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Phylogeography and evolutionary history of dengue virus type 3

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ABSTRACT

In this study, we revisited the phylogeography of the three of major DENV-3 genotypes and estimated its rate of evolution, based on the analysis of the envelope (E) gene of 200 strains isolated from 31 different countries around the world over a time period of 50 years (1956–2006). Our phylogenetic analysis revealed a geographical subdivision of DENV-3 population in several country-specific clades. Migration patterns of the main DENV-3 genotypes showed that genotype I was mainly circumspect to the maritime portion of Southeast-Asia and South Pacific, genotype II stayed within continental areas in South-East Asia, while genotype III spread across Asia, East Africa and into the Americas. No evidence for rampant co-circulation of distinct genotypes in a single locality was found, suggesting that some factors, other than geographic proximity, may limit the continual dispersion and reintroduction of new DENV-3 variants. Estimates of the evolutionary rate revealed no significant differences among major DENV-3 genotypes. The mean evolutionary rate of DENV-3 in areas with long-term endemic transmissions (*i.e.*, Indonesia and Thailand) was similar to that observed in the Americas, which have been experiencing a more recent dengue spread. We estimated the origin of DENV-3 virus around 1890, and the emergence of current diversity of main DENV-3 genotypes between the middle 1960s and the middle 1970s, coinciding with human population growth, urbanization, and massive human movement, and with the description of the first cases of DENV-3 hemorrhagic fever in Asia.

1. Introduction

Dengue virus (DENV) (family *Flaviviridae*) has a single-stranded positive-sense RNA genome of approximately 11 kb and is transmitted by *Aedes aegypti* mosquitoes among humans. Infection with DENV may cause an acute "influenza-like" febrile disease called classic dengue fever (DF), or the potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The four known distinct antigenic groups (*i.e.*, serotypes) of DENV (DENV-1 to DENV-4) contain well-defined phylogenetic clusters (*i.e.*, genotypes) that are causing human pandemics. The incidence, geographic distribution and severity of DENV epidemics have dramatically increased since the mid-1950s, probably due to the rapid increase in human population size, uncontrolled urbanization, and the advent of massive human movement which facilitates the spread and proliferation of mosquitoes and infected people (Gubler, 1998, 2002, 2004; Zanotto et al., 1996). It has been estimated that 50–100 million infections occur annually, and more than 2.5 billion people live in areas of risk for DENV infection (Gubler, 1998, 2002, 2004).

Using a maximum likelihood framework, Twiddy et al. (2003) extended and re-evaluated the first comprehensive estimates of the rate and time frame of DENV evolution (Zanotto et al., 1996). It was inferred that the current global genetic diversity in the four serotypes of DENV appeared around the last 100 years. Moreover, Twiddy et al. (2003) suggested that all serotypes may be evolving according to a molecular clock; but, for reasons that remain unclear, DENV-3 and the DENV-2 American/Asian genotype had significantly higher substitutions rates when compared to other DENV strains. It has been suggested that under epidemic conditions (*i.e.*, when a new variant is introduced into a susceptible population) the viral transmission rate is higher than under endemic conditions, thus increasing the overall diversity and evolutionary rate of the new variant in the population (Twiddy et al., 2003). A higher evolutionary rate could also be a consequence of the emergence of DENV variants with particular

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biological properties, such as increased transmissibility, infectiousness, and/or virulence (Cologna and Rico-Hesse, 2003; Holmes and Twiddy, 2003; Messer et al., 2003; Rico-Hesse, 2003; Rico-Hesse et al., 1997). Alternatively, the lineage-specific rate differences in DENV evolution described by Twiddy et al. could be also caused by the low number of sequences used, particularly for DENV-1 ($n = 9$), DENV-3 ($n = 21$), and DENV-4 ($n = 20$) serotypes.

DENV-3 was isolated for the first time during an epidemic outbreak in Philippines in 1956 (Hammon et al., 1960), and since then several DF/DHF outbreaks caused by this serotype have been described world-wide. Five distinct genotypes of DENV-3 have been identified to date (Lanciotti et al., 1994; Wittke et al., 2002). Genotypes I to III (GI to GIII) are responsible for most DENV-3 infections and have been associated with DF/DHF epidemics in Southeast Asia, Indian subcontinent, South Pacific, East Africa, and the Americas. Genotypes IV and V (GIV and GV) were not associated with DHF epidemics and are only represented by a few early sequences from the Americas, South Pacific, and Asia. Many phylogenetic studies on DENV-3 have documented the viral spread within individual countries (Aquino et al., 2006; Chungue et al., 1993; Diaz et al., 2006; Islam et al., 2006; Kobayashi et al., 1999; Peyrefitte et al., 2003, 2005; Podder et al., 2006; Raekiansyah et al., 2005; Rodriguez-Roche et al., 2005; Usuku et al., 2001; Uzcategui et al., 2003; Wittke et al., 2002; Zhang et al., 2005) or specific regions (Messer et al., 2003), but we still have an incomplete understanding of the global dispersion and evolutionary history of the distinct DENV-3 genotypes.

The objective of the present study was to revise the global phylogeography and evolutionary history of the main DENV-3 genotypes based on the analysis of a large number ($n = 200$) of envelope (E) gene sequences of DENV-3 strains isolated from 31 different countries around the world over a time period of 50 years (1956–2006).

2. Materials and methods

2.1. Sequence datasets

Complete E gene sequences (1479 bp in length) with known date of isolation and representing the full extent of genetic diversity in DENV-3 were collected from GenBank (www.ncbi.nlm.nih.gov). Sequences were excluded from the analysis if they were previously identified as recombinant (Worobey et al., 1999), or were 100% similar to any other strain in the data set. For those genotypes where there were more than 75 sequences available, such as for GII and GIII, a maximum of six sequences randomly chosen from a particular country in a given year were included. This resulted in a final data set of 200 DENV-3 E sequences from 31 countries spanning a 50-year period. This primary dataset was further divided into six subsets comprising sequences from distinct genotypes and geographical origin. Table 1 shows the number, date of isolation range, and origin of the DENV-3 E sequences included in each dataset. Nucleotide sequences were aligned using CLUSTAL X program (Thompson et al., 1997) and later hand edited. All alignments are available from the authors upon request.

Table 1
Sequence datasets.

