



Dengue virus surveillance in Singapore reveals high viral diversity through multiple introductions and *in situ* evolution

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ABSTRACT

Dengue fever, a vector-borne disease, has caused tremendous burden to countries in the tropics and sub tropics. Over the past 20 years, dengue epidemics have become more widespread, severe and frequent. This study aims to understand the dynamics of dengue viruses in cosmopolitan Singapore. Envelope protein gene sequences of all four dengue serotypes (DENV-1–DENV-4) obtained from human sera in Singapore (2008–2010) revealed that constant viral introductions and *in situ* evolution contribute to viral diversity in Singapore and play important roles in shaping the epidemiology of dengue in the island state. The diversity of dengue viruses reported here could be a reflection of the on-going dengue situation in the region given Singapore's location in a dengue hyperendemic region and its role as the regional hub for travels and trade. Though cosmopolitan genotype of DENV-2 has remained as the predominant strain circulating in Singapore, we uncovered evidence of *in situ* evolution which could possibly result in viruses with improved fitness. While we have previously shown that a switch in the predominant dengue serotype could serve as a warning for an impending outbreak, our current data shows that a replacement of a predominant viral clade, even in the absence of a switch in predominant serotype, could signal a possible increase in dengue transmission. The circulating dengue viruses in Singapore are highly diverse, a situation which could offer ample opportunities for selection of strains of higher fitness, thus increasing the risk of outbreaks despite a low *Aedes* population.

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1. Introduction

Dengue virus (DENV) is a mosquito-borne flavivirus that has expanded its distribution in Asia, Africa, the South Pacific, Central and South America (Gubler, 1998), and even transmitted autochthonously in temperate places like France (Gould et al., 2010), Croatia (Gjenero-Margan et al., 2011) and Ningbo (China) (Xu et al., 2007). The virus is transmitted primarily by *Aedes aegypti* mosquito, which is present in most part of the tropical and sub-tropical regions of the world; and less efficiently by *Aedes albopictus* (Lambrechts et al., 2010). There are four distinct DENV serotypes (DENV-1–4) and infection with any of the four serotypes results in a broad spectrum of illness ranging from non-severe infections with or without warning signs to severe infections with clinical signs such as plasma leakage, hemorrhage and organ impairment (WHO, 2009). It is estimated that 50–100 million cases of DF were reported annually. Of these, approximately 500,000 cases develop into severe forms of the disease (Guzman and Kouri, 2002). Increased rapid and frequent international travel, global urbanization, population growth, geographical habitat expansion of the mosquito vector, coupled with

inadequate mosquito control and poor public health measures in endemic countries, have facilitated the growing emergence of dengue incidences (Ito et al., 2007; Ng et al., 2009; Stephenson, 2005).

DENV consists of a single-stranded, positive-sense RNA genome approximately 10,700 nucleotides in length. The single open reading frame (ORF) in the genome encodes a polyprotein consisting of three structural proteins: capsid (C), membrane-associated (prM) and envelope (E); and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Chambers et al., 1990). Epidemiologic and phylogenetic studies have revealed the extensive diversity within each of the DENV serotypes, which have led to the recognition of different genotypes. Dengue genotypes are phylogenetically distinct clusters of viruses and often associated with specific geographical regions (Kyle and Harris, 2008; Lanciotti et al., 1997, 1994; Rico-Hesse, 1990; Twiddy et al., 2002). They are often characterized based on comparative analysis of E gene or the E-NS1 junction sequences using 6% divergence as the cut-off value (Rico-Hesse, 1990). Certain genotypes within each serotype have been linked to varying epidemic potentials (Rico-Hesse, 2003). For instance, the Asian DENV-2 and DENV-3 genotypes, both originating from Southeast Asia, correlated with increased incidences of DHF/DSS in the Americas (Gubler, 1998; Leitmeyer et al., 1999; Rico-Hesse et al., 1997; Rosen, 1977). Thus, molecular epidemiologic studies by employing phylogenetic and epidemiologic analyses is

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the key to revealing virulent or epidemic strains, and offers in-depth understanding of the epidemiology of the disease.

Singapore, a tropical country with hot and humid weather conditions ideal for *Aedes* habitation, is not spared from the burden of dengue. Dengue fever has become one of the major public health problems with cycles of epidemics occurring every 5–6 years (Lee et al., 2010), usually peaking in the hottest months of July and August. Singapore is hyperendemic for dengue, with all 4 serotypes circulating in the country. In 2005 and 2007, Singapore was challenged by two major outbreaks, caused by DENV-1 and DENV-2, respectively (Koh et al., 2008; Lee et al., 2010). A switch in predominant serotype was associated with each of the two outbreaks (Koh et al., 2008; Lee et al., 2010; Schreiber et al., 2009). A similar displacement of serotype event has been shown in other studies (Thu et al., 2004; Uzategui et al., 2003). Interestingly, the switch in predominant serotype in 2007 was also accompanied by clade replacement; clade I replaced by a closely related clade II within the cosmopolitan genotype (Lee et al., 2010). In view of this, a better understanding of the disease epidemiology at molecular level is essential for a better risk assessment and development of effective control programs. A laboratory-based dengue virus surveillance programme established since 2005 provides an opportunity to study the circulating dengue viruses in this island state. By utilizing the phylogenetic approach, the E gene of dengue viruses collected from 2008 to 2010 were sequenced and analyzed.

