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Short communication

Modeling gene sequence changes over time in type 3 dengue viruses from Ecuador

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ABSTRACT

Dengue virus (DENV) is a member of the genus *Flavivirus* of the family *Flaviviridae*. DENV-3 re-emerged in Central America in 1994, and continues to expand into the South American region. Little is known about the evolutionary rates, viral spread and population dynamics of this genotype in the Latin American region. In order to gain insight into these matters, we used a Bayesian Markov chain Monte Carlo (MCMC) approach, to analyze envelope (E) gene sequences of the DENV-3 genotype III of strains included in a monophyletic cluster composed by Ecuadorian as well as strains from Cuba, Puerto Rico and Peru. The results of these studies revealed that the expansion population growth model was the best fit to the data. The most common recent ancestor (MRCA) was placed around 1989, in agreement with the first reports of the emergence of this new DENV-3 type. A mean rate 1.033×10^{-3} nucleotide substitution per site per year was obtained. This rate is comparatively higher than the ones obtained for DENV-2 and DENV-4 in the same region. Faster population growth and greater population dispersal may have contributed to the vigorous initial transmission dynamics of this genotype in the Latin American region.

Dengue virus (DENV) is a member of the genus *Flavivirus* of the family *Flaviviridae*.

DENV are mosquito-borne flaviviruses with a single-stranded, nonsegmented, positive-sense RNA genome of approximately 11 kb in length (Rice, 1996). Dengue viruses are comprised of four distinct serotypes (DENV1 through DENV4), which are transmitted to humans through the bites of two mosquito species: *Aedes aegypti* and *Aedes albopictus* (Clyde et al., 2006).

DENV causes a wide range of diseases in humans, from the acute febrile illness dengue fever (DF) to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Dengue has spread throughout tropical and subtropical regions worldwide over the past several decades, with an estimated 100 million infections and tens of millions of cases occurring annually (Thomas et al., 2003). DHF/DSS is one of the leading causes of pediatric hospitalization in Southeast Asia, and has become endemic to many Latin American countries over the last 25 years (Thomas et al., 2003). During 2001, more than 600,000 cases of dengue infection were reported in the Americas, including 15,000 cases of DHF/DSS (WHO, 2002). Just in Brazil, a total of 4,243,049 dengue cases have been reported between 1981 and 2006, including 5817 cases of DHF/DSS (Nogueira et al., 2007).

Based on sequence analysis of the E/NS1 region, and using a cut-off of 6% divergence, each DENV serotype can be divided in different genotypes (DENV-1–4) (Rico-Hesse, 1990). In the case of DENV-3, this serotype has been divided into four genotypes (I–IV) (Holmes and Twiddy, 2003; Lanciotti et al., 1994; Messer et al., 2003), sometimes including a genotype V (Diaz et al., 2006).

Recent findings have demonstrated the emergence and global spread of DENV-3 genotype III (Messer et al., 2003). The emergence of DHF in Sri Lanka in 1989 coincided with the appearance that of a new DENV-3, genotype III variant, which spreads from the Indian subcontinent into Africa and Latin America (Messer et al., 2003). Recent studies have revealed a close genetic relation among DENV-3 strains recently isolated in Ecuador and DENV-3 isolated in the Caribbean region (Regato et al., 2008). Sri Lankan DENV-3 genotype III and associated American isolates have been linked to severe disease epidemics (Silva et al., 2008).

Despite the importance of these epidemics, little is known about the evolutionary rates, viral spread and population dynamics of DENV-3 genotype III in the South American region.

In order to gain insights into these matters, sera samples from 23 Ecuadorian patients presenting dengue-like syndromes were obtained at Instituto Nacional de Higiene y Medicina Tropical

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"Leopoldo Izquieta Perez" at Guayaquil, Ecuador. All samples from these patients were found to be positive for dengue infection by the presence of immunoglobulin M (IgM), elevation of specific IgG, or both, using an "in house" dengue virus-specific enzyme-linked immunosorbent assay (ELISA).

