Re-emergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships

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Chikungunya (CHIK) virus is a member of the genus *Alphavirus* in the family *Togaviridae*. Serologically, it is most closely related to o'nyong-nyong (ONN) virus and is a member of the Semliki Forest antigenic complex. CHIK virus is believed to be enzootic throughout much of Africa and historical evidence indicates that it spread to other parts of the world from this origin. Strains from Africa and Asia are reported to differ biologically, indicating that distinct lineages may exist. To examine the relatedness of CHIK and ONN viruses using genetic data, we conducted phylogenetic studies on isolates obtained throughout Africa and Southeast Asia. Analyses revealed that ONN virus is indeed distinct from CHIK viruses, and these viruses probably diverged thousands of years ago. Two distinct CHIK virus lineages were delineated, one containing all isolates from western Africa and the second comprising all southern and East African strains, as well as isolates from Asia. Phylogenetic trees corroborated historical evidence that CHIK virus originated in Africa and subsequently was introduced into Asia. Within the eastern Africa and southern Africa/Asia lineage, Asian strains grouped together in a genotype distinct from the African groups. These different geographical genotypes exhibit differences in their transmission cycles: in Asia, the virus appears to be maintained in an urban cycle with *Aedes aegypti* mosquito vectors, while CHIK virus transmission in Africa involves a sylvatic cycle, primarily with *Ae. furcifer* and *Ae. africanus* mosquitoes.

Introduction

Chikungunya (CHIK) virus, a member of the *Alphavirus* genus in the family *Togaviridae*, was first isolated from the serum of a febrile human in Tanganyika (Tanzania) in 1953 (Karabatsos, 1985). Between the 1960s and 1980s, the virus was isolated repeatedly from numerous countries in central and southern Africa as well as in Senegal and Nigeria in western Africa. During this same period, the virus was also identified in many areas of Asia. Since 1953, CHIK virus has caused numerous well-documented outbreaks and epidemics in both Africa and Southeast Asia, involving hundreds of thousands of people (Halstead *et al.*, 1969*a*, *b*; Rao, 1966). CHIK virus

infection produces an illness in humans that is characterized by fever, headache, nausea, vomiting, myalgia, rash and arthralgia. Because the clinical symptoms of CHIK infection often mimic those of dengue fever and because CHIK virus circulates in regions where dengue virus is endemic, it has been postulated that many cases of dengue virus infection are misdiagnosed and that the incidence of CHIK virus infection is much higher than reported (Carey, 1971).

In Africa, CHIK virus appears to be maintained in a sylvatic cycle involving wild primates and forest-dwelling *Aedes* spp. mosquitoes. Serological studies have repeatedly demonstrated the presence of antibodies in humans and wild primates throughout the moist forests and semi-arid savannas of Africa (Adesina & Odelola, 1991; Jupp & McIntosh, 1988; Rodhain *et al.*, 1989; Salim & Porterfield, 1973; Karabatsos, 1975). To date, a vertebrate reservoir or sylvan transmission cycle has not been identified outside Africa, supporting the historical evidence (Carey, 1971) that CHIK virus originated in Africa

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and was subsequently introduced into Asia, where it is now typically associated with *Ae. aegypti* mosquitoes. Strains from Africa and Asia are reported to differ biologically (Jupp & McIntosh, 1988), indicating that distinct lineages may exist.

In 1996, a closely related alphavirus, o'nyong-nyong (ONN) virus, caused a major epidemic in southern Uganda (Lanciotti *et al.*, 1998). This was the first epidemic of ONN virus infection since 1959, when a large epidemic swept across East Africa involving over 2 million reported cases (Johnson, 1988). Unlike CHIK and all other alphaviruses, ONN virus is unique in its transmission patterns: the virus is not transmitted by culicine mosquitoes, but rather by anophelines, typically *Anopheles funestus* and *An. gambiae*. A vertebrate reservoir for ONN virus has not yet been identified. The transmission of ONN virus by two common vectors that inhabit much of tropical Africa and that live in close association with humans may be a factor in the rapid spread of the virus during epidemics.

