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Short Communication

Comparative complete genome analysis of dengue virus type 3 circulating in India between 2003 and 2008

Shashi Sharma, Paban Kumar Dash, Surekha Agarwal, Jyoti Shukla, M. M. Parida and P. V. L. Rao

Division of Virology, Defence R&D Establishment (DRDE), Jhansi Road, Gwalior 474002, MP, India

Correspondence
Paban Kumar Dash
pabandash@rediffmail.com

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Dengue is endemic in most parts of the tropics including India. So far, complete genome information for Indian dengue isolates is not available. In the present study, we characterized the genome of three dengue type 3 viruses isolated from India. The genomes of all three viruses were found to be 10 707 bp long with an ORF encoding 3390 aa. Extensive molecular phylogenetic analysis based on comparison of the complete genome and envelope gene classified the recent Indian viruses into genotype III (lineage III), revealing a shift of lineage from lineage V. The sequence analysis revealed several non-conservative changes in major structural proteins. This study clearly indicates that the genotype III (lineage III) dengue type 3 viruses have been continuously circulating in major parts of India since 2003 and are responsible for the recent major outbreaks all over India. This is the first extensive study on complete genome analysis of dengue type 3 viruses in India.

Dengue/dengue haemorrhagic fever (DF/DHF) has emerged as the most important mosquito-borne human viral infection of the 21st century. An estimated 100 million dengue infections are now reported annually around the world. Dengue is caused by four antigenically and genetically distinct, single-stranded positive-sense RNA viruses designated dengue virus types 1–4 (DENV-1–4). Dengue viruses belong to the genus *Flavivirus* of the family *Flaviviridae*. The genome of dengue virus is approximately 10.7 kb in length and contains a single ORF that encodes three structural [capsid (C), premembrane (prM) and envelope (E)] and seven non-structural (NS) proteins (NS1, Ns2a, NS2b, NS3, NS4a, NS4b and NS5) (Gubler, 1998).

Dengue infection is characterized by a spectrum of illness ranging from mild, self-limiting dengue fever to life-threatening dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The pathogenesis of DHF and DSS is poorly understood. However, several hypotheses, such as antibody dependent enhancement in heterotypic secondary dengue infections, involvement of a virulent viral genotype and host factors, have been suggested to explain this mechanism (Leitmeyer *et al.*, 1999; Rico-Hesse *et al.*, 1997; Messer *et al.*, 2003).

All the DENV serotypes are further classified into multiple subtypes/genotypes based on their genomic diversity (Weaver & Vasilakis, 2009). Five distinct genotypes of DENV-3 have been identified to date (Lanciotti *et al.*, 1994; Wittke *et al.*, 2002; Uzcategui *et al.*, 2003). Genotypes I–III are responsible for most DENV-3 infections and have been associated with DF/DHF epidemics in South East Asia, the Indian subcontinent, the south Pacific, East Africa and the Americas. Genotypes IV and V were not associated with DHF epidemics and are only represented by a few early sequences from the Americas, the south Pacific and Asia, with the exception of recent reports regarding genotype V-related cases from Brazil and Colombia (Aquino *et al.*, 2009).

In India, the frequency of DHF outbreaks have increased at an alarming rate over the past 15 years. Dengue received national attention in 1996 when a major DHF outbreak occurred in Delhi and DENV-2 was identified as the aetiology of this outbreak (Dar *et al.*, 1999). DENV-2 continued circulating in major parts of northern India during the subsequent period (Dash *et al.*, 2004). However, in 2003 a major DHF outbreak swept most parts of northern India and, surprisingly, DENV-3 was identified as the aetiology of this epidemic (Kumar *et al.*, 2004a). Phylogenetic analysis based on a short region of the C–prM gene junction classified these DENV-3 into genotype III (Dash *et al.*, 2006). Since then DENV-3 has remained a

The GenBank accession numbers for the complete genomes of the Indian DENV-3 sequences reported in this paper are AY770511, FJ644564 and GQ466079.