In order to assign each Ecuadorian DENV strain to an appropriate serotype, serum samples from patients tested positive in the serology assays underwent reverse transcription-PCR (RT-PCR), using a multiplex PCR method according to Harris et al. (1998). By these means, it was possible for us to assign 8 of the 23 Ecuadorian strains to DENV-3 serotype (not shown). Then, serum samples from patients assigned to DENV-3 underwent RT-PCR according to Aquino et al. (2006) in order to obtain amplicons containing the full-length sequences from the DENV envelope (E) gen. To avoid false positive results, the recommendations of Kwok and Higuchi (1989) were strictly adhered to. Amplicons were purified using QIAquick PCR Purification Kit from QIAGEN, according to instructions from the manufacturers. The sequence reaction was carried out using the Big Dye DNA sequencing kit (PerkinElmer) on a 373 DNA sequencer apparatus (PerkinElmer). Both strands of the PCR product were sequenced in order to avoid discrepancies. Envelope (E) gene nucleotide sequences from the DENV-3 Ecuadorian strains, corresponding to position 1014 through 2413 of the DENV genome (relative to the sequence of DENV-3 strain NC001475) were obtained. These sequences were deposited in the EMBL Database under accession numbers FM246466 through FM246473, (see also Table 1). To study the degree of genetic variability of the DENV strains isolated in Ecuador, the DENV-3 E code sequences obtained from Ecuadorian patients were aligned with 18 comparable sequences of DENV-3 genotype III strains isolated in the Latin American region for whom their genotype was previously described, and 3 DENV-3 genotype II comparable sequences from strains isolated in Thailand (as an outgroup), using the CLUSTAL W program (Thompson et al., 1994). All these sequences were obtained by the use of the Flavitrack database (Misra and Schein, 2007; available at: http://carnot.utmb.edu/flavitrack/resultIndex.php).

Table 1
Origins of the DENV strains.

Name	Year of Isolation	Country of Isolation	Accession number
D3BR/BV4/02	2002	Brazil	DQ118865
D3BR/CU6/02	2002	Brazil	DQ118866
D3BRD3BR/GO5/03	2003	Brazil	DQ118867
D3BR/IG10/03	2003	Brazil	DQ118868
D3PY/AS9/03	2003	Brazil	DQ118885
D3BR/BR8/04	2004	Brazil	DQ118864
D3BR/PP15/04	2004	Brazil	DQ118878
Cuba116/00	2000	Cuba	AY702032
Cuba580/01	2001	Cuba	AY702030
Cuba21/02	2002	Cuba	AY702031
EC8241	2000	Ecuador	FM246468
EC5080	2001	Ecuador	FM246467
EC9110	2003	Ecuador	FM246470
EC8801	2004	Ecuador	FM246469
EC15082	2004	Ecuador	FM246472
EC9266	2005	Ecuador	FM246473
EC9233	2005	Ecuador	FM246471
EC4860	2008	Ecuador	FM246466
D3PY/AS12/02	2002	Paraguay	DQ118884
D3PY/FM11/03	2003	Paraguay	DQ118886
BID V1075	1998	Puerto Rico	EU482563
BID V1090	1998	Puerto Rico	EU529703
BID V1077	2000	Puerto Rico	EU529697
BID V1091	2004	Puerto Rico	EU529704
BID V1415	2007	Puerto Rico	EU596492
OBT412/Tumbes	2000	Peru	DQ177903
FSP581/Piura	2001	Peru	DQ177890
FSL706/Loreto	2002	Peru	DQ177889
IQD1728/Iquitos	2002	Peru	DQ177895
IQD5132/Iquitos	2003	Peru	DQ177896
FST289/Tumbes	2004	Peru	DQ177892
FSL1212/Yurimaguas	2004	Peru	DQ177888
ThD3_0012_90	1990	Thailand	AY676361
ThD3_0472_93	1993	Thailand	AY676381
ThD3_0396_94	1994	Thailand	AY676382

For strain names, country and year of isolation, and accession numbers see Table 1.

Once aligned, we first tested whether a recombination event occurred on any of the sequences used in these studies. We used two approaches implemented in the SimPlot Program (Lole et al., 1999): (1) a sliding window analysis of distances and (2) the bootscanning (Salminen et al., 1995). No recombinant strains were found in the dataset (not shown).

The program Modelgenerator (Keane et al., 2006) was used to identify the optimal evolutionary model (Akaike Information Criteria and Hierarchical Likelihood Ratio Test indicated that the GTR+Γ model fit the sequence data). Using this model, maximum likelihood trees were constructed using software from the PhyML program (Guindon et al., 2005, available at: http://www.phylogeny.fr/phylo_cgi/phyml).

As a measure of the robustness of each node, we employed an approximate Likelihood Ratio Test (aLRT), which assesses that the branch being studied provides a significant likelihood gain, in comparison with the null hypothesis that involves collapsing the branch but leaving the rest of the tree topology identical (Anisimova and Gascuel, 2006). aLRT was calculated using three different approaches: (a) by minimum of Chi2-based calculations; (b) a Shimodaira-Hasegawa-like procedure (SH-like) (Shimodaira and Hasegawa, 2001; Shimodaira, 2002), which is non-parametric, and (c) a combination of both (SH-like and the minimum Chi2-based calculations), which is the most conservative option for these calculations. The results of these studies are shown in Fig. 1.