With the exception of information derived from a limited number of serosurveys, little is known about the relationships of CHIK and ONN viruses (Chanas *et al.*, 1979; Karabatsos, 1975; Porterfield, 1961). ONN is considered to be a subtype of CHIK virus: serological tests reveal a one-way antigenic cross-reactivity between the two agents. Antibody to CHIK virus reacts almost equally with both CHIK and ONN viral antigens while ONN virus antibodies react weakly against CHIK virus antigen (Blackburn *et al.*, 1995; Chanas *et al.*, 1979; Lee *et al.*, 1997; Karabatsos, 1985). It was once postulated that mutations in CHIK virus led to the emergence of ONN virus and its ability to be transmitted by anopheline mosquitoes (Johnson, 1988). However, genetic studies by Lanciotti *et al.* (1998) as well as the phylogenetic analyses presented here clearly demonstrate that ONN and CHIK viruses are genetically distinct. The phylogenetic and serological studies presented here were designed to help elucidate the evolutionary relationships of these viruses and to aid in understanding their epidemic and maintenance transmission patterns.

Methods

■ **Virus preparation.** The CHIK and ONN virus strains used in this study are described in Table 1. Viruses were diluted and grown on either BHK-21 or Vero 76 cells at an m.o.i. less than 1. After approximately 75 % of the cells exhibited cytopathic effects, the virus present in the supernatant was concentrated by polyethylene glycol precipitation (Killington *et al.*, 1996). The virus pellet was resuspended in 150 µl TEN buffer and 2 ml Trizol LS (Gibco-BRL) was added in preparation for RNA extraction according to the manufacturer's protocol.

■ **RNA extraction and RT–PCR.** RNA was extracted from the virus/Trizol suspension according to manufacturer's protocols as described previously (Cilnis *et al.*, 1996). cDNA was synthesized from the RNA using a poly(T) oligonucleotide primer (either $T_{19}V$ or $T_{25}V$-*Mlu*; 5′ TTACGAATTCACGCGT$_{25}$V 3′). PCR amplification was performed on the first strand cDNA using the poly(T) primer and a forward primer designed to anneal to genome positions 10344 to 10360 (5′

TACCCNTTYATGTGGGG 3′) of ONN strain SG650, covering the carboxy-terminal portion of the E2 envelope glycoprotein gene. The following parameters were used: 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 50 °C for 30 s, and extension at 72 °C for 3 min. A 10 min final extension was used to ensure complete product synthesis.

■ **Sequencing/phylogenetic analyses.** PCR products ranging from 1·2 to 1·7 kb were isolated from 1 % agarose gels. The cleaned DNA fragments were cloned into the pCR2.1 TA cloning vector (Invitrogen) and white bacterial colonies screened for plasmids containing inserts of the correct size. Selected clones were sequenced using the plasmid-specific T7 promoter and m13 reverse primers combined with internal, CHIK virus-specific primers (C3205, 5′ GCRACAAACCCSGTAAG 3′; C3152, 5′ ACTGGCTRAAAGAACGAGG 3′). Sequencing was performed using an Applied Biosystems Prism 377 sequencer and automated DNA sequencing kit. The deduced amino acid sequences were aligned by using the PILEUP program in the Wisconsin Package (Genetics Computer Group) with default parameters, and the nucleotide sequences were aligned manually based on codon homology. Phylogenetic analyses were performed using maximum parsimony, neighbour joining and maximum likelihood programs implemented in the PAUP 4.0 software (Swofford, 1998). Distance analyses used the Kimura 2-parameter formula to correct for multiple substitutions of the same nucleotides. Unordered and ordered characters (transition/transversion ratio of 4:1; based on previous alphavirus estimates) were used in the parsimony analysis. Alphaviruses in the Venezuelan equine encephalitis, Barmah Forest and eastern equine encephalitis antigenic complexes were used as an outgroup. Bootstrap analysis (Felsenstein, 1985) was performed with 1000 replicates to determine confidence values on the clades within trees.