A supplementary table giving details of the full-length genome sequences of the DENV-3 isolates investigated in the genome analysis in this study is available with the online version of this paper.

dominant serotype in most parts of India and has been associated with all the major dengue outbreaks over the past decade (Kukreti, 2010). In spite of dengue being the most important arboviral infection in India, no complete genome information for any of the Indian DENVs is available. The lack of adequate genome information continued to serve as a hindrance towards understanding the true picture of DENV circulating in India. In view of the facts above, the present study was undertaken to elucidate the sequence information of the complete genomes of three recently circulating DENV-3 isolated from different parts of India during 2003–2008. We also performed an extensive phylogenetic analysis to understand the origin and dispersal of Indian DENV-3.

Three DENV-3 viruses isolated from viraemic human patients from different parts of India (GW25 from Gwalior in 2003, ND143 from Hyderabad in 2007 and RR72 from New Delhi in 2008) were selected for this study. These DENV-3 isolates at passage level 2 in C6/36 cells were used in the present study (Igarashi, 1978). The viral RNA was extracted from 140 µl of infected culture supernatant using a QIAamp viral RNA mini kit (Qiagen) in accordance with the manufacturer's instructions. A total of 18 overlapping amplicons spanning the complete genome were amplified using 36 primers designed for this study. The primers were designed using the PrimerDesign module of LASERGENE5 (DNASTAR) and the sequences of primers are available upon request. The one-step RT-PCR was carried out using an AccessQuick RT-PCR System (Promega). PCR amplification was carried out in a final volume of 50 µl with the thermal profile comprised of: a reverse transcriptase reaction step at 48 °C for 45 min and a denaturation step at 95 °C for 2 min followed by 35 cycles of 94 °C for 1 min, 55–59 °C for 1 min and 72 °C for 1 min. A final extension stage was carried out at 72 °C for 10 min. The amplicons were gel purified and used as template in sequencing reactions.

Double-pass sequencing was carried out by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems) following the manufacturer's instructions. The cycle-sequenced products were column purified and analysed on an ABI 3.1 automated DNA sequencer (Applied Biosystems). The nucleotide sequences were retrieved, edited and analysed using the SeqScape (Applied Biosystems) EditSeq and MEGALIGN modules of LASERGENE5. Multiple sequence alignment was carried out by employing MUSCLE (Edgar, 2004). The percentage nucleotide identity and percentage amino acid identity values were calculated as pairwise *P* distances. The best fit model of nucleotide substitution was selected under the hierarchical likelihood ratio test by using MODELTEST version 3.7 (Posada, 2006). Phylogenetic analysis based on the nucleotide sequences of complete ORFs of 66 DENV-3 was conducted using MEGA version 2.1 (Kumar *et al.*, 2004b) (Supplementary Table S1, available in JGV Online). A Tamura Nei model of nucleotide substitution with a gamma distribution of among-site rate variation was used to construct the neighbour-joining tree (Saitou & Nei, 1987). Another extensive phylogenetic analysis based on complete E gene sequences was carried out by including 96 globally diverse DENV-3 (genotype III) sequences using MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). The Bayesian tree was inferred by running a Markov-chain Monte Carlo algorithm for 1 500 000 generations, sampling at every 100th generation with a burn in setting of 10 % of generations. Convergence was assessed using mean SD in partition frequency values by using a threshold of 0.01.