All DENV strains cluster in the tree according to their genotype. Inside genotype III cluster of Latin American strains, different genetic lineages can be observed. One lineage is composed mainly by strains isolated in Brazil and Paraguay (see Fig. 1, top), while another lineage includes all strains isolated in Ecuador and strains isolated in Cuba, Puerto Rico and Peru (Fig. 1, middle). This is in agreement with very recent analysis of DENV-3 genotype III circulating in northern South America (Kochel et al., 2008). A third cluster, exclusively composed of strains isolated in Puerto Rico, is also observed (Fig. 1, bottom). All of these branches have very high values of aLRT, and suggest a diversification of DENV-3 genotype III in the Latin American region, in agreement with very recent results (Kochel et al., 2008).

In order to gain insight into the evolutionary rate and mode of evolution of DENV-3 genotype III circulating the Latin American region, we used a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the BEAST package (Drummond and Rambaut, 2007; BEAST v1.4.8, available from http://evolve.zoo.ox.ac.uk/beast), to analyze envelope (E) gene sequences of DENV-3 genotype III of strains included in the cluster composed by Ecuadorian as well as strains from Cuba, Puerto Rico and Peru (see Fig. 1). Using the GTR+Γ model and 20 million steps of MCMC, different population dynamic models were tested (constant population size, exponential population growth, expansion population growth, logistic population growth and Bayesian skyline). Consistently, all the analyses revealed similar evolutionary rates. Statistical uncertainty in the data is reflected by the 95% highest probability density (HPD) values. Results were examined using the TRACER program from the BEAST package. Convergence was assessed with ESS (Effective Sample Size) values, after a burning of 2 million steps. Comparison of the values obtained for likelihood as well as ESS of these models revealed that the expansion population growth model was the best fit to the data.

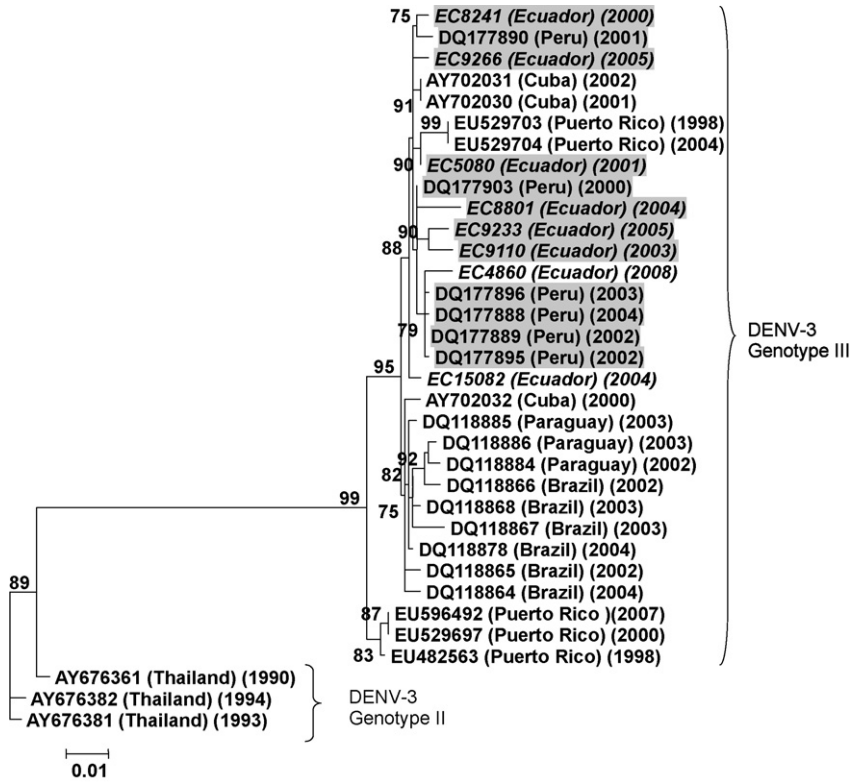

Fig. 1. Maximum likelihood phylogenetic tree analysis DENV-3 isolated in Ecuador. Strains in the trees are shown by their accession number for strains previously described followed by their year and geographic location of isolation between parentheses. Ecuadorian strains are shown by name in italics. Strains included in the Bayesian coalescent inference studies are highlighted in grey. Numbers at the branches show aLRT values obtained using a combination of SH-like and minimum Chi2-based calculations according to Anasimova and Gascuel (2006), as implemented in the PhyML program (Guindon et al., 2005). The scale bar indicates nucleotide substitutions per site. For results found for aLRT using Chi2-based or SH-like calculations see Supplementary Material Fig. 1.

