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BRIEF REPORT

Identification of a recombinant dengue virus type 1 with 3 recombination regions in natural populations in Guangdong province, China

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Abstract Using recombination analysis, we identified a recombinant dengue virus type 1 strain, namely, GD23/95, with three recombination regions, located within the sequences of the prM/E junction, NS1, and NS3, respectively. The recombinant dengue virus was further confirmed by phylogenetic analysis based on its recombination and non-recombination regions. This appears to be the first study to confirm the existence of three recombination regions in a single dengue virus isolate and to report recombination between parent virus strains isolated from the same geographic area (Guangdong province, China). It is also the first to report breakpoints within the NS3 gene of dengue viruses.

Both dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are severe arboviral diseases caused by dengue viruses (DENV), with more than 100 million cases of infection and estimated 25,000 deaths recorded annually around the world [5]. DENV belongs to the genus *Flavivirus* of the family *Flaviviridae*. There are four distinct serotypes of DENV—DENV1, DENV2, DENV3, and DENV4. The dengue virus genome is a single-stranded, positive-sense RNA virus of approximately 11 kb that encodes three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [13]. Recombination events have been proven to occur in RNA viruses such as polioviruses [3], feline calicivirus [4], Western equine encephalitis virus [6], severe acute respiratory syndrome coronavirus (SARS-CoV) [20, 21, 25], and hepatitis C virus (HCV) [2, 9, 10, 12, 17]. Recombination events also occur in DENV, which are one of the most important mosquito-borne RNA viruses in tropical and subtropical areas. In defined recombinant DENV, all confirmed recombination breakpoints ("hot spots") are located just within the C, prM, E, and NS1 sequences [7, 22–24] rather than in other regions of the dengue genome.

Guangdong province is the most severely affected epidemiological area for dengue in China. Between 1991 and 2007, there were ten major dengue epidemics (1991, 1993, 1995, 1997, 1998, 1999, 2000, 2001, 2002, and 2006) mainly caused by DENV1 in Guangdong province. Among them, the most severe outbreak occurred in 1995, when 6,812 people were infected. In this study, DENV1 isolates from Guangdong province were analyzed to investigate the existence of a recombinant virus in the area.

The DENV strains used in this study are as follows: GZ01/04, 71/02GZ, GD05/99, GD14/97, GZ01/95, GD23/95, GZ/80, Mochizuki, 16007, A88, 98901530, ZJ01/2004, NB04, Fj231/04, 98901518, WestPac 74, ARG9920, BR90, FGA/89, DENV2-43, DENV3-80-2, and DENV4-B5. The first seven strains were isolated from Guangdong province. GD23/95 was recovered from C6/36 cells (that showed distinct cytopathic effect) infected by the acute serum of dengue patients in Guangdong province in 1995. It was identified by serotype assays, nested reverse transcription-polymerase chain reaction (RT-PCR) [15], and sequence analysis. All the data demonstrated that GD23/95 was a dengue virus type 1 strain.