■ **Estimation of divergence times.** An average divergence rate for CHIK and ONN virus lineages was estimated by identification of sister-sequence pairs that were robust (bootstrap values > 90 %), closely related and isolated at least 7 years apart in the same geographical region. The number of differences in synonymous changes depicted in branch lengths separating each sister sequence from the predicted common ancestor's sequence was divided by the number of years between isolations to yield rates expressed as changes per nucleotide per year, and several estimates were compared to provide an estimated mean and standard deviation. Synonymous nucleotide divergence estimates for pair-wise sequence comparisons were generated using the formula of Li *et al.* (1985) to correct for multiple substitutions of the same nucleotides.

■ **Production of immune sera.** Syrian golden hamsters and BALB/C mice were used to generate immune sera to three strains of CHIK virus (37997, Ross and 1455/75) and one strain of ONN virus (Igbo Ora, IbH12628). Animals received a single injection of virus (∼ 10⁵ p.f.u./ml), either intraperitoneally (i.p.) alone or subcutaneously with a mixture of virus and an *Ae. aegypti* mosquito salivary gland suspension to enhance the infection. Approximately 4 weeks post-inoculation, blood was obtained from the rodents from the retroorbital sinus and tested for antibody to CHIK virus by an immunofluorescent antibody assay (IFA) or by neutralization test (NT). Mice that were positive for CHIK virus antibody by IFA were injected i.p. with sarcoma 180 cells to produce hyperimmune ascitic fluid. Abdominal fluid was removed between 1 and 2 weeks after injection of the sarcoma cells and was used in IFA and plaque reduction neutralization tests (PRNT) to determine homologous titres.

■ **Titration of neutralizing antibody.** Three of the four viruses (37997, Ross and 1455/75) generated a detectable homologous antibody response as determined by IFA (in mice) or NT (in hamsters). Only two of these, 37997 and Ross, had IFA titres sufficient to perform additional serological analyses. These two viruses were used in 80 % PRNTs to

Table 1. Viruses used in phylogenetic analyses

Strain	Virus type*	Location	Date†	Host	Passage history‡	Accession no.
RSU1	CHIK	Maluku, Ambon Island, Indonesia	1985	Human	V-3	AF192894
Ross	CHIK	Newala District, Tanzania	1953	Human	SM-175, V-1	AF192905
IPD A234	ONN	Bandia, Senegal	1963	Sentinel mouse	V-1, BHK-1	AF192890
B448	SIN	Yunan, China	Unknown	Unknown	V-1, BHK-1	AF192888
IBH35	CHIK	Ibadan, Nigeria	1964	Human	SM-?, V-1, BHK-1	AF192893
CAR 256	CHIK	Central African Region	Unknown	Unknown	SM-1, BHK-1	AF192906
PH H15483	CHIK	Bocolod, Philippines	1985	Human	V-5	AF192895
Gibbs 63-263	CHIK	Calcutta, India	1963	Human	SM-16, V-2	AF192901
PM 2951	CHIK	Ndofore, Senegal	1966	*Ae. aegypti*	SM-3, V-2	AF192891
UG AG41855	CHIK	Mukono District, Uganda	1982	Human	SM-3, V-2	AF192907
37997	CHIK	Kedougou, Senegal	1983	*Ae. furcifer*	AP61-1, V-2	AF192892
1455/75	CHIK	Bangkok, Thailand	1975	Human	C6/36-3, V-1	AF192898
PO 731460	CHIK	Barsi, India	1973	Human	V-2, M-1	AF192902
AR 18211	CHIK	South African Republic	1976	*Ae. furcifer*	M-2, SM-3, V-1	AF192903
SA H2123	CHIK	South African Republic	1976	Human	M-1, SM-2, BHK-1	AF192904
181/25	CHIK	Thailand	1962	Human	MRC5-18, V-1	AF192908
IbH12628	ONN	Igbo Ora, Nigeria	1966	Human	p-6, V-1	AF192889
6441/88	CHIK	Suin Province, Thailand	1988	Human	RMK-1, V-1	AF192896
C-0392/95	CHIK	Bangkok, Thailand	1995	Human	RMK-1, V-1	AF192897
SV-0451/96	CHIK	Payoa Province, Thailand	1996	Human	RMK-1, V-1	AF192900
3412/78	CHIK	Bangkok, Thailand	1978	Human	RMK-3, V-1	AF192899
DAK Ar Mg812	SIN	Perinet, Madagascar	1979	*Culex* sp.	SM-3, BHK-1	AF192887
DAK Ar B16878	SF	Bouboui, Central African Region	1980	*An. funestus*	SM-5, BHK-1	AF192909
SG650	ONN	Rakai District, Uganda	1996	Human	V-1	AF079456

* CHIK, chikungunya; ONN, o'nyong-nyong; SIN, Sindbis; SF, Semliki Forest.