The complete genome of all three Indian DENV-3 was found to be 10 707 nt in length with an ORF of 3390 aa. The sequences were deposited in GenBank with accession numbers AY770511, FJ644564 and GQ466079 for GW25, ND143 and RR72, respectively. A BLAST search of these DENV-3 sequences against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) revealed maximum sequence identities (98 %) with a 2009 Chinese isolate (GenBank accession. no. GU363549). Analysis of multiple sequence alignment with the complete genomes of 66 representative DENV-3 from throughout the world revealed base substitutions scattered throughout the alignment. However, no deletion, insertion or premature polyprotein stop codon was observed within the ORF. The nucleotide sequence identity among all the global DENV-3 viruses was 92.6–99.9 %. Among Indian DENV-3 isolates, GW25 revealed maximum sequence identity (94.5 %) with the prototype strain, H-87. All of the three Indian DENV-3 isolates sequenced in this study were found to be closely related (99.5 %). In comparison with the E gene sequence of an earlier Indian DENV-3 isolate from 1984, these three viruses revealed 98 % nucleotide sequence identity. Compared to H-87, few mutations (one in the 5′ UTR and eight in the 3′ UTR) were seen. In addition, an 11 nt insertion (AGTGAAAAAGA) in the 3′ UTR was also observed. The pairwise comparison indicated high amino acid similarity (99.2–99.7 %) among the three DENV-3 isolates sequenced in this study. Compared with the prototype DEN-3 strain (H-87), these three DENV-3 isolates revealed 97.6–98.0 % amino acid sequence identity. All the cysteine residues (six in prM, 12 in E and 12 in NS1) remained positionally conserved among all the DENV-3 isolates owing to their role in maintaining the conformation of the protein. Most of the amino acid changes were conservative, involving interchange of amino acids having similar physico-chemical properties. However, few major non-conservative amino acid substitutions among DENV-3 (sequenced in this study) vis-à-vis H-87 were seen (Table 1). The E protein of the three Indian isolates showed 14 sites with variable amino acid residues with respect to H-87. Out of these 14 sites, 3, 5 and 4 shifts were observed in domains I, II and III, respectively. Two substitutions were also found in the transmembrane region. Compared with H-87, strongly basic amino acid lysine was replaced by glutamic acid at positions 225 and 291 of the E protein in all the three Indian DENV-3 isolates. Two important non-conservative

Table 1. Description of important non-conservative amino acid substitutions among the Indian DENV-3 compared with the prototype DENV-3 strain (H-87)

Major non-conservative changes involving basic to acidic amino acid are underlined.

Amino acid position		H-87	GW25	ND143	RR72
ORF	Protein				
Envelope					
449	169	A	T	T	T
505	225	K	E	E	E
571	291	K	E	E	E
581	301	L	T	T	T
NS1					
866	93	I	T	T	T
893	120	L	K	K	K
1044	271	G	G	E	E
NS2A					
1177	52	G	D	D	D
1237	112	A	T	T	T
NS3					
1588	115	I	T	T	T
1844	371	V	D	D	D
1892	419	I	K	K	K
1949	476	M	T	T	T
NS4B					
2350	108	I	T	T	T
NS5					
2970	480	A	A	S	S

substitutions involving hydrophobic alanine to polar threonine at E (domain I)-169 and leucine to threonine at E (domain III)-301 were also recorded in the E protein. The E (domain I)-169 was identified earlier as a positively selected site and is reported to be located within a murine T- and B-cell epitope (Leclerc *et al.*, 1993; Twiddy *et al.*, 2002). Since the envelope plays a crucial role in virus attachment to the host receptor, these substitutions might have played important role in making these isolates more transmissible, as similar viruses are now circulating and causing major outbreaks around the world (Messer *et al.*, 2003). Similar non-conservative substitutions involving shifts to polar amino acids were also recorded in other non-structural proteins, particularly in NS1 and NS3. However, the exact functions of these substitutions need to be confirmed in future studies.