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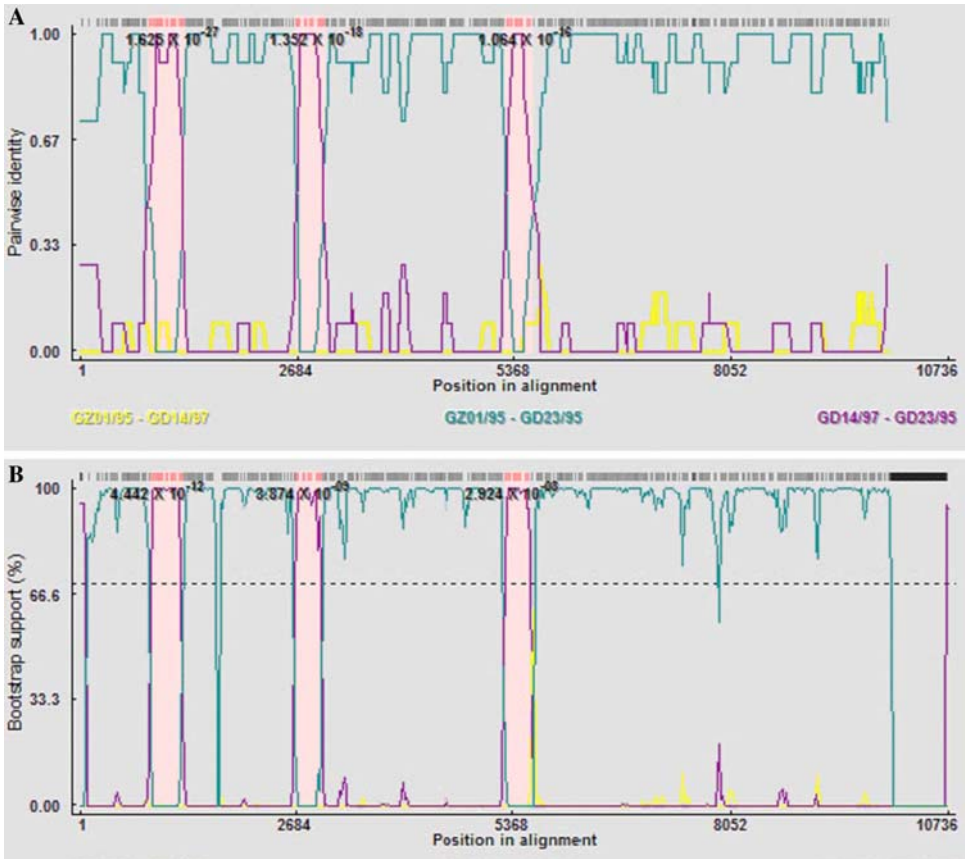
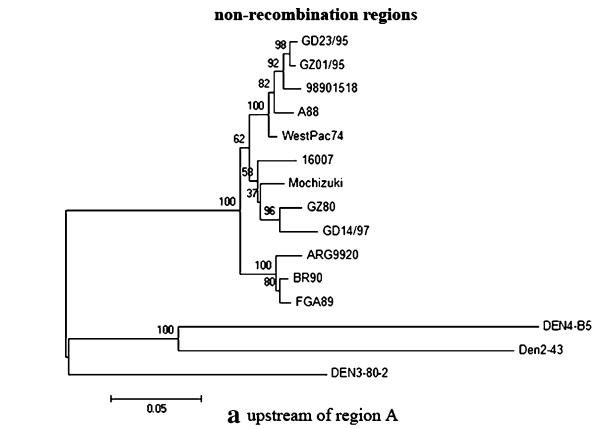


Fig. 1 Determination of recombination regions using RDP (**a**) and bootscan (**b**) as implemented in Recombinant Detection Program version 2. Plots for comparing GD14/97 and GD23/95, GZ01/95 and GD23/95, and GZ01/95 and GD14/97 are in *purple*, *blue*, and *yellow*, respectively. The *highlighted regions* are recombination regions (regions A, B, and C) between GZ01/95 and GD14/97. The virus strains and GenBank accession numbers are GZ01/04 (EF032589), 71/02GZ (EF025110), GD05/99 (AY376738), GD14/97 (AY376737), GZ01/95 (EF032590), GD23/95 (AY373427), GZ/80 (AF350498), 98901530 (AB189121), ZJ01/2004 (AY835999), NB04 (DQ836632), and Fj231/04 (DQ193572)

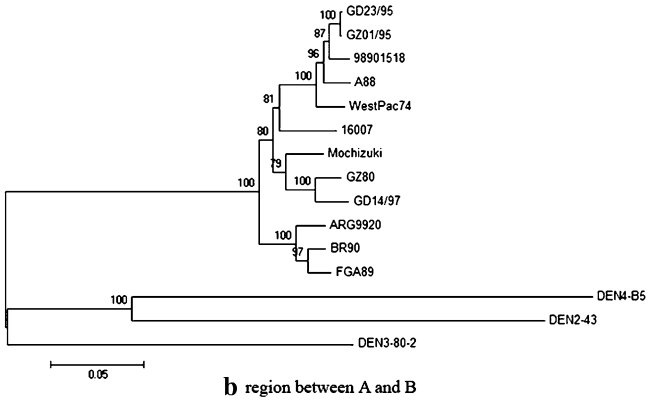
The sequences of the DENV1 isolates from Guangdong province (GD23/95, GZ01/95, 71/02GZ, GD05/99, GD14/97, GZ/80, and GZ01/04) and those of other reference isolates (98901530, ZJ01/2004, NB04, and Fj231/04) were firstly aligned using ClustalW as implemented in the MEGA3.1 (Molecular Evolutionary Genetics Analysis, Pennsylvania State University, PA, USA) [11] software with default parameters. The aligned sequences were then analyzed by RDP as implemented in Recombinant Detection Program version 2 (RDP2, http://darwin.uvigo.es/rdp/rdp.html) [14] with default parameters. The results showed that GD23/95 was a recombinant (daughter) virus and that the major and minor parent viruses were GZ01/95 and GD14/97, respectively. The three regions of recombination (termed regions A, B, and C) between GZ01/95 and GD14/97 were distributed in the structural and nonstructural-coding regions. Region A (between nucleotides 851 and 1,241 in GD23/95), region B (between nucleotides 2,672 and 2,975 in GD23/95), and region C (between nucleotides 5,264 and 5,555 in GD23/95) were located within the sequences of the prM/E junction, NS1, and NS3, respectively. The *P* value for each recombination region was significantly lower than 0.01 (the *P* values were 1.625×10^{-27}, 1.352×10^{-18}, and 1.064×10^{-16} for regions A, B, and C, respectively) (Fig. 1a). The recombination events detected by RDP were further investigated by bootscan [19] as implemented in RDP2. The bootscan analysis also confirmed the three recombination regions between GZ01/95 and GD14/97 (the *P* value was 4.442×10^{-12}, 3.874×10^{-9}, and 2.924×10^{-8} for regions A, B, and C, respectively) (Fig. 1b), which were consistent with the RDP results. This is the first study to confirm recombination breakpoints within the NS3 gene in DENV, although they have been confirmed in HCV [9, 10, 17].