2. Material and methods

2.1. Sample collection and serotyping

As part of the laboratory-based dengue virus surveillance program, clinical blood samples, received by the Environmental Health Institute (EHI) Diagnostics, were collected from an extensive network of hospitals and general practitioner (GP) clinics located throughout Singapore. These samples were collected from dengue-suspected patients, which required laboratory-confirmation testing. Real-time PCR (RT-PCR) for dengue RNA detection and serotyping was carried out in EHI according to its in-house protocol (Lai et al., 2007). This study was approved by the NEA Bioethics Committee (IRB003.1).

2.2. PCR amplification and sequencing of E-gene

PCR product amplified directly from patient serum was used for sequencing. Samples with low viral load were subjected to virus isolation prior to PCR amplification following procedures as previously described (Lai et al., 2007). Viral RNA was extracted from 140 µl of patient's serum or culture supernatant by using the QIAmp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was converted to cDNA using the Superscript TM III First-strand Synthesis System (Invitrogen, Carlsbad, USA). The (~1.4 kb) envelope protein (E) gene fragment was amplified by PCR using DENV serotype-specific primers (Supplementary Table S1) and GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA). PCR-amplified products were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Sequencing of purified PCR products was outsourced to a commercial sequencing facility (1st BASE Pte Ltd., Singapore).

2.3. Complete genome amplification and sequencing

A total of 20 DENV-2 viral isolates from Clade I ($n = 9$) and Clade II ($n = 11$) of the Cosmopolitan genotype, were selected for complete genome sequencing. Six overlapping fragments, which cov-

ered the entire genome, were amplified by PCR using in-house designed DENV-2 genome primers (Supplementary Table S2) and GoTaq DNA polymerase (Promega, Madison, WI, USA). PCR amplified fragments were purified using QIAquick PCR Purification kit (Qiagen, Hilden, Germany), followed by direct sequencing at a commercial sequencing facility (1st BASE Pte Ltd., Singapore).

2.4. Phylogenetic analysis

Sequences of the E gene were aligned using ClustalX program (Larkin et al., 2007), and compared with published sequences from global dengue isolates in Genbank database. Phylogenetic analysis of each DENV serotype was performed using the maximum-likelihood method, as implemented in the MEGA5 program (Tamura et al., 2011). The maximum-likelihood trees were reconstructed using the GTR model with gamma distribution and invariant sites. A bootstrap analysis of 1000 replicates was included to assess the robustness of branch topology and derived phylogeny. For complete genome of DENV, contiguous sequence for each isolate was assembled from sequence data of overlapping fragments using the Lasergene package (DNASTAR Inc., Madison, WI, USA), after which multiple DENV genome sequences were aligned using the ClustalX program (Larkin et al., 2007).

3. Results

3.1. Circulating dengue serotypes (2008–2010)

Of a total of 6515 samples received between January 2008 and December 2010 from general practitioners and hospitals throughout the country, 994 samples were positive for dengue by real-time RT-PCR. All 4 serotypes were detected during this study period, with DENV-2 (80.5%) continuing as the predominant serotype following the major dengue outbreak in 2007. DENV-1 (8.7%) and DENV-3 (8.2%) were also regularly detected while DENV-4 was rare (2.4%).

To determine the genetic diversity of DENV genotypes circulating in Singapore, a total of 380 (38.2%) samples were sequenced and analyzed for the E gene. The 380 samples comprised 51 DENV-1, 255 DENV-2, 59 DENV-3 and 15 DENV-4. The E gene sequences generated in this study and those reported previously (Genbank accession GQ357666–GQ357892) (Lee et al., 2010) were analyzed and compared with a representative global DENV sequences for each genotype obtained from Genbank database. Sequences generated in this study were deposited in Genbank database with the following accession numbers: JF960210–JF960234, JN019816–JN019830, JN030160–JN030345, JN196565–JN196617 and JN380803–JN380862.

Interestingly, in 2009–2010 years, we detected a significant number of strains that were not previously detected. In 2009, about 26 new strains were found in the virus surveillance, and they account for 26% of cases collected through the system. In 2010, new strains account for 20% of cases sampled.