† Year of isolation.

‡ Cell type followed by number of passages in that cell. BHK, baby hamster kidney cells; V, Vero cells; SM, suckling mouse; AP61, *Ae. pseudoscutellaris* mosquito cells; C6/36, *Ae. albopictus* mosquito cells; M, mosquito; MRC5, human lung cells; p, unknown cell type; RMK, rhesus monkey kidney (LLC-MK2) cells.

determine both the homologous and heterologous neutralizing antibody titres.

Results

Phylogenetic analysis

Cloned PCR products ranging from approximately 1200 to 1700 nucleotides of the E1 envelope glycoprotein gene and the entire 3′ noncoding region (NCR) were sequenced and aligned using the PILEUP program in the GCG software package. Because alignment of the 3′NCR was poor, only the E1 coding nucleotides were used in the phylogenetic analyses. Both distance matrix programs and maximum parsimony generated trees with the same basic topology, differing only in the arrangement of the CHIK virus isolates from the Asian clade.

Initial parsimony analyses revealed that several isolates, previously designated as CHIK virus, were genetically quite distinct from the prototype strain and from all other isolates examined. Inclusion of representative members of the Semliki Forest, Venezuelan equine encephalitis, Barmah Forest and eastern equine encephalitis virus antigenic complexes showed that these viruses were actually ONN (strain IPD A234), Semliki Forest (DAK ArB16878) and Sindbis-like (ArMg812 and B448) viruses. Additionally, CHIK virus strains 3412/78 and C-0392/95 were isolated from patients in Thailand suspected of having dengue virus infection, reinforcing the uncertainties of viral diagnosis based upon clinical presentation.

All of the CHIK and ONN virus isolates examined formed a monophyletic group within the Semliki Forest virus antigenic complex (Fig. 1), supported by a 100 % bootstrap value. The ONN virus isolates formed a robust, distinct clade (100 % bootstrap support) apart from all isolates of CHIK virus. ONN and CHIK virus sequences were approximately 28 % and 13 % divergent at the nucleotide and amino acid levels, respectively, underscoring the distinct nature of the two virus groups. Igbo Ora virus (strain IbH12628) grouped closely with the other strains of ONN, supporting previous reports that this is indeed an antigenic variant of ONN virus (Lanciotti *et al.*, 1998).