To determine the phylogenetic relationships between GD23/95, GZ01/95, GD14/97, and other defined reference

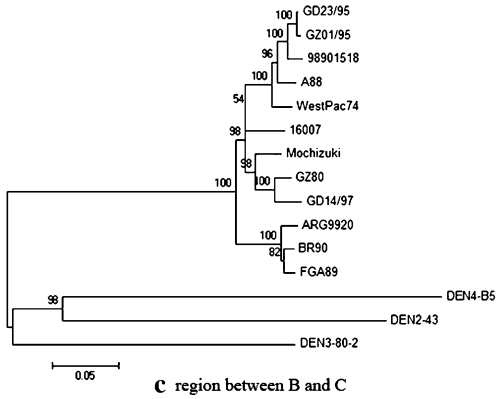
Fig. 2 Phylogenetic trees of GD23/95, GZ01/95, and GD14/97 based ▶ on putative recombination regions (nucleotides (nt) 851–1,241, nt 2,672–2,975, and nt 5,264–5,555) and non-recombination regions (nt 1–850, nt 1,242–2,671, nt 2,976–5,263, and nt 5,556–10,014). Bootstrap values are shown at each node. The phylogenetic trees in the left panels correspond to the non-recombination regions, and the phylogenetic trees in the right panels correspond to the recombination regions. DENV2–43, DENV3-80-2, and DENV4-B5 were used to root the trees. The virus strains and GenBank accession numbers are GD14/97 (AY376737), GZ01/95 (EF032590), GD23/95 (AY373427), GZ/80 (AF350498), Mochizuki (AB074760), 16007 (AF180818), A88 (AB074761), 98901518 (AB189120), WestPac 74 (U88535), ARG9920 (AY277664), BR90 (AF226685), FGA/89 (AF226687), DENV2-43 (AF204178), DENV3-80-2 (AF317645), and DENV4-B5 (AF289029)