Dataset	Number of sequences	Date range	Geographic origin
DENV-3	200	1956–2006	Asia, America, South Pacific, and Africa
GI	43	1973–2005	Asia and South Pacific
GI-ID	24	1973–2005	Indonesia
GII	75	1973–2005	Asia
GII-TH	56	1973–2002	Thailand
GIII	75	1981–2006	Asia, America, and Africa
GIII-AM	63	1994–2005	America

2.2. Phylogenetic analysis

A Bayesian phylogenetic tree for the complete data set of 200 DENV-3 E sequences was inferred with MrBayes program (Ronquist and Huelsenbeck, 2003), under a General Time Reversible model of nucleotide substitution (Rodriguez et al., 1990) with gamma-distributed rate variation and a proportion of invariable sites (GTR + Γ + I). Two runs of 4 chains each (one cold and tree heated, temperature = 0.20) were run for 10×10^6 generations, with a burn-in of 2.5×10^6 generations. Convergence was assessed using the average standard deviation in partition frequency values across independent analyses, using a threshold value of 0.01. Convergence of parameters was also confirmed by calculating the Effective Sample Size (ESS) using TRACER v1.4 program (Rambaut and Drummond, 2007), excluding an initial 10% for each run. All parameters estimates showed ESS values >200.

2.3. Migration analysis

To investigate the migratory patterns of DENV-3 we examined each of the three main genotypes separately, since the isolation of viruses from different genotypes at the same locality would sum splits due to cladogenetic and migratory events, causing inferential errors. Geographical origin of each sample was coded as a set of terminal unordered character states for each dengue time-stamped, geo-referenced sample, represented as a single capital letter. The most parsimonious reconstructions (MPRs) sets of changes at each internal state in the viral phylogeny was calculated with PAUP v4.0b10 (Swofford, 2002) and MacClade v4.07 (Maddison and Maddison, 2005) and taken as surrogate for migration events. To help resolve among equally parsimonious reconstructions that leads to ambiguities in character tracing, we used assignments that delay (DELTRAN) or accelerate (ACCTRAN) character transformations (Swofford and Maddison, 1987). The phylogenies used for each genotype came from the global maximum posterior probability (MAP) tree obtained with Bayesian inference (BI) with BEAST v1.4.7 (Drummond et al., 2002; Drummond and Rambaut, 2006) for the entire dataset of 200 DENV-3 E sequences from which, subtrees for each genotype were analyzed in separate. For comparison, phylogenetic trees for each genotype were also inferred with the maximum likelihood (ML) criterion as implemented in the program GARLI v0.95 (Zwickl, 2006) that estimates simultaneously the best topology, branch lengths and the best values for the parameters for the GTR + Γ + I model of nucleotide evolution. Independent random runs were conducted with GARLI and the tree with highest likelihood was subsequently used as input for further optimization in both GARLI and PAUP, since both programs calculate the same likelihood score for a tree under the same model. The topology used for calculating the MPRs was the fully resolved consensus of 100 bootstrap replicates with GARLI.

2.4. Estimation of evolutionary rates and dates

Seven different datasets were used to estimate the evolutionary rate (μ, units are nucleotide substitutions per site per year; subs/site/year), and the time of the most recent common ancestor (T_{mrca}, years) of the viruses sampled. First, we used the

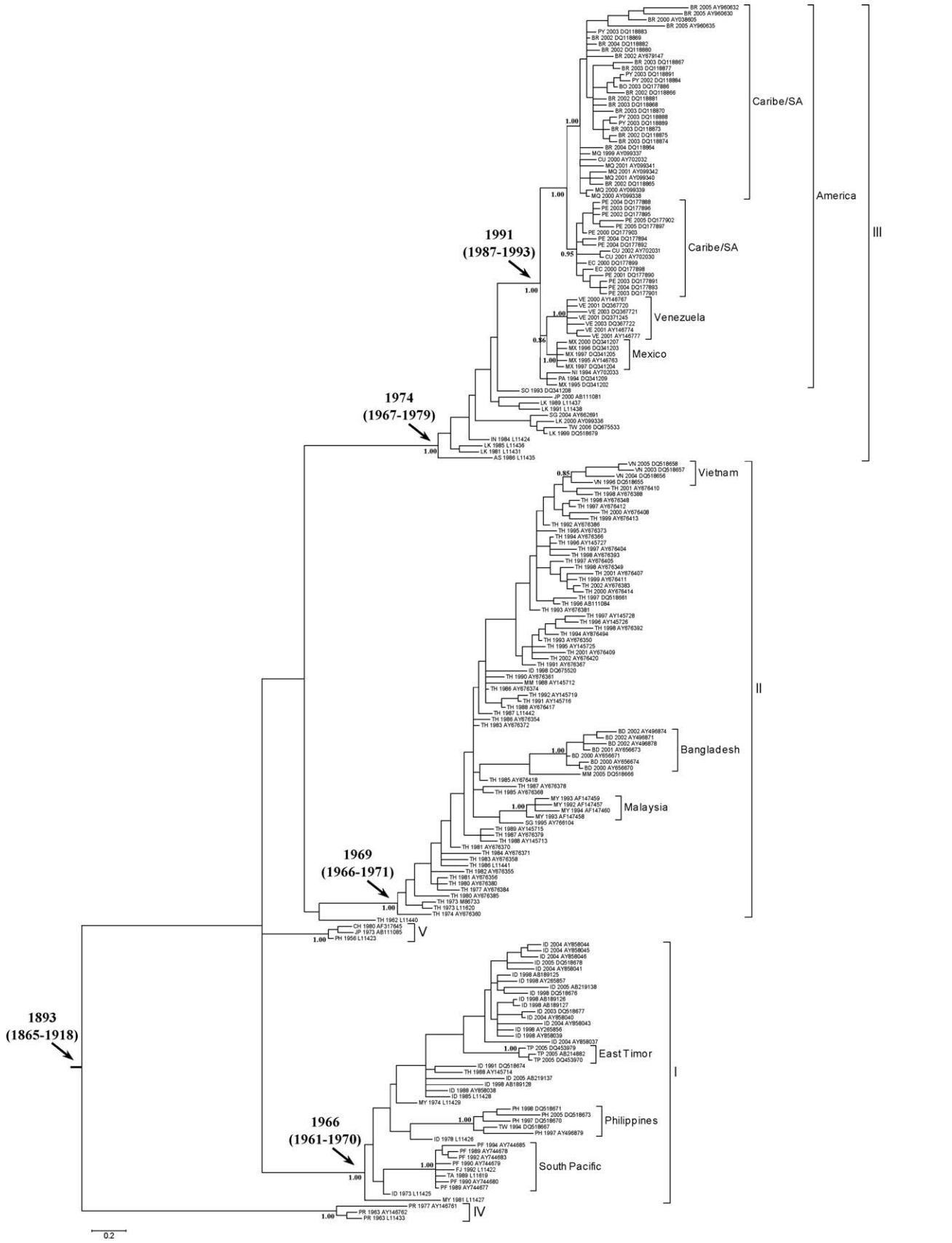

Fig. 1. Majority-rule Bayesian consensus tree of 200 E gene sequences representing the global diversity of DENV-3. Genotypes (roman numerals) and country-specific clades are indicated. Estimates for the age of some relevant nodes on the tree (point to by arrows) are also highlighted. Posterior probabilities are shown for key nodes. The names of DENV-3 isolates include reference to country origin, year of isolation, and GenBank accession number. Country represented are American Samoa (AS), Bangladesh (BD), Bolivia (BO), Brazil (BR), China (CH), Cuba (CU), Ecuador (EC), Fiji (FJ), Indonesia (ID), India (IN), Japan (JP), Sri Lanka (LK), Myanmar (MM), Martinique (MQ), Mexico (MX),