The phylogenetic analysis based on the complete coding region classified the geographically diverse DENV-3 isolates into four different genotypes (Fig. 1). All three Indian DENV-3 isolates sequenced in this study were grouped into genotype III, along with isolates from around the world, including Asian, Pacific island and South American countries. This genotype was also considered a cosmopolitan genotype, owing to its wide geographical distribution. Furthermore, critical examination of the branching pattern enabled us to classify genotype III isolates into five lineages. Lineage I is represented by large number of DENV-3 isolates primarily from the Americas, including Puerto Rico (1998–2006), Venezuela (2000–2007), Colombia (2002–2007), Brazil (1997–2007), Martinique (1999) and Saint Lucia (2001). Lineage II is represented by two isolates from Sri Lanka (1989–1997) and a DENV-3 isolated in Mozambique in 1985. Three Indian DENV-3 (sequenced in this study) were grouped into lineage III, along with a Chinese isolate from 2009. Lineage IV is represented by DENV-3 from Sri Lanka (1993–2000), Taiwan (1999) and Singapore (2004–2005). Three Sri Lankan isolates of the 1980s formed a close branching pattern and were grouped into lineage V. Genotype I is represented by DENV-3 from Indonesia (1978–2004), Taiwan (1995), French Polynesia (1989–1994), the Philippines (1997) and East Timor (2005). Genotype II is represented by DENV-3 from Asian countries including Thailand (1973–2001), Singapore (1995), Taiwan (1998), Cambodia (2003–2008) and Vietnam (2006). The prototype virus, H-87, isolated in the Philippines in 1956 was found belong to genotype V, along with isolates from China (1980) and Brazil (2002). However, this phylogenetic tree based on complete ORFs was found to be less informative owing to a lack of complete genome information for representative DENV-3 from all the dengue affected areas. A separate tree based on the 1479 nt sequence of the complete E gene was constructed that included the maximum number of representative DENV-3 (genotype III) from diverse geographical origins (Fig. 2). The branching pattern was found to be similar to the tree based on the complete ORF sequences, with lineage I represented by DENV-3 isolated from the Americas in the past 15 years (Mexico, Panama, Colombia, Venezuela, Brazil, Peru, Nicaragua, Puerto Rico, Cuba, Saint Lucia and Martinique). Lineage II is represented by DENV-3 from Mozambique, Sri Lanka and Somalia. All three Indian DENV-3 viruses (sequenced in this study) were grouped into lineage III along with DENV-3 isolates from various Asian countries including Cambodia (2000), Saudi Arabia (1997–2004), Bhutan (2006–2007), Japan (2008) and China (2009), along with a recent isolate from Tanzania (2010). However, an earlier Indian isolate from 1984 belonged to lineage V, along with isolates from Sri Lanka from the 1980s. The existence of two distinct lineages of genotype III (isolated before and after 1989) in Sri Lanka and the association with post-1989 isolates from DHF epidemics has already been reported (Messer *et al.*, 2003). The shift of lineage from V to III might have played an important role in the emergence of DHF outbreaks in India post-1990. The sharing of a close branching pattern between GW25, isolated from the first major DENV-3-associated outbreak in India in the last decade, and isolates from Saudi Arabia (since 1997) raises important questions regarding the origin and circulation of these viruses. Recently, it has been suggested that Haj pilgrims to Saudi Arabia might play an important role in the importation and exportation of dengue virus between Saudi Arabia and their home countries (Zaki *et al.*, 2008). However, the precise origin of Indian DENV-3 could not be confirmed because of a lack of sequence information from Indian DENV-3 during