All phylogenetic methods divided the CHIK virus isolates into three distinct genotypes, based primarily on geographical
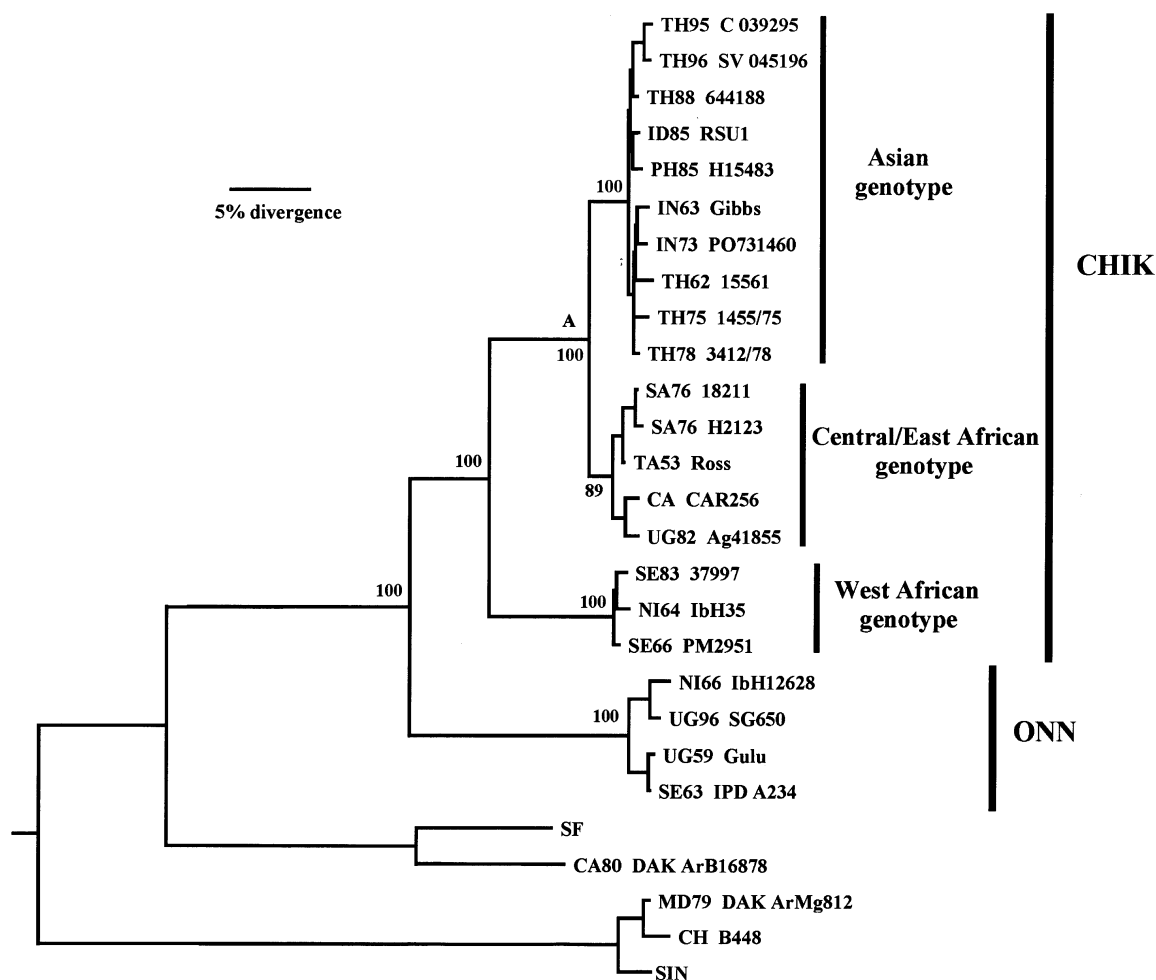
Fig. 1. Phylogenetic analysis of CHIK and ONN viruses generated by performing a PAUP analysis on the 1050 bp partial E1 gene sequence. To correct branch lengths for multiple substitutions, the Neighbor distance program was used to draw the tree utilizing the topology of the PAUP phylogram. Numbers indicate bootstrap values for the groups to the right. Letter A indicates the hypothetical ancestor used to estimate the time of divergence of Asian CHIK isolates from the African progenitor. The bar indicates horizontal distance corresponding to 5% nucleotide sequence divergence.

origins. One CHIK virus clade consisted of the isolates from Senegal and Nigeria, forming the West Africa genotype (Fig. 1). These were quite distinct from the remaining CHIK virus isolates having only 78 to 85% nucleotide sequence identity over the fragment analysed. The remaining CHIK virus isolates formed two clades: one contained strains from central and eastern Africa, while the other contained solely Asian isolates. The paraphyletic grouping of the African CHIK viruses supports the historical evidence that the virus was introduced into Asia from Africa.

Estimated divergence times

An attempt was made to estimate the average rate of evolution of the CHIK and ONN viruses by comparison of sequences of sister taxa from the same geographical areas. Analysis of individual lineages was not possible because too few strains were available. Sister pairs were chosen that had

bootstrap values > 90% and were isolated at least 7 years apart. Using these sequences, an estimated rate of evolution was determined to be 6×10^{-4} substitutions per nucleotide per year with a standard deviation of 4×10^{-4}. The same estimate was obtained when pair-wise comparisons included distance corrections using the Kimura two-parameter formula or maximum likelihood (see below). The synonymous rate was 5×10^{-4} (standard deviation 3×10^{-4}) and the non-synonymous rate was 6×10^{-5} (standard deviation 5×10^{-5}). Although these estimates were based on only six sister-pair sequences and therefore had a high degree of error, they are similar to those previously determined for neotropical alphaviruses (Weaver *et al.*, 1993, 1997; Powers *et al.*, 1997). Using the synonymous rate and the K_s values computed for CHIK strain comparisons (ranging from 0·12 to 0·25 with standard deviations of 0·03), the Asian genotype evolved from a hypothetical African ancestor (node A, Fig. 1) an estimated 50