200 E gene sequences to estimates the overall rate and T_{mrca} for DENV-3. The age of relevant internal nodes, such as those corresponding to the MRCA of distinct genotypes, were also estimated by setting up specific taxon subsets within the global DENV-3 dataset. Second, we analyzed the GI, GII, and GIII datasets to obtain rates and divergence times for individual genotypes separately. Third, separate analyses were also performed on the viruses collected from Indonesia (GI-ID dataset), Thailand (GII-TH dataset), and the Americas (GIII-AM dataset) to estimate rates and divergence times for DENV-3 in these specific localities. Evolutionary parameters were estimated by using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST v1.4.7 (Drummond et al., 2002; Drummond and Rambaut, 2006). Analyses were carried out with a Bayesian Skyline coalescent tree prior (Drummond et al., 2005) under the GTR + Γ + I model, and using both a strict and a relaxed (uncorrelated lognormal) (Drummond et al., 2006) molecular clock. MCMC chains were run for 1–3 $\times$ 10^7 generations for each data set, with a burn-in of 1–3 $\times$ 10^6. BEAST outputs were inspected with TRACER v1.4, with uncertainty in parameter estimates reflected by their 95% Highest Posterior Density (HPD) values. All parameters estimates showed ESS values >100. Molecular clock models were compared by calculating the Bayes Factor (BF) (Suchard et al., 2001) from the posterior output of each of the models using TRACER v1.4 as explained in BEAST website (http://beast.bio.ed.ac.uk/Model_comparison). A log BF (natural log units) >2.3 indicates strong evidence against the null model.

3. Results

3.1. Phylogeography of DENV-3

Our phylogenetic analysis of 200 DENV-3 E gene sequences recovered the five genotypes previously described for this serotype (Lanciotti et al., 1994; Wittke et al., 2002). It also suggested the existence of a strong geographical subdivision of DENV-3 population with no evidences of significant co-circulation of distinct genotypes in a single locality (Fig. 1). Genotype I contained most of the Indonesian strains from 1973 to 2004, along with two early Malaysian isolates (1974 and 1981), one Thai isolate (1988), and three well-supported monophyletic groups of strains isolated in South Pacific (1989–1994), Philippines (1997–2005), and East Timor (2005). Genotype II included almost all DENV-3 strains isolated in Thailand between 1973 and 2002, along with two Myanmar strains (1988 and 2005), one isolate from Singapore (1995), one isolate from Indonesia (1998), and three strongly supported groups of strains from Malaysia (1992–1994), Bangladesh (2000–2002), and Vietnam (1996–2005). Genotype III was composed by Sri Lankan isolates from 1981 to 2000, along with single isolates from India (1984), Samoa (1986), Somalia (1993), Japan (2000), Singapore (2004), and Taiwan (2006). Moreover, all American strains sampled after 1994 were monophyletic, suggestive of a single introduction of this genotype into the continent, consistent with previous studies (Aquino et al., 2006; Diaz et al., 2006; Messer et al., 2002; Peyrefitte et al., 2005; Rodriguez-Roche et al., 2005). The American cluster was further subdivided into four well-supported lineages containing isolates from: (1) Mexico, (2) Venezuela, (3) Cuba/Martinique/Brazil/Paraguay/Bolivia, and (4) Cuba/Peru/Ecuador. Genotype IV was the most divergent group and included three early Puerto Rican strains from 1960s and 1970s. Genotype V was represented by the oldest prototype strain Philippines/1956, and two Asian isolates from 1973 and 1980. Finally, one isolate sampled from Thailand in 1962 did not fall into any established genotype and was located toward the common ancestral node of GII and GIII.

3.2. Migration patterns of DENV-3 genotypes

Before reconstructing the MPRs along the trees for each genotype we tested for panmixis by calculating with MacClade the expected number of changes along 25,000 equiprobable trees and again over other 25,000 trees obtained by random partition. The observed number of character state changes for each DENV-3 genotype was much lower than expected by chance ($P < 0.00004$ for all analyses). By increasing the number of random trees we could never sample the observed values in the random distribution (data not shown). Therefore, the DENV-3 genotypes are under high constraints for geographical gene flow and the reconstruction of the observed character state changes along the trees was justified.

Because the viral-based tree did not use geographical information at the inference step, it was used for the reconstruction of the migratory history of samples. Sub-trees for each of the three main DENV-3 genotypes, derived from the global MAP tree obtained with BI, had congruent topologies and supported the same sets of MPRs for each genoptype. All the most parsimonious states at each node are shown in Fig. 2. Nine steps were required to trace the MPRs for GI (Fig. 2a). The character tracings were unequivocal and had a single MPR for all nodes, irrespective of the resolving option. The node connecting it to the global MAP tree had Indonesia as point of radiation, since it was the ancestral state at the root. From there, independent lineages of the virus spread to the French Polynesia, reaching Fiji and Tahiti; to the Philippines, also reaching Taiwan; and more recently to the East Timor. Three distinct lineages related to Indonesia were also found in Malaysia and in Thailand.

Seven steps were required to trace the MPRs for GII (Fig. 2b). The rooting position in the MAP tree and the ancestral state for GII unambiguously suggested an origin in Thailand (T). From there, the virus appeared to have gone into Myanmar (M) and Bangladesh (B). The ancestral state at that node in the tree is ambiguous, including T, B, or M. Using DELTRAN, the state would resolve into T indicating that the virus went into the two places at different occasions. However, ACCTRAN would result on either B or M, indicating that the virus may have moved serially following either T > B > M, T > M > B (*i.e.*, stepping-stone model) or alternatively, B < T > B. Likewise, the independent movement of the GII into Singapore (S) and Malaysia (Y) from Thailand (T) had an ambiguous set of MPRs, including T, S, or Y. DELTRAN suggested Thailand broadcasting to both places and ACCTRAN indicated a stepping-stone process T > Y > S, T > S > Y, or simultaneously from Thailand Y < T > S.