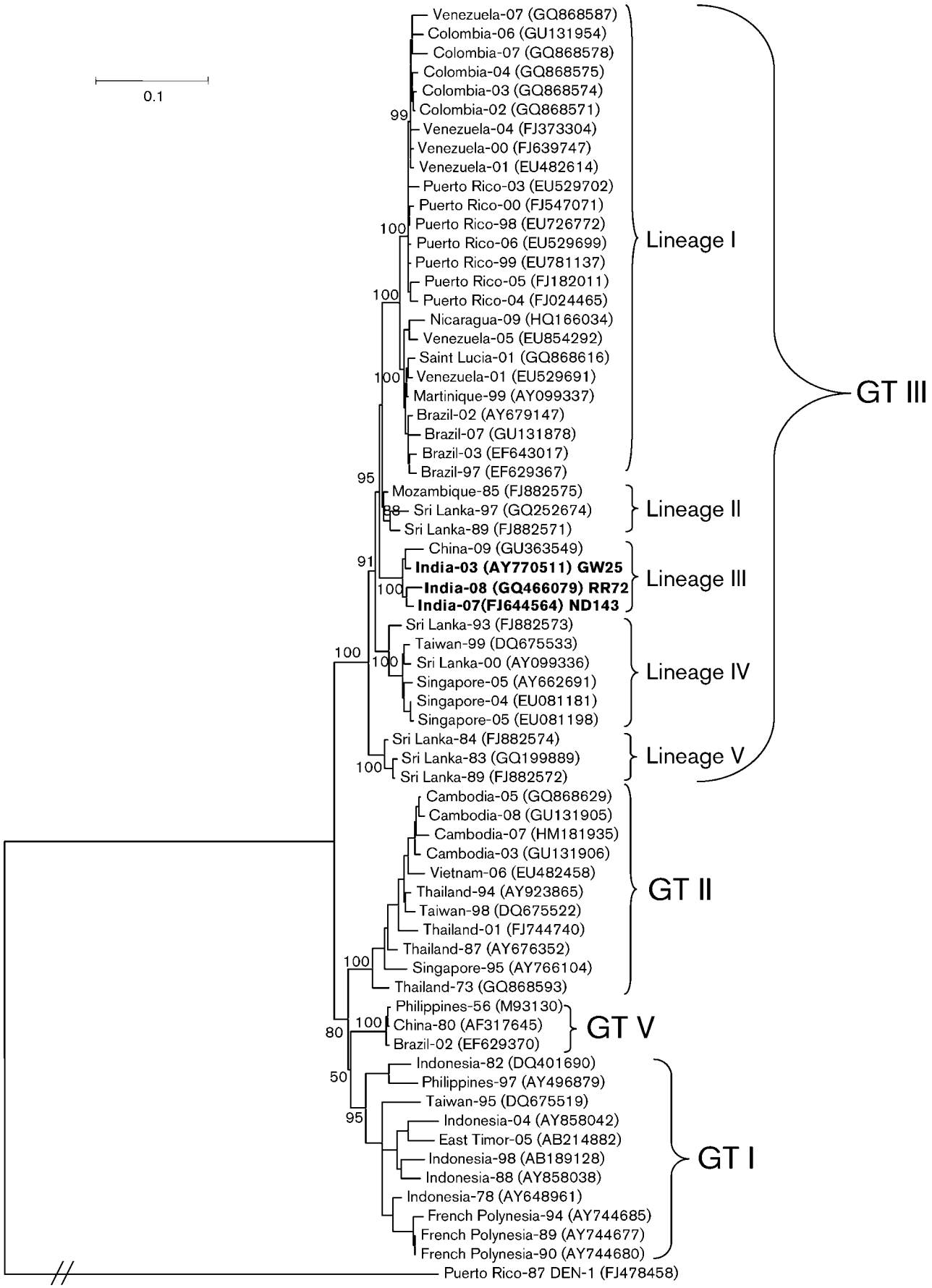
0.1
Venezuela-07 (GQ868587)
Colombia-06 (GU131954)
Colombia-07 (GQ868578)
Colombia-04 (GQ868575)
Colombia-03 (GQ868574)
Colombia-02 (GQ868571)
Venezuela-04 (FJ373304)
Venezuela-00 (FJ639747)
Venezuela-01 (EU482614)
Puerto Rico-03 (EU529702)
Puerto Rico-00 (FJ547071)
Puerto Rico-98 (EU726772)
Puerto Rico-06 (EU529699)
Puerto Rico-99 (EU781137)
Puerto Rico-05 (FJ182011)
Puerto Rico-04 (FJ024465)
Nicaragua-09 (HQ166034)
Venezuela-05 (EU854292)
Saint Lucia-01 (GQ868616)
Venezuela-01 (EU529691)
Martinique-99 (AY099337)
Brazil-02 (AY679147)
Brazil-07 (GU131878)
Brazil-03 (EF643017)
Brazil-97 (EF629367)
Lineage I
Mozambique-85 (FJ882575)
Sri Lanka-97 (GQ252674)
Sri Lanka-89 (FJ882571)
Lineage II
China-09 (GU363549)
India-03 (AY770511) GW25
India-08 (GQ466079) RR72
India-07(FJ644564) ND143
Lineage III
Sri Lanka-93 (FJ882573)
Taiwan-99 (DQ675533)