3.2. Phylogeny of DENV-2

Cosmopolitan DENV-2 has played a major role in dengue transmission in Singapore in the last decade. The virus of this genotype was first isolated in 2000 (Genbank accession GQ357788). Though circulating at a lower level, DENV-2 was found to be the predominant virus in Singapore in 2003 and the beginning of 2004, when virological surveillance was first initiated. During the 2005 outbreak, though DENV-1 gained dominance, cosmopolitan genotype of DENV-2 continued to circulate. By 2007, DENV-2 (cosmopolitan genotype) replaced DENV-1 as the predominant virus, an event

that was followed by an outbreak of 8637 cases. The serotype replacement was found to be associated with a clade replacement within the DENV-2 cosmopolitan genotype; designated here as clade I and clade II (Fig. 1) respectively. Analysis of the complete genome sequences revealed that the two clades had 96.7–97.8% nucleotide similarity and were separated by nine fixed amino acid changes (Supplementary Fig. S1). It remains unclear whether clade II was evolved from the viruses circulating prior to 2007 (clade I), or it was newly introduced. Interestingly, clade I viruses was not detected by our surveillance system from 2007 to 2009, but subsequently re-emerged in 2010.

Phylogenetic analysis of the 255 E gene sequences obtained between 2008 and 2010 revealed that all but 22 of the DENV-2 viruses were closely related to clade II of the cosmopolitan genotype that were associated with the 2007 dengue outbreak. More remarkable is the observation that clade II lineage has further expanded since 2007, into two separate newer clades with strong bootstrap support; designated here as clades III and IV (Fig. 1). Notably, by 2010, the two newer clades (III and IV) replaced clade II to be the predominant virus and were involved in the larger clusters occurring in Singapore, particularly at the end of 2010 (Supplementary Table S3). Although there were 11 non-synonymous and 32 synonymous changes between clades III and IV, there were only one fixed amino acid changes at position L53P. While it is highly probable that clade III was derived through *in situ* evolution in Singapore, the source of clade IV remains unclear. Clade IV viruses first appeared in 2009 and formed the basal clade to clade II (Fig. 1). Analysis of E gene sequences of clades II and IV showed 6 non-synonymous and 24 synonymous mutations, also with one fixed amino acid changes at L53P. Also of interest is the detection of the clade I viruses (circulating virus before 2007 outbreak), which returned in 2010.

The 22 DENV-2 viruses not belonging to clade I–IV fell into other lineages within the cosmopolitan genotype. Majority of these samples ($n = 17$) had the closest virus relatives previously sampled in the Indian sub-continent (Fig. 1). Our analysis indicates that several importations of this virus lineage might have taken place in 2010. Specifically, 11 isolates sampled between May and July 2010 were clustered with isolates sampled in India and Sri Lanka, and 6 isolates sampled between October and December 2010 were more closely related to the Bangladeshi sequences. These isolates were associated with small clusters in Singapore (Supplementary Table S3). The remaining one sample of DENV-2 detected in 2008 belonged to the Asian I genotype. It is highly likely that Asian genotype DENV was introduced. Thus far these viruses have not contributed to any major local outbreak in Singapore.

3.3. Phylogeny of DENV-1

DENV-1 was the predominant serotype involved in the 2005 outbreak in Singapore (Koh et al., 2008; Schreiber et al., 2009), but has not been associated with major clusters (10 or more cases) in Singapore in the last 3 years. After 2005, DENV-1 has been circulating at lower levels, approximately 8.7% of viruses sampled at EHI. Phylogenetic analysis of 51 E gene sequences of DENV-1 obtained between 2008 and 2010 revealed 3 circulating genotypes with diverse lineages in Singapore in the last three years (Fig. 2). Forty-seven of 51 sequences were identified as genotype I. Of these, 11 were clustered with DENV-1 obtained in 2005 (Schreiber et al., 2009), indicating that viruses associated with the 2005 dengue outbreak in Singapore were still circulating. This cluster of viruses also formed the basal clade to 33 sequences obtained during the study period. Interestingly, these 33 sequences clustered into 3 distinct sub-clades within genotype I and supported by bootstrap values of at least 98% (Fig. 2).

DENV-1 strains that were uncommon to Singapore were also detected in this surveillance. These may have been introduced into Singapore in the last 3 years. Within genotype I, 4 sequences were found to be closely related to a DENV-1 sequence previously reported in China. Sequences from 2 samples, one of which had recent travel history to Vietnam, clustered with a Cambodian sequence (Fig. 2). Apart from genotype I, 4 sequences were identified as genotype III. Two of these were closely related to an isolate previously sampled in Brunei and the remainders were clustered with isolates from South Korea.