Table 2. Initial serological analysis of CHIK/ONN viruses

Antibody*	Virus species†	Genotype	Source	Serological test	Homologous titre
37997H	CHIK	West African	Hamster	NT	320
37997M	CHIK	West African	Mouse	IFA	640
37997 HIAF	CHIK	West African	Mouse	IFA	200
RossH	CHIK	Central/East African	Hamster	NT	160
RossM	CHIK	Central/East African	Mouse	IFA	80
Ross HIAF	CHIK	Central/East African	Mouse	IFA	200
1455/75M	CHIK	Asian	Mouse	IFA	20
1455/75H	CHIK	Asian	Hamster	NT	< 10
IbH12628M	ONN	ONN	Mouse	IFA	< 10
IbH12628H	ONN	ONN	Hamster	NT	< 10

* Identification number indicates virus strain used to immunize hamsters (H) or mice (M). HIAF indicates hyperimmune ascitic fluid; the remainder of the antibodies were prepared by a single injection of virus (antigen).
† As Table 1.

to 430 ($\pm$ 1 standard deviation) years ago. K_s values for strains from the West African vs East African/Asian genotypes ranged from 0·75 to 0·86 with standard deviations of 0·10 to 0·12. Using these values, the ancestor of all of the CHIK virus strains is estimated to have emerged between 150 and 1350 years ago. Although the divergence time of ONN virus from CHIK virus could not be estimated reliably due to excessive variance in the K_s values resulting from near saturation of synonymous changes, divergence of CHIK and ONN viruses probably occurred at least thousands of years ago.

A potential flaw in these time estimates is that substitution rates may vary across nucleotide sites, including synonymous sites, as has been reported for human immunodeficiency virus (Leitner *et al.*, 1997). Unequal substitution rates across sites could result in an underestimation of true sequence divergence because the sites undergoing more change may accumulate more sequential mutations than are estimated by traditional formulas that assume equal rates. Therefore, we estimated the gamma distribution shape parameter for unequal rates using maximum likelihood analysis applied to all equally parsimonious tree topologies as well as the topology generated by neighbour joining. The gamma distribution shape parameter estimate was 0·42, and the transition/transversion ratio estimate was 4·3, similar to previous alphavirus estimates using substitution data from parsimony analyses (Cilnis *et al.*, 1996; Weaver *et al.*, 1994, 1997). We used these values to generate trees with maximum likelihood branch lengths applied to tree topologies generated using maximum parsimony and neighbour joining methods. Using this approach, similar divergence time estimates were obtained, with the Asian CHIK virus genotype emerging between 50 and 310 years ago, and the West and East African genotypes diverging 100 to 840 years ago.

Antigenic analysis

To determine the antigenic relatedness of viruses in the CHIK and ONN virus clades, one virus from each CHIK and ONN genotype was selected and used to generate antibodies in hamsters and mice (Table 2). Four weeks after a single injection of virus, animals were bled, and their sera tested for antibodies by IFA. The three CHIK viruses all produced specific antibodies while the ONN virus-infected mice and hamsters produced no detectable antibody response. The homologous IFA titre of CHIK virus strain 1455/75 was too low to be useful in neutralization assays; however, strains 37997 and Ross produced adequate antibody titres and were tested by 80% PRNT (Table 3). Results indicated that these viruses have a greater than 4-fold difference in one direction suggesting that they are distinct enough to be classified as antigenic subtypes (Calisher & Karabatsos, 1988; Calisher *et*

Table 3. Serological analysis of CHIK viruses (80% PRNT titres)

	Antiserum			
	Hamster		Mouse	
Virus	RossH	37997H	RossM	37997M
Ross	80 (−)*	160 (2)	40 (−)	320 (1)
37997	10 (8)	320 (−)	10 (4)	320 (−)

* Number in parentheses represents the -fold difference in homologous versus heterologous titre for that antiserum.

al., 1980). While no antibody against ONN virus was generated here, eliminating the possibility of performing two-way cross neutralization tests between CHIK and ONN viruses, it would be reasonable to assume that because distinct genotypes of CHIK virus are sufficiently different antigenically to be considered subtypes, the ONN virus lineage would be more likely to be considered a distinct group of viruses within this antigenic complex.