Sri Lanka-00 (AY099336)
Singapore-05 (AY662691)
Singapore-04 (EU081181)
Singapore-05 (EU081198)
Lineage IV
Sri Lanka-84 (FJ882574)
Sri Lanka-83 (GQ199889)
Sri Lanka-89 (FJ882572)
Lineage V
GT III
Cambodia-05 (GQ868629)
Cambodia-08 (GU131905)
Cambodia-07 (HM181935)
Cambodia-03 (GU131906)
Vietnam-06 (EU482458)
Thailand-94 (AY923865)
Taiwan-98 (DQ675522)
Thailand-01 (FJ744740)
Thailand-87 (AY676352)
Singapore-95 (AY766104)
Thailand-73 (GQ868593)
GT II
Philippines-56 (M93130)
China-80 (AF317645)
Brazil-02 (EF629370)
GT V
Indonesia-82 (DQ401690)
Philippines-97 (AY496879)
Taiwan-95 (DQ675519)
Indonesia-04 (AY858042)
East Timor-05 (AB214882)
Indonesia-98 (AB189128)
Indonesia-88 (AY858038)
Indonesia-78 (AY648961)
French Polynesia-94 (AY744685)
French Polynesia-89 (AY744677)
French Polynesia-90 (AY744680)
GT I
Puerto Rico-87 DEN-1 (FJ478458)
99
100
100
100
95
88
91
100
100
100
100
100
80
50
95