3.4. Phylogeny of DENV-3

We have observed that the population of DENV-3, though at lower apparent transmission rate (8.2% of viruses sampled), is growing in diversity in Singapore between 2008 and 2010. Introductions of multiple strains of genotype I, II and III, were evident based on the phylogenetic analysis of Singaporean DENV-3 (Fig. 3). In 2005, only two clades, belonging to genotype I and III of DENV-3 were detected in Singapore. Viruses closely related to the Sri Lankan strain within genotype III had been circulating in Singapore at least since 2003, and remained as the predominant DENV-3 genotype till 2008. Interestingly, isolates of genotype III sampled from 2007 onwards formed a distinct lineage that shared a common ancestor with those previously identified in 2004 and 2005. This suggests that the genotype III viruses circulating post 2007 could be due to an independent importation, which subsequently established its transmission locally. DENV-3 genotype III was involved in 2 small clusters in 2008 (Supplementary Table S3). In addition, 3 samples, including 2 from 2007, were closely related to DENV-3 from Saudi Arabia (99.3% sequence similarity) and this evidently represents another importation of the DENV into Singapore (Fig. 3).

Recently, DENV-3 genotype I appeared to have gained a foothold in Singapore. Genotype I consists of viruses mainly from Southeast Asia; Indonesia, Malaysia, East Timor and the Philippines. Within genotype I, the Singaporean DENV-3 formed 2 distinct clades that were closely related to the Indonesian/Malaysian or the Philippines DENV strain (Fig. 3). The Indonesian/Malaysian strain has been circulating in the country since at least 2005. In 2009, the newly emerged Philippines strain of DENV-3 was also found to be responsible for a major cluster in the western region of Singapore (Supplementary Table S3).

Five Singaporean isolates were identified as genotype II. This group comprised mainly Asian viruses previously reported in Thailand, Bangladesh, Myanmar, Malaysia and Vietnam. Importation of this genotype into Singapore is highly likely since the isolates were uncommon throughout the surveillance period and they were closely related to those from Bangladesh, Myanmar and Thailand. Genotype II of DENV-3 was last detected in Singapore in 1995 and 2006. However, 2 samples were found to be associated with a dengue cluster in 2008 (Supplementary Table S3).

3.5. Phylogeny of DENV-4

The molecular epidemiology of DENV-4 in Singapore has not been described previously. DENV-4 is uncommon and only a small proportion of cases was detected since the implementation of dengue surveillance program in 2005. Between 2008 and 2010, a total of 24 (2.4%) samples were detected as DENV-4. DENV-4 consists of 2 main genotypes; genotype I (Southeast Asia/India) and genotype II (Southeast Asia/Oceania/Americas). Phylogenetic analysis showed that genotype II was the predominant circulating DENV-4 genotype in Singapore. These sequences clustered with those previously reported in Indonesia and shared at least 99% sequence similarity in the E gene. This suggests that virus importation may