The results shown in Table 2 are the outcome of the analysis for 20 million steps of the MCMC, using the GTR+Γ model, a relaxed clock (Drummond et al., 2006) and the expansion population growth model (Drummond et al., 2005).

As can be seen in Table 2, our results suggest that DENV-3 genotype III, which currently circulates in South America, evolved from ancestors that existed around 1989. This is in agreement with the first reports of the emergence of this new DENV-3 variant in Sri Lanka in 1989, which spread to Africa and into the Latin American region (Messer et al., 2003, see also Table 2). Despite large geographic distances and marine barriers, the results of these studies show that these viruses can quickly spread throughout a region and to expand to other regions of the world.

When the GTR+Γ model is used, a mean rate of 1.033×10^{-3} nucleotide substitution per site per year was obtained for the DENV-3 genotype III cluster studied (Table 2). This rate is comparatively higher than those previously estimated for dengue virus by Twiddy et al. (2003) using maximum likelihood method and for mosquito-borne flaviviruses (Zanotto et al., 1996). Moreover, this rate is higher than the ones obtained for DENV-2 genotype III and DENV-4 genotype II (8.0×10^{-4} and 8.3×10^{-4} substitutions/site/year, respectively), obtained in similar studies done in DENV populations circulating in the Americas (Carrington et al., 2005). Besides, the logistic model of population growth was the best fit to the data for both DENV-2 and 4 serotypes in the same study (Carrington et al., 2005). This is in contrast with the results found in these studies for DENV-3 genotype III, in which the expansion population growth model is the most appropriate.

Why DENV-3 genotype III might have a different population dynamics in comparison with DENV-2 and DENV-4? One possible

Table 2
Bayesian coalescent inference of DENV3 genotype III sequences isolated in the Latin American region.

Group[a]	Parameter	Value[b]	HPD[c]	ESS[d]
DENV3 genotype III	Log likelihood	−1847.67	−1839.75 to −1856.06	6232.26
	Prior	50.74	22.73 to 77.75	315.73
	Posterior	−1796.93	−1768.51 to −1825.90	322.57
	Mean Rate[e]	1.03×10^{-3}	3.18×10^{-4} to 1.77×10^{-3}	2681.65
	Expansion Growth Rate[f]	34.27	0.026 to 80.76	5799.08
	Root age (yr)	11.81	5.46 to 22.27	1856.40
	MRCA[g]	1989		

[a] See Fig. 1 and Table 1 for strains included in this analysis.
[b] In all cases, the mean values are shown.
[c] HPD, high probability density values.
[d] ESS, effective sample size.
[e] Mean Rate is expressed in substitutions/site/year.
[f] Expansion Growth Rate is expressed in number of new infections/individual/year.
[g] MRCA, year of the most common recent ancestor.

explanation is that the difference might not correspond to underlying differences in rates of evolutionary change. DENV-2 and DENV-4 in the Americas seem to follow a similar demographic pattern: an "invasion" phase, characterized by a rapid increase in the number of DENV lineages, followed by a "maintenance" phase, during which relative genetic diversity remains approximately constant (Carrington et al., 2005). It is probable that in the case of DENV-3 genotype III we are still observing the "invasion" phase.

It is possible that the more rapid initial increase in DENV-3 genotype III lineages reflects a more rapid geographic dispersal within the Latin American region and therefore a more rapid implementation of population structure during the invasion phase. Faster population growth and greater population dispersal may have contributed to the more vigorous initial transmission dynamics of DENV-3 genotype III in the region.

Another possible explanation for the differences of population dynamics among DENV-3 genotype III and other DENV serotypes in our region might involve the possible differences in the level of immunity within host populations (Carrington et al., 2005). After an absence of 17 years from the Latin American region, DENV-3 re-emerged in Central America in 1994 (Usuku et al., 2001), and continue to expand into South America (Peyrefitte et al., 2003, 2005; Messer et al., 2003; Uzcategui et al., 2003; Balmaseda et al., 1999; Nogueira et al., 2001; Aquino et al., 2006; Barrero and Mistchenko, 2008; Usme-Ciro et al., 2008). It is possible that the transmission dynamics of DENV-3 genotype III might also reflect the immunological landscape of host populations, after lack of exposure to this DENV type for relative long periods, although cross-neutralization between DENV serotypes has been observed (Kochel et al., 2002).

More studies on the evolution of DENV-3 genotype III in the Latin America region will be needed to gain insight into the degree of diversification, spread to other countries and the population dynamics of DENV in this region. The results of this study suggest that DENV-3 genotype III may continue to expand throughout the South America region, and that more DENV-3 genotype III outbreaks might take place in this region. This speaks of the importance of more in-depth studies on DENV-3 genetic variability and evolution in the Latin American region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2009.01.003.

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