GIII required the highest number of reconstruction steps (23 on average, Fig. 2c). Moreover, its rooting and internal MPRs were problematic. The MAP tree obtained with BI indicated a strain from American Samoa isolated in 1986 at the root of the genotype, but older sequences from 1981 to 1985 were sampled in Sri Lanka. Both rooting options did not alter either the cost or the character transformations (and therefore implied migration events) across the tree, but rooting at American Samoa (as shown in Fig. 2c) leaves the state at the root of the tree undefined. In any case, the virus apparently spread from Sri Lanka into nearby India and more

Malaysia (MY), Nicaragua (NI), Panama (PA), Peru (PE), French Polynesia (PF), Philippines (PH), Puerto Rico (PR), Paraguay (PY), Singapore (SG), Somalia (SO), Tahiti (TA), Thailand (TH), East Timor (TP), Taiwan (TW), Venezuela (VE), Vietnam (VN). The tree is rooted using GIV, which have been shown to represent an appropriate outgroup. Horizontal branch lengths are drawn to scale with the bar at the bottom indicating 0.2 nucleotide substitutions per site.

recently into Japan, Singapore and Taiwan. An interesting event, which epitomized the cosmopolitan nature of GIII, was its sampling in Somalia in 1993 making the elucidation of the entry into the Americas problematic. DELTRAN suggested that the virus was broadcasted into Africa and the Americas from Sri Lanka. ACCTRAN pointed to a scenario in which the virus may have gone from Sri Lanka into Africa, and then into the Americas.

The spread of GIII into the Americas appears to have had Mexico as hub, since the data at hand allowed the unequivocal reconstruction of the ancestral state into the Americas there. Moreover the data resolved well the movement of the virus from Mexico into Venezuela and certainly, two more independent entries into South America that had several possible routes. DELTRAN resolved additional entries into South America via Mexico passing the virus via two routes. The first route went into the Pacific side of the Andes hitting Ecuador and Peru, and the second one went via the Caribbean, with Martinique passing the virus into Brazil and from there into Bolivia and Paraguay. ACCTRAN did not resolve if Mexico or Martinique, was the origin of the virus that went into the Pacific side of South America. However, there was no issue with the reconstruction of the entry into Brazil from Martinique. Moreover, both DELTRAN and ACCTRAN were unanimous in that a virus coming from Pacific side of the Andes moved back from South America into Cuba.

3.3. Estimation of evolutionary rates and dates

The phylogenetic tree of globally sampled DENV-3 isolates was characterized by a clear temporal structure, with the oldest sampled viruses tending to fall closest to the root of the tree, while those sampled more recently were located at the most distal tips (Fig. 1). This temporal structure allowed us to estimates the rate of molecular evolution and the T_{mrca} for different DENV-3 datasets. The substitution rate was first estimated for the complete DENV3 data set of 200 sequences, using both strict and relaxed molecular clock models. The BF analysis clearly favored a relaxed molecular clock model over a strict clock model (Table 2), indicating detectable variation in evolutionary rates among branches. The coefficient of rate variation was estimated at 0.28 (95% HPD, 0.15–0.41). Despite this variation, the median evolutionary rate (E gene) and T_{mrca} of DENV-3 estimated under both strict ($\mu = 8.7 \times 10^{-4}$ subs./site/yr; $T_{mrca} = 1891$) and relaxed ($\mu = 8.9 \times 10^{-4}$ subs./site/yr; $T_{mrca} = 1893$) molecular clock models were very close (Table 3). This result was also similar to that previously obtained by Twiddy et al. ($\mu = 9.0 \times 10^{-4}$ subs./site/yr, $T_{mrca} \sim 1900$) using a much smaller data set of DENV-3 sequences ($n = 21$) (Twiddy et al., 2003). By setting up specific taxon sub-groups within the global DENV-3 phylogeny, we also estimated the age of the major genotypes resulting in a median T_{mrca} estimate of 1967 for GI and GII, and 1975 for GIII, under either strict or relaxed molecular clock models (Table 3).

The detected variation in evolutionary rates among DENV-3 lineages could reflect rate differences among distinct genotypes (Twiddy et al., 2003), and/or rate heterogeneity within a single genotype. To test these hypotheses, we analyzed each genotype separately. The BF analysis showed that the relaxed clock method was favored over the strict clock method in the GII and GIII data sets (Table 2), indicating significant rate heterogeneity within these genotypes. The coefficient of rate variation for GII and GIII was estimated at 0.25 (95% HPD, 0.01–0.44) and 0.37 (95% HPD, 0.13–0.61), respectively. On the other hand, the median evolutionary rate estimates of the distinct genotypes were very similar and displayed a considerable overlap of HPD intervals (Table 3), clearly suggesting that there are no major differences in evolution rate among main DENV-3 genotypes. Accordingly, the median T_{mrca} estimated for each genotype was almost equal to that previously obtained using the complete DENV-3 data set (Table 3).

It has been suggested that under epidemic conditions, such as when a new variant is introduced into a susceptible population, the mean viral evolutionary rate could be higher than under endemic conditions (Twiddy et al., 2003). To test this hypothesis, we compared the DENV-3 in Indonesia and Thailand, where GI and GII have been evolving since the early 1970s, with DENV-3 lineages from the Americas, where GIII only emerged in the early 1990s. Significant rate heterogeneity was detected within GIII American lineages (Table 2). The median evolutionary rate of DENV-3 lineages circulating in different regions, however, was very similar and displayed a considerable overlap of HPD intervals (Table 4), indicating no major differences in rates of DENV-3 among regions with endemic or epidemic patterns of dengue transmission. The median T_{mrca} estimated for GI-ID and GII-TH datasets were close to that previously estimated using all GI and GII sequences (Table 4), supporting the notion that Indonesia and Thailand are the epicenters for these DENV-3 lineages; whereas the median T_{mrca} for the GIII-American clade was estimated around 1991 (Table 4).