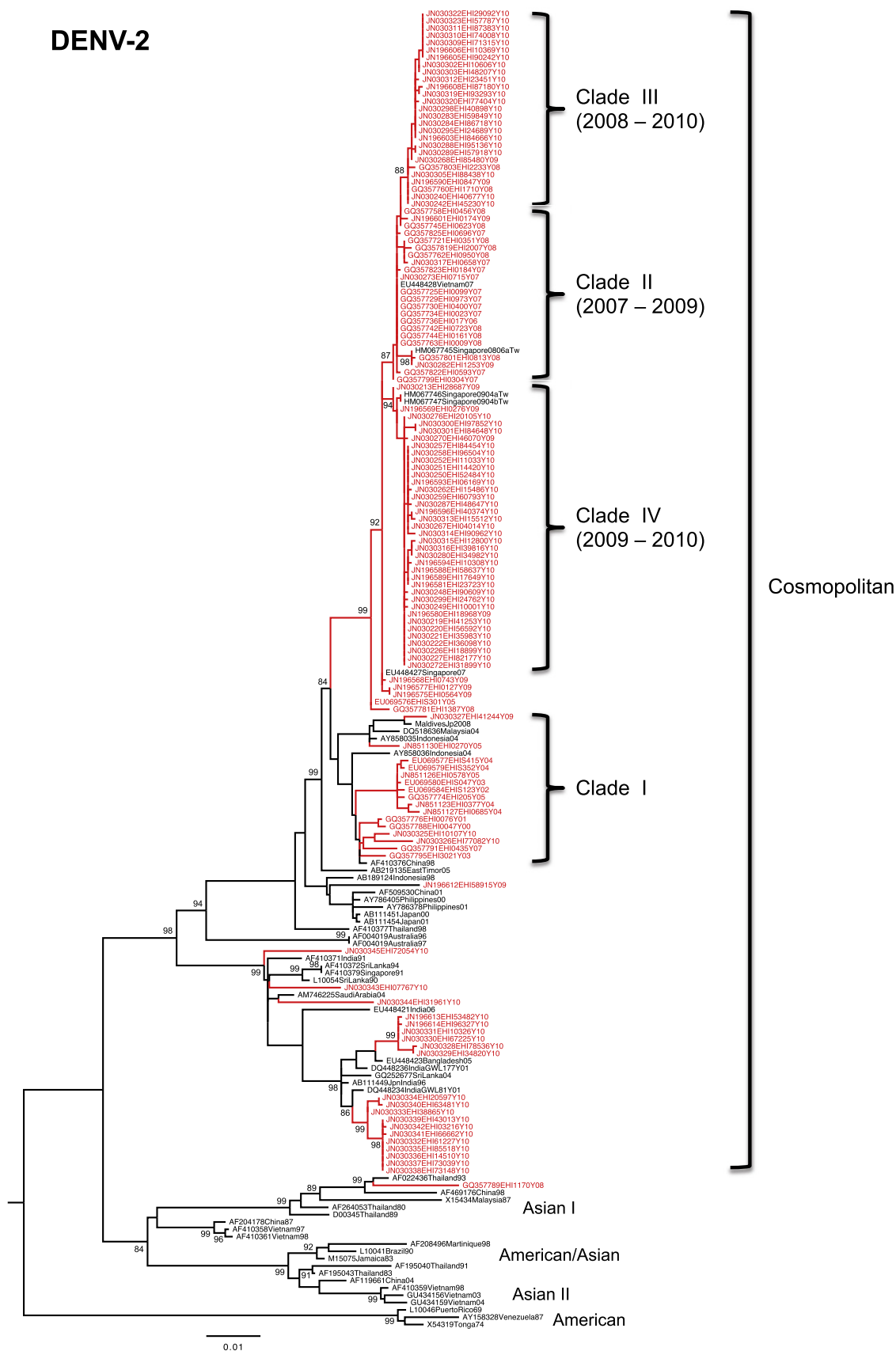


Fig. 1. Phylogenetic relationship of global isolates of DENV-2 determined based on the maximum-likelihood method. Sequence data generated at Environmental Health Institute are shown in red. Numbers on branches represent significant bootstrap percentages (>80%) and scale bar indicates substitutions per site. Not all Singaporean DENV-2 sequences were included due to the space constraints. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