Discussion

Results of our analyses support the hypothesis and historical accounts that CHIK virus probably originated in tropical Africa and subsequently was imported into southern Asia. In Africa, evidence that the virus circulates continually in sylvatic cycles has been documented for decades. The virus has been isolated from sylvatic mosquito species in several countries including Senegal, Cote d'Ivoire, Central African Republic and South Africa (Diallo et al., 1999; Jupp & McIntosh, 1990; McCarthy et al., 1996). The mosquito species involved vary geographically and with ecological conditions. In Senegal, for example, *Ae. furcifer*, *Ae. taylori*, *Ae. luteocephalus*, *Ae. africanus* and *Ae. neoafricanus* are the species determined to be of major importance in maintaining CHIK virus transmission cycles. Interestingly, several of these are the same mosquito species involved in maintaining yellow fever virus, perhaps suggesting that outbreaks of CHIK virus infection could be concomitant with sylvan yellow fever (Traore-Lamizana et al., 1996). In addition, it has been reported that different populations of *Ae. aegypti* in Senegal have distinct suscepti-bilities to CHIK virus (Diallo et al., 1999), suggesting vector strain specificity for CHIK viruses. Further characterization of the mosquito vectors present in the endemic areas and their vector competence for CHIK viruses could provide valuable information regarding the potential for re-emergence of the viruses in human populations.

In contrast to the numerous species involved in main-tenance of CHIK virus infection in Africa, *Ae. aegypti* and *Ae. albopictus* are the only vector species known to transmit CHIK virus in Asia. These are urban and peridomestic, anthropophilic mosquitoes that maintain close associations with humans. It is therefore not surprising that outbreaks of CHIK virus infection are noted more frequently in Asia than in Africa. Several studies have demonstrated the varying susceptibility of different Asian mosquito strains for CHIK viruses (Banerjee et al., 1988; Mourya & Banerjee, 1987; Mourya et al., 1987; Tesh et al., 1976). Because CHIK and dengue viruses are transmitted by the same mosquito species in Asia and because the clinical symptoms of the two viral diseases are similar, the two diseases are difficult to differentiate. Furthermore, there have been documented cases of simultaneous coinfection with CHIK and dengue viruses (Halstead, 1966; Myers & Carey, 1967), further complicating the characterization of CHIK virus maintenance, evolution and emergence in Asia.

Another question concerning the transmission of CHIK virus in Asia relates to the high degree of genetic similarity among Asian genotype viruses. Although our sampling of the Asian virus was limited, sequences from viruses spanning a wide geographical range and isolated over a period of almost 35 years showed less than 3% nucleotide sequence divergence (Fig. 1). This genetic conservation in Asia is intriguing for a virus that is known to be maintained only between humans and peridomestic mosquitoes. A similar, high degree of sequence conservation is observed within several other groups of alphaviruses: the North American eastern equine encephalitis viruses (Weaver et al., 1994; Brault et al., 1999), Highlands J virus from North America (Cilnis et al., 1996), western equine encephalitis viruses (Weaver et al., 1997) and the Sindbis-like viruses distributed throughout Australia (Sammels et al., 1999). As an example, North American eastern equine encephalitis viruses are maintained by an avian reservoir host; therefore, the increased movement of the virus due to migration of the birds may effectively increase the virus population size and decrease founder effects and genetic drift. This may explain their sequence conservation (Weaver, 1995; Weaver et al., 1992; Brault et al., 1999). It is unknown whether such an avian transmission cycle exists for CHIK viruses in Asia. Migratory patterns of both passerines and shorebirds do encompass much of Southeast Asia ranging from the Yellow Sea and South China Sea across the Philippines and Indonesia to Australia. Additionally, migration routes from India across the Indian Ocean to East Africa have been documented (Williams & Williams, 1990). Serological testing of passerines and shore-birds in Southeast Asia could reveal if this is a plausible means of virus dispersal. Alternatively, dispersal of the virus by travel of humans could account for the presence of virtually identical viruses in areas as distant as Indonesia and the Philippines to Barsi in central India, as well as the introduction of the virus into Asia from Africa.