4. Discussion

This study represents the largest phylogeographic and evolutionary analysis reported for DENV-3 to date. Our phylogenetic analysis of 200 DENV-3 E sequences with world-wide distribution revealed a clear geographical subdivision of viral strains. Genotypes I, II, and III have been evolving independently in Indonesia, Thailand, and Sri Lanka, respectively, over the last 30–40 years. Our data supports the notion that these countries not only sustain the oldest DENV-3 epidemics but also were sources for dengue lineages that have subsequently spread over the world. Strains more recently isolated in South Pacific (1989–1994), Philippines (1997–2005), East Timor (2005), Malaysia (1992–1994), Bangladesh (2000–2002), Vietnam (1996–2005), and the Americas (1994–2006) segregated into distinct monophyletic clusters within the main genotypes; indicating that each country formed a geographically distinct mostly self-contained region with regard to DENV-3 viruses, with few instances of repeated gene flow among regions.

The plausible routes of DENV-3 migration are described in Fig. 3. According to our analyses the spread of GI was mainly circumspect to the maritime portion of Southeast-Asia (East Timor, Malaysia, and Philippines) and South Pacific, where most migrant strains appeared to have been broadcasted from Indonesia. By contrast, most GII strains appeared to have been broadcasted from Thailand and stayed within continental areas in South-East Asia (Bangladesh, Myanmar, Singapore, and Vietnam), with the exception of Malaysia. GIII was the most widely spread of all DENV-3 genotypes, and most GIII strains found in Asia, East Africa and the Americas appeared to have been transmitted from or near from Sri Lanka. It is unclear if the American GIII lineage came from Africa or Asia. The oldest GIII sequences in the Americas were identified in Panama and Nicaragua in 1994 (CDC, 1995; Guzman et al., 1996), but our migration data suggested that the GIII was introduced into the Americas through Mexico where the first GIII strains were identified in 1995 (Briseno-Garcia et al., 1996). More sampling in those countries will be necessary to elucidate the precise point of introduction of GIII to the Americas. In any case, GIII viruses rapidly spread to others countries in the region (Nogueira et al., 2001; Peyrefitte et al., 2003; Rigau-Perez et al., 2002; Usuku et al., 2001; Uzcategui et al., 2003), using several independent routes from Central America to the Caribbean and South America.

Our phylogeographic analysis also revealed that the co-circulation of different DENV-3 genotypes in a single location is

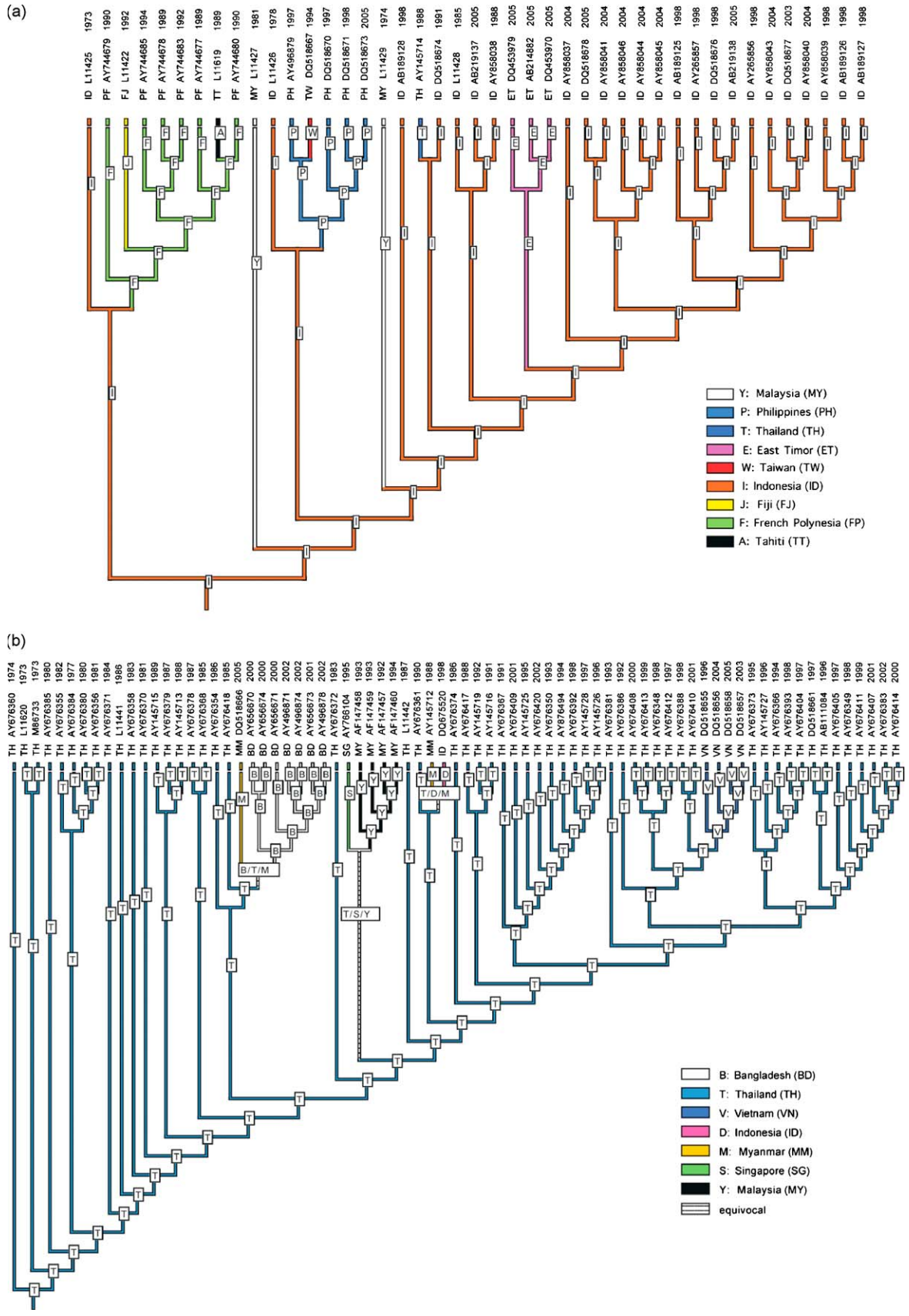

Fig. 2. Migration patterns of genotypes I (a), II (b), and III (c). The names of DENV-3 isolates include reference to country origin, GenBank accession number, and year of isolation. The color of each branch represents the country of origin of the sequence corresponding to that branch, according to the figure legend.