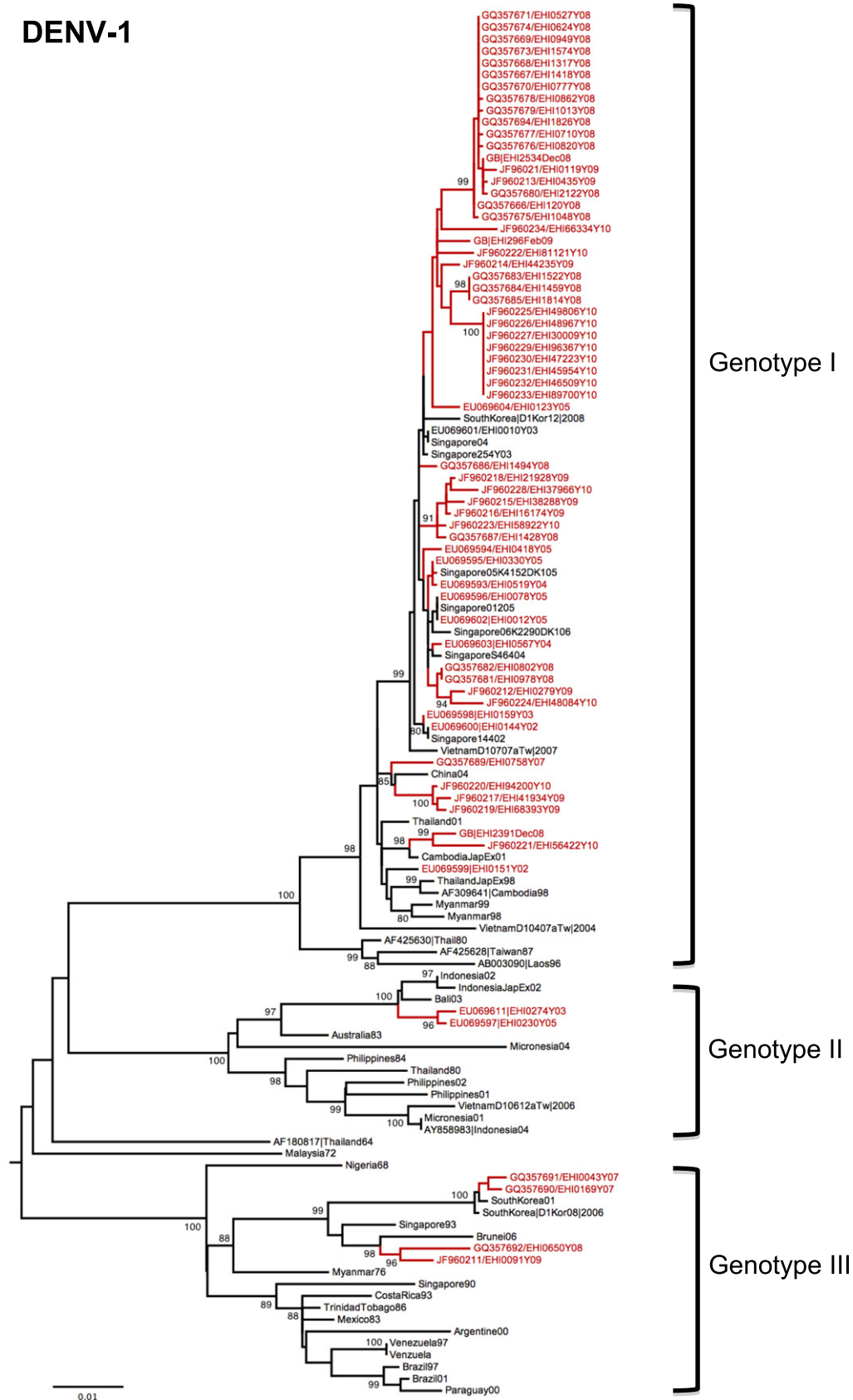
DENV-1

Fig. 2. Phylogenetic relationship of global isolates of DENV-1 determined based on the maximum-likelihood method. Sequence data generated at Environmental Health Institute are shown in red. Numbers on branches represent significant bootstrap percentages (>80%) and scale bar indicates substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DENV-3