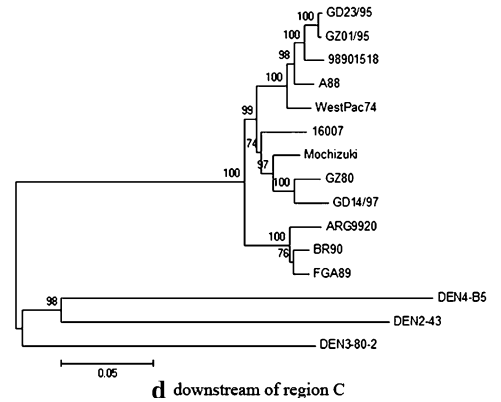
a upstream of region A



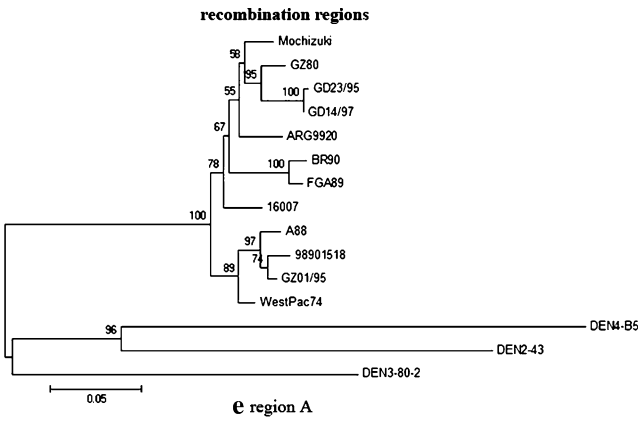
b region between A and B



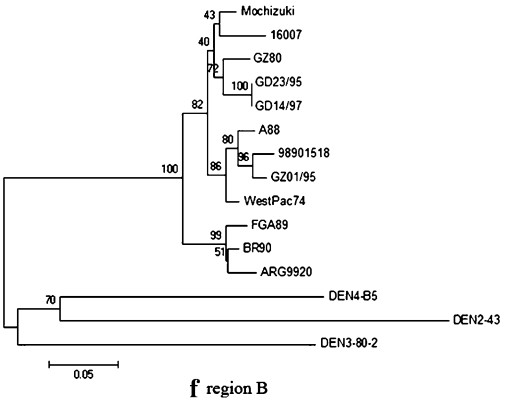
c region between B and C



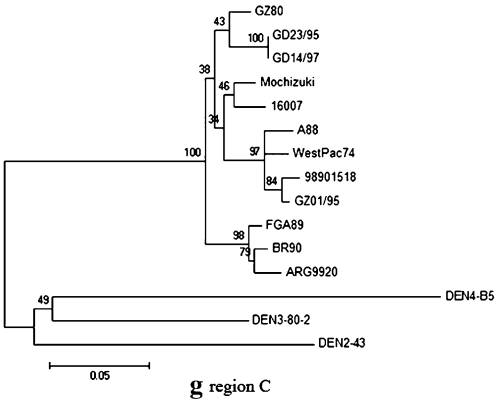
d downstream of region C



e region A



f region B



g region C

isolates (GZ/80, Mochizuki, 16007, A88, 98901518, WestPac 74, ARG9920, BR90, and FGA/89) [18], phylogenetic trees, based on the putative recombination and non-recombination regions described above, were generated and visualized by the neighbor-joining method using MEGA3.1. Bootstrap analysis was performed using 1,000 replicates. As seen in Fig. 2, the left panels represent non-recombination regions and the right ones, recombination regions. We compared these phylogenetic trees and found that the topology of the phylogenetic trees in the left panels (non-recombination regions) was very different from that of the trees in the right panels (recombination regions). For example, with regard to the non-recombination regions (the region upstream of A, the region between A and B, the region between B and C, and the region downstream of C), GD23/95 and GZ01/95 were clustered into the same group as WestPac74, 98901518, and A88, but GD14/97 fell into the same group as GZ80. On the contrary, with regard to the recombination regions (regions A, B, and C), GD23/95 and GD14/97 fell into the same group as GZ80, and GZ01/95 were clustered into the same group as WestPac 74, 98901518, and A88 (Fig. 2). Thus, phylogenetic analysis further confirmed that GD23/95 was a recombinant virus, and that the regions A, B, and C in GD23/95 were inherited from the minor parent virus GD14/97, while the other regions were inherited from the major parent virus GZ01/95.

The recombination events observed in this study are unusual in natural populations. It is unclear whether the recombination events took place in a human host or a mosquito vector co-infected by multiple virus strains. However, when two different virus strains simultaneously infect a single cell, it is theoretically possible for recombination to occur through a copy-choice mechanism [1] wherein recombination results from template switches during viral genome replication. The existence of six breakpoints (two breakpoints per recombination region) in GD23/95 implies six template switches. Recombination might occur during synthesis of the positive strand of the viral genome as observed in the case of polioviruses [8] and pestiviruses [16]. During the recombination processes observed in this study, no insertions, deletions, or duplications occurred. However, the precise mechanism underlying the recombination events observed in the present study is unknown. A better understanding of the recombination process requires the development of experimental models for co-infection and the generation of recombinant DENV.

In conclusion, we confirm a recombinant DENV1 strain, namely, GD23/95, with three regions of recombination between the parent virus strains GZ01/95 and GD14/97. This appears to be the first study that confirms the existence of three recombination regions in a single dengue virus isolate, that identifies breakpoints within the NS3 gene, and that reports recombination between parent virus strains isolated from the same geographic area (Guangdong province). The present study also contributes toward the understanding of the pathogenesis, evolution, vaccine development, treatment, and diagnosis of DENV.

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