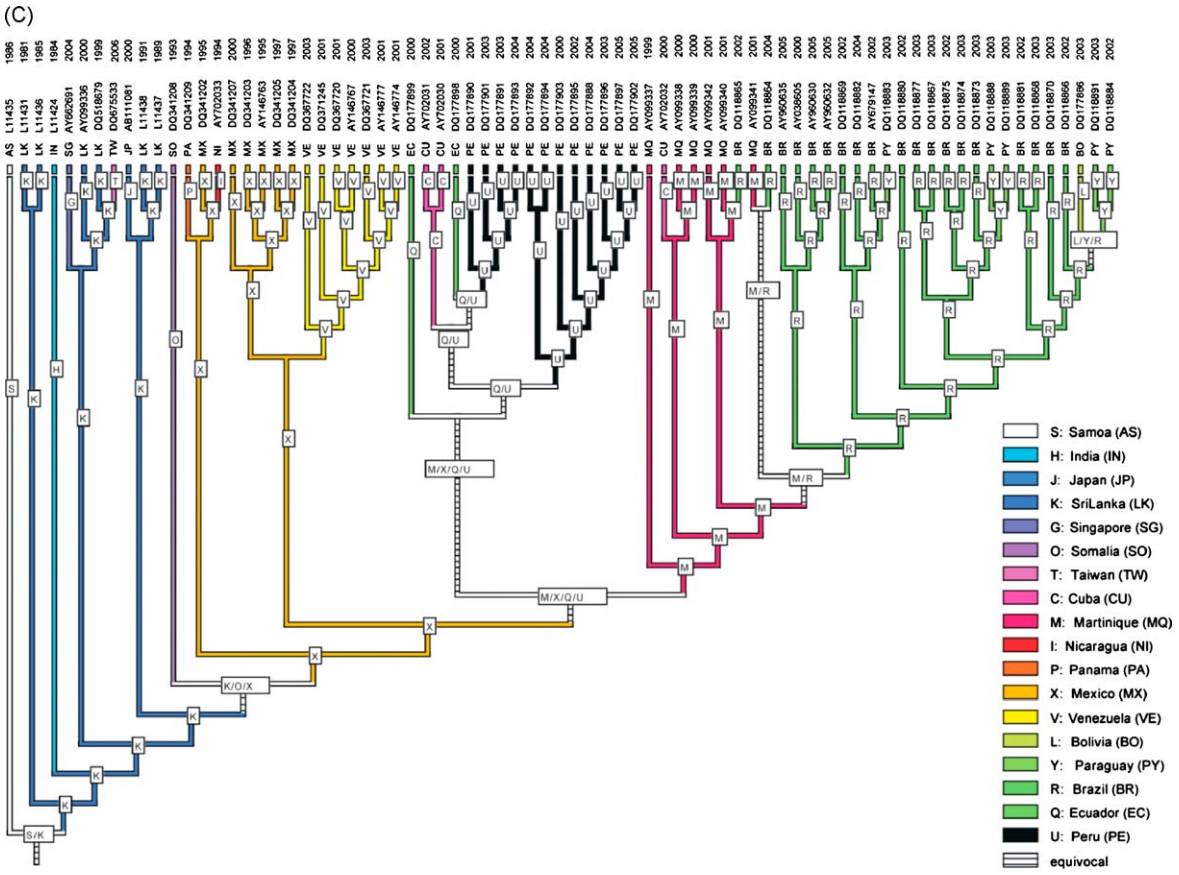

Fig. 2. (*Continued*).

a rare event. This could result from a limited gene flow among distantly geographic regions. However, genotype differences were observed even between neighboring countries like Indonesia and Thailand, where DENV-3 epidemics have been consistently dominated by GI and GII, respectively, since the 1970s. Notwithstanding, there is evidence of incursions into Indonesia by "Thai-like" GII strains in 1988 (represented by strain DQ675520) (Raekiansyah et al., 2005), as well as into Thailand by "Indonesian-like" GI strains in 1998 (represented by strain AY145714) (Wittke et al., 2002). Nevertheless, these incursions seem to have failed to become established, since none of the later Indonesian and Thai isolates grouped within GII and GI, respectively. These facts suggested that several factors, other than geographic proximity, could have had a significant impact on the observed spatial dispersion patterns of DENV-3.

It is hard to envisage a general vicariance mechanism that would explain a lack of genotype overlapping at the same geographic locality. Possibly competition among genotypes, and/or regional differences in mosquito vector competence for each genotype (Anderson and Rico-Hesse, 2006; Armstrong and Rico-Hesse, 2001; Cologna et al., 2005) may be involved. Another possible explanation for the observed patterns could involve viral neutralization by cross-immunity among closely related strains caused by a pre-exposed human population. This would allow for distinct serotype co-circulation but make it difficult for intra-serotype (*i.e.*, genotype) co-circulation, due to a reduction in numbers of the available susceptible human hosts to levels below that necessary to sustain significant epidemics (Adams et al., 2006). This would help explain why the evolution of DENV-3 is characterized by phylogenetic trees with a strong temporal

Table 2
Bayes factors between different molecular clock models for DENV-3.

Dataset	Model comparison	log BF[a]	Evidence against H_0[b]
DENV-3	Strict (H_0) vs. relaxed (H_1) clock	22.8 (0.4)	Decisive
GI	Strict (H_0) vs. relaxed (H_1) clock	−0.8 (0.4)	Negative
GII	Strict (H_0) vs. relaxed (H_1) clock	5.0 (0.5)	Decisive
GIII	Strict (H_0) vs. relaxed (H_1) clock	10.8 (0.5)	Decisive
GI-ID	Strict (H_0) vs. relaxed (H_1) clock	−0.4 (0.3)	Negative
GII-TH	Strict (H_0) vs. relaxed (H_1) clock	1.6 (0.4)	Weak
GIII-AM	Strict (H_0) vs. relaxed (H_1) clock	12.8 (0.4)	Decisive

[a] log BF (Bayes factor) is the difference (in natural log units) of the marginal likelihood of null (H_0) and alternative (H_1) model. The standard error of the estimates is given in parenthesis.

[b] Evidence against H_0 is assessed in the following way: ln BF <0 indicates no evidence against the null model; ln BF between 0 and 2.3 indicates weak evidence against the null model, ln BF between 2.3 and 3.4 indicates strong evidence against the null model; ln BF between 3.4 and 4.6 indicates very strong evidence against the null model; ln BF >4.6 indicates decisive evidence against the null model.

Table 3
Estimated substitutions rates and dates for DENV-3 genotypes.