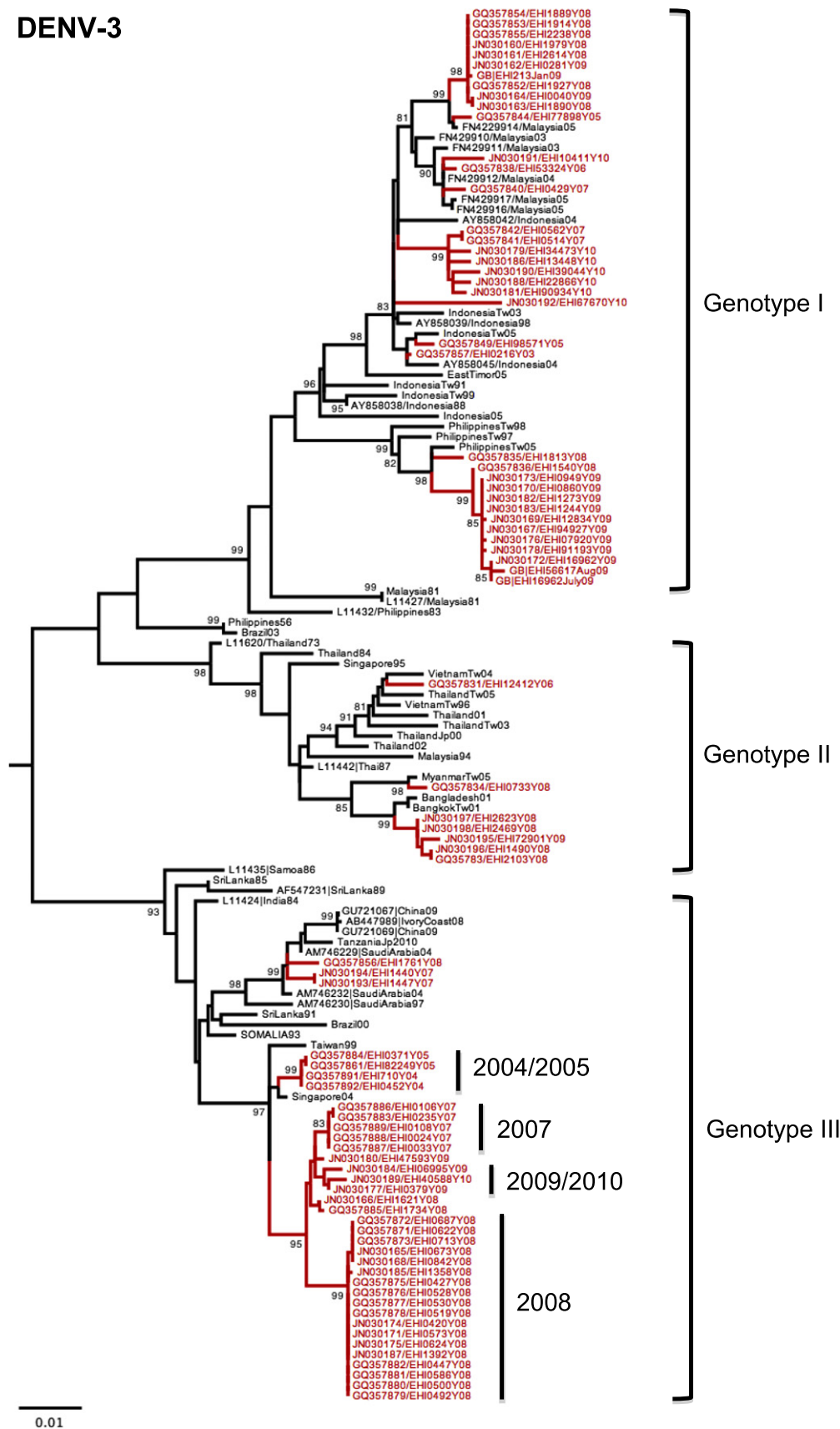


Fig. 3. Phylogenetic relationship of global isolates of DENV-3 determined based on the maximum-likelihood method. Sequence data generated at Environmental Health Institute are shown in red. Numbers on branches represent significant bootstrap percentages (>80%) and scale bar indicates substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have occurred at some point and subsequently led to a localized transmission. In addition, the inclusion of Singaporean sequences that resulted in the multiple branching of lineages previously undetected and uncommon to Singapore is highly likely due to multiple importations of DENV-4. The importations of DENV-4 were evident with 2 samples forming the basal clade to the commonly circulating DENV-4 within genotype II and a sample closely related to those in Thailand and Cambodia within genotype I (Fig. 4). However, it is interesting to note that though the same DENV-4 strain has been circulating at low level for 3 years in the north-eastern part of Singapore, the cases have been scattered with no clear clustering effect. There is a possibility that silent transmission of DENV-4 may be taking place in Singapore.

4. Discussion

Over the last two decades, the epidemiology of dengue in Singapore has changed dramatically with an increase in frequency and intensity of outbreaks, despite an intensive vector control program (Koh et al., 2008; Ooi et al., 2006). It was only after 2005 that a reviewed control programme succeeded in reversing the trend of dengue in Singapore. Although dengue has been a major public health problem since its resurgence in the early 1990s, the virological factors driving its recent epidemiology in Singapore remained unclear. This study documents the molecular epidemiological aspect of dengue and provides insight into the diversity of dengue viruses in Singapore.