Fig. 1. Phylogenetic tree of DENV-3 viruses generated by a neighbour-joining method based on the complete nucleotide sequence the ORF region (10 173 nt). Each strain is identified by its country of origin and the last two digits of the year it was isolated, followed by the GenBank accession number in parentheses. The DENV-3 viruses sequenced in this study (GW25, ND143, RR72) are shown in boldface type. Bootstrap values are indicated at the major branch points. GT, Genotype. Bar, 0.1 substitutions per site.

1984–2003. The sequence information from this period will enable us to understand whether the origin is through *in situ* evolution or via importation of strains. Similar viruses were also recovered from Bhutan during 2006–2007 outbreaks, where it was attributed to importation from northern India (Dorji *et al.*, 2009). However, surprisingly, in neighbouring Bangladesh and Myanmar, genotype II viruses were implicated in DF/DHF outbreaks (Podder *et al.*, 2006).

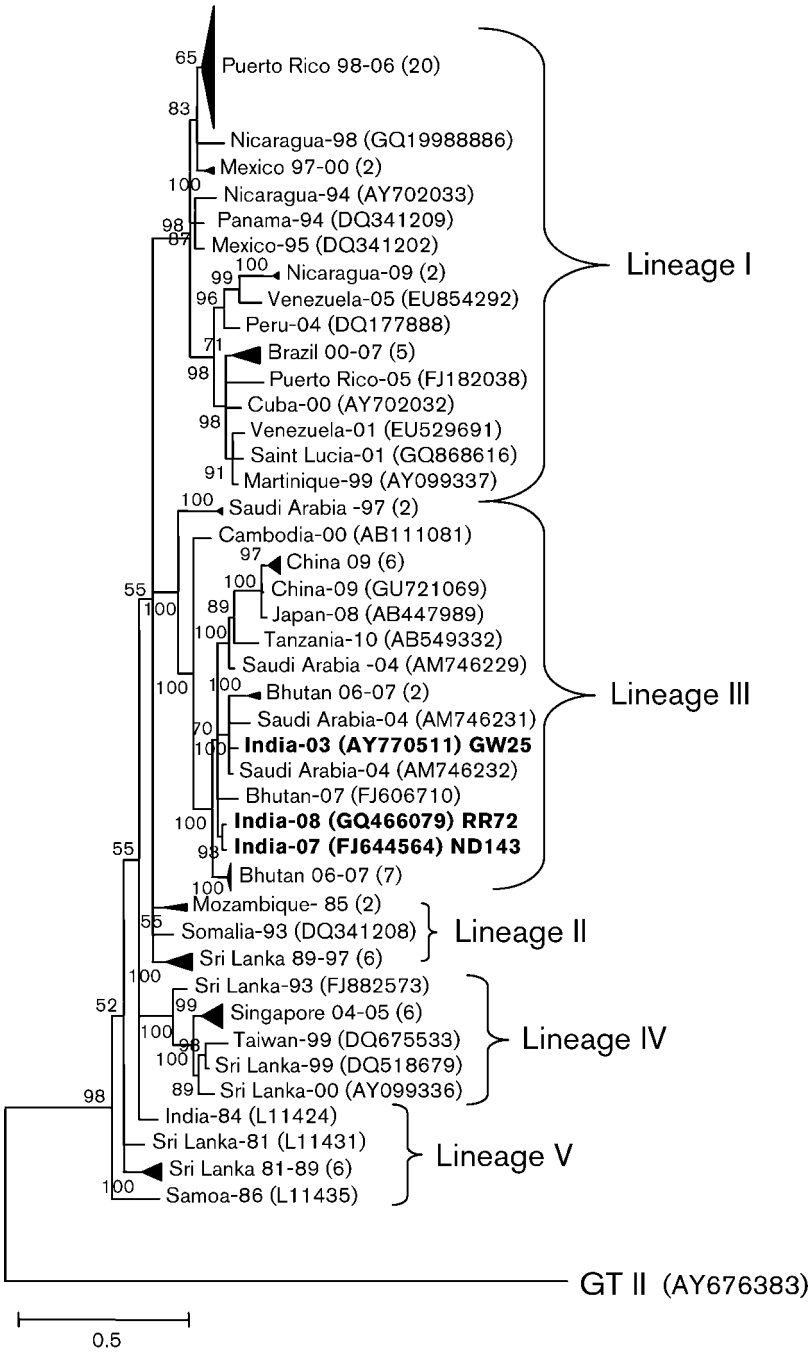

Fig. 2. Phylogenetic tree among DENV-3 (genotype-3) viruses generated by a Bayesian method based on the complete nucleotide sequence of the E gene (1479 nt). Each strain is identified by its country of origin and the last two digits of the year it was isolated, followed by the GenBank accession number in parentheses. Groups of phylogenetically closely related viruses from a specific country and specific year(s) were grouped into clusters and are designated by country of origin and the last two digits of the year of isolation(s) followed by the number of isolates in parentheses. The DENV-3 viruses sequenced in this study (GW25, ND143, RR72) are shown in boldface type. Posterior probability values are indicated at the major branch points. Bar, 0.5 substitutions per site.

The present study is the largest and first systematic study carried out to characterize the true genetic nature of recently circulating Indian DENV-3. This study clearly revealed that genotype III (lineage III) of DENV-3 viruses are circulating predominantly and are the major cause of dengue infection in India. Though the exact time of introduction of this genotype into India could not be ascertained, this study revealed that DENV-3 (genotype III) has been circulating in India since at least 1984. The associations between genotype III DENV-3 from DHF outbreaks in many parts of the world is a major source of concern for the public health authorities. The complete genome sequences of Indian dengue virus isolates elucidated for the first time in this study will serve as baseline data for future epidemiological surveillance in the Indian subcontinent and abroad.

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