Dataset	Molecular clock	μ	T_{mrca} DENV-3	T_{mrca} GI	T_{mrca} GII	T_{mrca} GIII
DENV-3	Strict	8.7×10^{-4} (7.7×10^{-4} to 9.7×10^{-4})	1891 (1876–1904)	1967 (1963–1970)	1967 (1965–1970)	1975 (1972–1978)
	Relaxed	8.9×10^{-4} (7.9×10^{-4} to 10.0×10^{-4})	1893 (1865–1918)	1967 (1963–1970)	1967 (1964–1970)	1975 (1972–1978)
GI	Strict	8.3×10^{-4} (6.8×10^{-4} to 9.8×10^{-4})	–	1966 (1961–1970)	–	–
	Relaxed	8.4×10^{-4} (6.9×10^{-4} to 10.1×10^{-4})	–	1966 (1961–1970)	–	–
GII	Strict	10.1×10^{-4} (8.7×10^{-4} to 11.7×10^{-4})	–	–	1969 (1966–1971)	–
	Relaxed	10.3×10^{-4} (8.7×10^{-4} to 12.0×10^{-4})	–	–	1969 (1966–1971)	–
GIII	Strict	8.1×10^{-4} (6.6×10^{-4} to 9.7×10^{-4})	–	–	–	1974 (1969–1978)
	Relaxed	8.2×10^{-4} (6.6×10^{-4} to 9.9×10^{-4})	–	–	–	1974 (1967–1979)

Estimates of the median evolutionary rate (μ, substitutions site^{-1} year^{-1}) and, median time for the most recent common ancestor (T_{mrca}, year). The age estimated for some relevant internal nodes within the global DENV-3 tree (corresponding to the T_{mrca} of major genotypes) are also shown. 95% HPD intervals are shown between parentheses.

Table 4
Estimated substitutions rates and dates for DENV-3 in Indonesia, Thailand and the Americas.

DENV	Genotype	Region	μ	T_{mrca}
3	I	Indonesia	8.4×10^{-4} (6.2×10^{-4} to 10.7×10^{-4})	1970 (1966–1973)
	II	Thailand	10.0×10^{-4} (8.2×10^{-4} to 11.8×10^{-4})	1969 (1966–1971)
	III	America	9.2×10^{-4} (6.9×10^{-4} to 11.5×10^{-4})	1991 (1987–1993)
2	Asian I[a]	Thailand	8.5×10^{-4} (7.2×10^{-4} to 9.9×10^{-4})	–
	Asian-American[b]	America	8.0×10^{-4} (6.6×10^{-4} to 9.5×10^{-4})	–
4	I[c]	Thailand	10.7×10^{-4} (8.4×10^{-4} to 13.1×10^{-4})	–
	II[b]	America	8.3×10^{-4} (6.8×10^{-4} to 10.0×10^{-4})	–

Estimates of the median evolutionary rate (μ, substitutions site^{-1} year^{-1}), and median time of the most recent common ancestor (T_{mrca}, year) for the GI-ID (strict molecular clock), GII-TH (relaxed molecular clock), and GIII-AM (relaxed molecular clock) datasets. 95% HPD intervals are shown between parentheses.
[a] Data taken from Zhang et al. (2006).
[b] Data taken from Carrington et al. (2005).
[c] Data taken from Klungthong et al. (2004).

structure as previously noted for this and other DENV serotypes (Goncalvez et al., 2002; Klungthong et al., 2004; Twiddy et al., 2003; Wittke et al., 2002; Zhang et al., 2005, 2006), which may indicate the strong pruning effect of DENV lineages by host immunity. Finally, we cannot also exclude the possibility that GI and GII are circulating as minor variants in Thailand and Indonesia, respectively, but have remained undetectable because the low number of sequences analyzed (sampling bias).

Our analyses of rates of nucleotide substitution in DENV-3 revealed that the relaxed clock model outperforms the strict clock model, indicating detectable variation in evolutionary rates among DENV-3 lineages. Significant rate variation among genotypes was described previously for DENV-2 (Twiddy et al., 2003), suggesting that rate variation detected in DENV-3 could be also consequence of differences among genotypes. However, the analysis of each DENV-3 genotype separately revealed that rate heterogeneity in DENV-3 can be mainly explained by rate differences within genotypes, particularly within GII and GIII, rather than among genotypes. The median evolutionary rate of the distinct DENV-3 genotypes analyzed was very similar (ranging from 8.3×10^{-4} subs./site/yr to 10.3×10^{-4} subs./site/yr) and displayed a considerable overlap of the HPD intervals, clearly suggesting no major differences in evolution rate among genotypes. It is also interesting to note that although the relaxed clock model outperforms the strict clock model in most DENV-3 datasets analyzed, the median value of estimates obtained under both relaxed and strict clock models were very close in all analyses. This is fully consistent with the concept that the substitution rate estimated from large data sets are reliable indicators of the average rate of evolution, even if rate heterogeneity is present (Jenkins et al., 2002).

A previous study suggested that DENV-3 is evolving at a rate ($\mu = 9.0 \times 10^{-4}$ [7.3×10^{-4} to 10.8×10^{-4}] subs./site/yr) significantly faster than other DENV serotypes; and proposed similar rates of substitutions for each DENV-3 genotype (GI = 7.5×10^{-4} [4.5×10^{-4} to 10.7×10^{-4}] subs./site/yr; GIII = 11.6×10^{-4} [7.8×10^{-4} to 15.9×10^{-4}] subs./site/yr) (Twiddy et al., 2003). Those estimates, however, were based on the analysis of very small DENV-3 datasets (total = 21, GI = 8, GIII = 8), and no estimations of the substitution rate for GII were provided. We re-estimated these evolutionary rates using much larger data sets of DENV-3 sequences (total = 200, GI = 43, GII = 75, GIII = 75). Our median rate estimates for DENV-3 (8.9×10^{-4} subs./site/yr) and GI (8.3×10^{-4} subs./site/yr) were similar to those previously reported by Twiddy et al. whereas the median rate estimated for GIII (8.2×10^{-4} subs./site/yr) was considerably lower, although within the large confidence interval of the previous estimate. The median evolutionary rate estimated for GII was 10.3×10^{-4} subs./site/yr. Overall, the confidence intervals of our estimates (6.6×10^{-4} to 12×10^{-4}) were significantly narrower than those described by Twiddy et al. (4.5×10^{-4} to 15.9×10^{-4}), probably due to the higher number of sequences used in the present study.