The phylogenetic results presented here clearly demon-strate that ONN virus did not emerge via a recent mutation of CHIK virus as was once postulated (Johnson, 1988). This hypothesis was based on serological evidence indicating that the viruses could only be distinguished by two-way specific antigenic tests (i.e. neutralization assay) or the use of monoclonal antibodies (Karabatsos, 1975; Porterfield, 1961). Antiserum raised against CHIK virus reacted with ONN virus but the reciprocal was not true, leading to the hypothesis that mutations in CHIK virus generated altered structural con-figurations in ONN virus affecting seroassay results (Johnson, 1988; Williams & Woodall, 1961; Williams et al., 1962). It was suggested that these same mutations were responsible for the novel ability of ONN virus to replicate in and be transmitted by anopheline mosquitoes. However, if ONN virus undergoes periodic emergence from CHIK virus progenitors, ONN virus isolates from the outbreak in Uganda in 1996 would be predicted to group phylogenetically with CHIK virus isolates rather than with the other strains of ONN virus as seen in our

analysis (Fig. 1). For example, repeated emergence from a common progenitor has been found with epidemic/epizootic Venezuelan equine encephalitis viruses, which emerge periodically from enzootic viruses and occupy clades nested within the enzootic ID lineage (Kinney *et al.*, 1992; Powers *et al.*, 1997; Weaver *et al.*, 1996).

In addition to the antigenic and sequence differences between CHIK and ONN viruses, differences in several other biological patterns exist. Studies examining the relative ability of various strains of CHIK and ONN to replicate in different cell types have shown clear distinctions between these two viruses. CHIK viruses can replicate in both *Ae. aegypti* cell lines and numerous *Aedes* spp. mosquitoes (Chanas *et al.*, 1979; Jupp & McIntosh, 1988; Mourya *et al.*, 1987) while ONN does not appear to replicate in *Ae. aegypti* cells (Chanas *et al.*, 1979). Interestingly, both CHIK and ONN viruses can replicate in *An. gambiae* cells; however, only ONN replicates in and is believed to be transmitted primarily by *An. gambiae* or *An. funestus* mosquitoes under natural conditions (Corbet *et al.*, 1961; Williams *et al.*, 1965). Differences between the plaque sizes of CHIK and ONN viruses on mammalian cells have also been described (Chanas *et al.*, 1979; Tesh *et al.*, 1976); however, among CHIK and ONN viruses, plaque size may be strain specific (Chanas *et al.*, 1979).

A putative explanation for the varying biological properties among CHIK and ONN viruses is differences in the 3′NCR. All alphaviruses sequenced have repeat sequence elements in the 3′NCR that vary in length and number, often according to serogroup (Pfeffer *et al.*, 1998; reviewed in Strauss & Strauss, 1994). Within some virus groups (e.g. the Sindbis-like viruses) very little sequence heterogeneity is detected in the 3′NCR (Shirako *et al.*, 1991) while other alphaviruses including Ross River virus, Venezuelan equine encephalitis and Semliki Forest complex viruses show high degrees of 3′NCR variation in both length and nucleotide composition (Faragher & Dalgarno, 1986; Pfeffer *et al.*, 1998). Kuhn *et al.* (1991) have shown that changes in the repeat sequence elements can affect virus replication in different cell types, suggesting that this region may be important in binding cellular proteins utilized during virus replication. Our sequences from 22 strains of CHIK and ONN viruses from a diverse geographical and temporal range demonstrated such a high degree of variability in the 3′NCR that nucleotide sequence alignments in this area were unreliable. This information, combined with the knowledge that the replicative ability of a given CHIK or ONN viral strain varies tremendously with different strains of mosquitoes, may support Kuhn's hypothesis.

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