating in the coastal areas of the Indian Ocean and in South-east Asia.

The sporadic cases in China comprise 2 variations of CHIKV strains. With the early strain of 2008, SD08pan, the genomic signature pattern and phylogenetic analysis have shown a pattern similar to that of the Sri Lanka group 2. This strain has been isolated from Chinese travelers who

stayed for six months in Sri Lanka and returned to China via Malaysia. Although the patient was transported via Malaysia, the genomic signature pattern was more similar to isolates from Sri Lanka than Malaysia confirming that this virus had been contracted in Sri Lanka. In contrast, the genomic signatures of the late 2008 strains were related to the 2008 strains from India, Singapore, Malaysia and Thailand. These cases were diagnosed among a Chinese group returning from Malaysia[20]. The genomic signature pattern confirmed that these viruses had been transmitted in Malaysia.

Due to lack of an efficient vaccine or antiviral therapy, the vector control and thus, transmission control is the only way to limit CHIKV transmission. The molecular tracking reported in this study will assist in understanding the epidemic transmission route taking into consideration epidemiology and molecular evolution of CHIKV. However, further surveillance will still be required to control emerging and re-emerging CHIKV infections.

Conflict of interest statement

We declare that we have no conflict of interest.

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