It has been suggested that the ecological conditions for DENV dissemination may alter the viral evolutionary rate, which could explain some rate differences among dengue lineages previously described (Twiddy et al., 2003). Our analyses revealed, however, that the median evolutionary rate of GI in Indonesia and GII in Thailand (areas with long-term endemic infections), was similar to that observed for GIII in the Americas (a region with a recent epidemic pattern of DENV-3 transmission). Our DENV-3 rate estimates were also comparable to those estimated for DENV-2 (genotype Asian I) and DENV-4 (genotype I) circulating in Thailand (Klungthong et al., 2004; Zhang et al., 2006), and for DENV-2 (genotype Asian-American) and DENV-4 (genotype II) circulating in the Americas (Carrington et al., 2005) (Table 4). These studies confirmed a lack of association between dengue substitution rate and ecological pattern of virus spread, and revealed no major

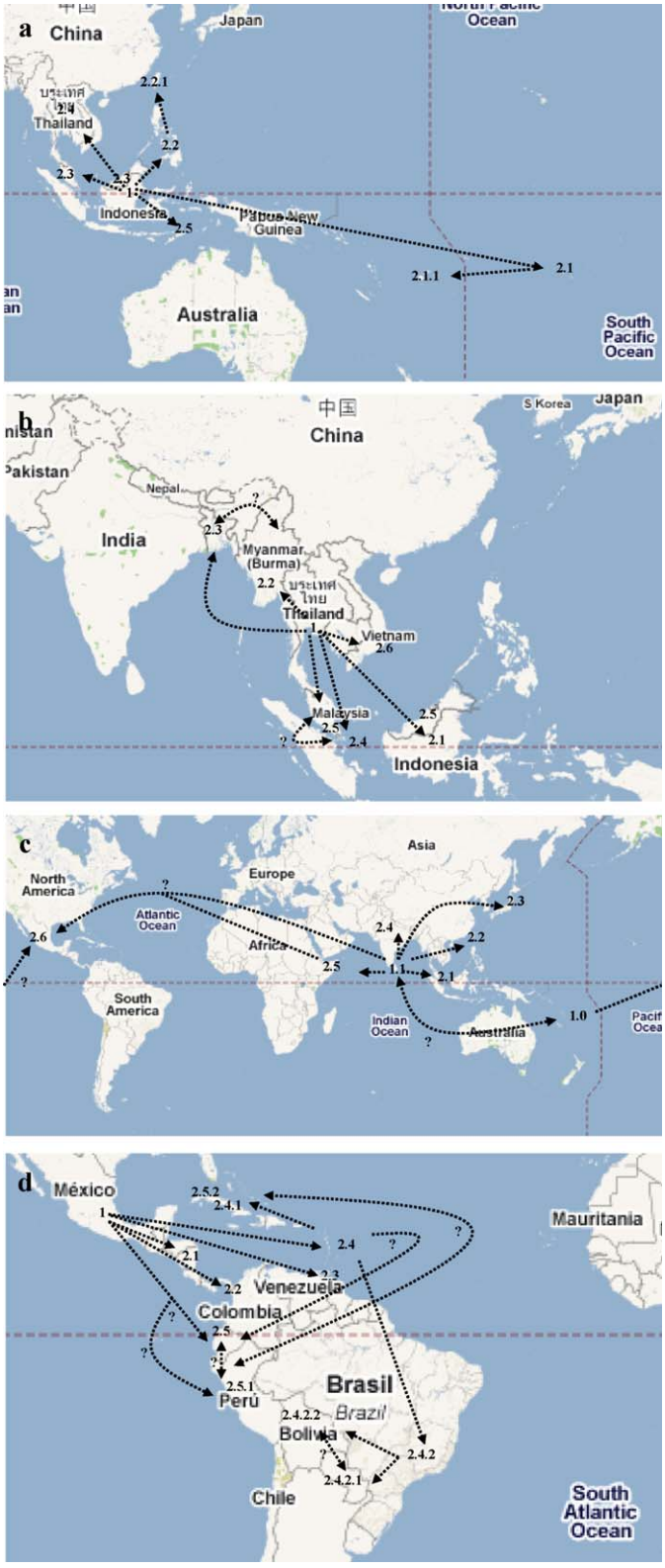

Fig. 3. Plausible sites of origin and migration routes of DENV-3 genotypes I (a), II (b), III (c) and III-AM (d). (a) (1) Indonesia, (2.1) French Polynesia/Tahiti, (2.1.1) Fiji, (2.2) Philippines, (2.2.1) Taiwan, (2.3) Malaysia, (2.4) Thailand, (2.5) East Timor. (b) (1) Thailand, (2.1) Indonesia, (2.2) Myanmar, (2.3) Bangladesh, (2.4) Singapore, (2.5) Malaysia, (2.6) Vietnam. (c) (1.0) Samoa, (1.1) Sri Lanka, (2.1) Singapore, (2.2) Taiwan, (2.3) Japan, (2.4) India, (2.5) Somalia, (2.6) Mexico. (d) (1) Mexico, (2.1) Nicaragua, (2.2) Panama, (2.3) Venezuela, (2.4) Martinica, (2.4.1) Cuba (2000), (2.4.2) Brazil, (2.4.2.1) Paraguay, (2.4.2.2) Bolivia, (2.5) Ecuador, (2.5.1) Peru, (2.5.2) Cuba (2001/2002). Inconclusive routes of DENV-3 were identified with the signal "?".

lineage-specific rate differences among DENV-2, DENV-3, and DENV-4. Whether lineage-specific rate differences in DENV evolution previously described (Twiddy et al., 2003) really exist, or simply reflects a previous use of much smaller datasets needs further investigation.

We estimated the T_{mrca} of DENV-3 at around 1890, fully consistent with previous estimation ($T_{mrca} \sim 1900$) (Twiddy et al., 2003). Our analysis also suggested that the current global genetic diversity of genotypes I, II, and III arose almost simultaneously within a short time period between the middle 1960s and the middle 1970s, coinciding with the description of the first cases of DHF by DENV-3 in Asia (Gubler et al., 1979; Nisalak et al., 2003; Sumarmo, 1987; Wallace et al., 1980) and the rapid increase in human population size, urbanization, and human movement. According with our estimations, GIII strains were probably introduced into Latin America around 1991, few years earlier than the initial detection of this genotype in the continent in 1994 (CDC, 1995; Guzman et al., 1996). Similar time intervals of few years between the estimated introduction and initial detection were also described for DENV-2 and DENV-4 in the Americas (Carrington et al., 2005).

In conclusion, this study proposes that global DENV-3 evolution could be well characterized as a collection of discrete, country-specific viral population bursts, with limited co-circulation of distinct genotypes in a single region. Despite this strong spatial subdivision, DENV-3 strains of distinct genotypes and from different localities have been evolving at roughly the same rate over time. Whether such similar evolutionary rate estimates translate into comparable biological properties (such as transmissibility, infectiousness, and/or virulence) across distinct DENV-3 lineages is still unclear. Our data also suggested that the current diversity of the three main DENV-3 genotypes arose within the last 30–40 years, coinciding with the emergence of large-scale DHF/DSS epidemics in Asia.

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