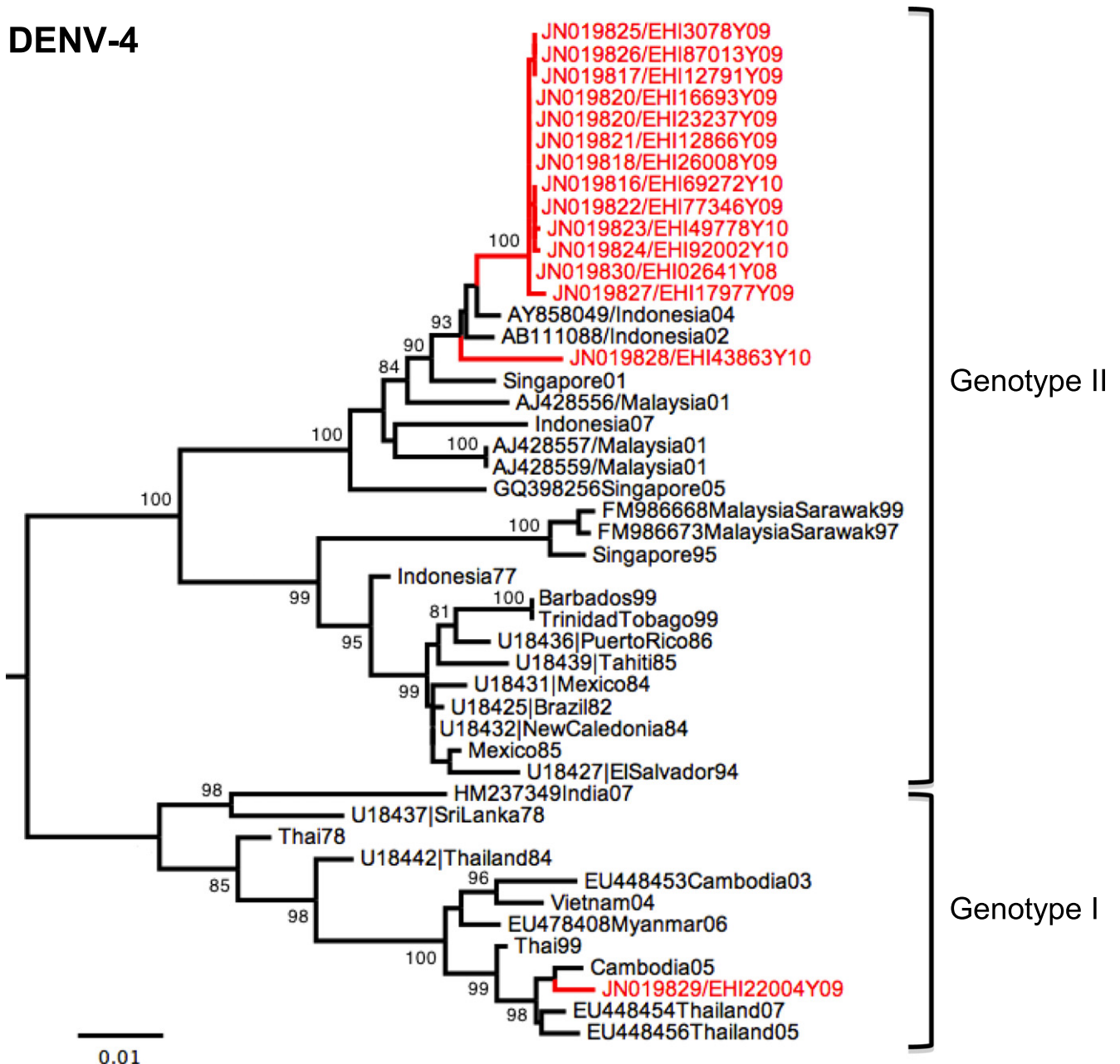


Fig. 4. Phylogenetic relationship of global isolates of DENV-4 determined based on the maximum-likelihood method. Sequence data generated at Environmental Health Institute are shown in red. Numbers on branches represent bootstrap percentages (>80%) and scale bar indicates substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The last two dengue epidemics in Singapore in 2005 and 2007 were each associated with a switch in predominant serotype (Koh et al., 2008; Lee et al., 2010). The 2005 outbreak occurred following a serotype switch from DENV-2 to DENV-1, and dengue outbreak in 2007 occurred about six month after a switch from DENV-1 back to DENV-2. In addition to the serotype switching in 2007, phylogenetic analysis of the E protein gene revealed a clade replacement (Clades I–II) within DENV-2 cosmopolitan genotype during the 2007 outbreak (Lee et al., 2010). Interestingly, the Aedes premise index in 2007, at 0.68, was significantly lower than that in 2002, 2003 and 2004, at 2.23%, 1.7% and 2%, respectively (Ministry of Health, Singapore, 2011), when the clade I of the cosmopolitan genotype were predominant but did not cause major outbreaks. Aedes premise index is the proportion of premises found breeding mosquitoes during routine premise checks. We hypothesize that the clade II virus, though closely related to clade I, had an improved fitness that enhanced its transmissibility despite lower mosquito population. It was tamed only when premise Aedes index was brought down to less than 0.4 from 2009 to 2010 (Supplementary Figure S2). Indeed, recent infection studies showed that clade II viruses had faster dissemination rate and higher viral titer in local *Ae. aegypti*, when compared with a clade I virus (Tan and Ng, unpublished data). The further development of clade II into clade III and IV in the last 2 years is being monitored closely, particularly because the tight clades have been associated with an increased number of dengue cases in several localities in late 2010 (Supplementary Table S3) and early 2011 (data not shown).

It has been previously suggested that the emergence of virus with better fitness may occur as a result of immunologic selection during periods of intense transmission during outbreaks (Rodriguez-Roche et al., 2011). Here, we propose that the increase in dengue transmission over the recent years have resulted in viral diversity, and the aggressive vector control measures in Singapore, which has led to a very low mosquito population, could have selected for the circulation of a virus lineage with better fitness.

At the point of writing (July 2011), Singapore was experiencing a significant rise in number of cases, which were predominantly due to clades III and IV of cosmopolitan DENV-2. Reaching about 260 cases per week, it represented the highest number of cases per week since the 2007 outbreak; and was equivalent to or more than twice the weekly numbers documented in the same period in 2008–2010. Whether the virus has further adapted to local entomological pressure, leading to increased fitness, remains to be elucidated with controlled experiments.

Nevertheless, while we have previously shown that a switch in the predominant dengue serotype could serve as a warning for an impending outbreak, our current data shows that a replacement of a predominant viral clade, even in the absence of a switch in predominant serotype, could signal a possible increase in dengue transmission. In the backdrop of low herd immunity of human population and low Aedes population in Singapore, it is highly plausible that a subtle adaptation of an existing virus could potentially pose a challenge to the control system.

It is also interesting to note that though Singapore is located in a region that is endemic with the Asian genotype, which has been found to be of high epidemic potential and virulence (Rico-Hesse, 2003), the Asian genotype [subsequently reclassified as Asian I and II (Twiddy et al., 2002)] has not replaced the cosmopolitan genotype in the last 10 years. We thus speculate that the cosmopolitan genotype may be as fit or even fitter than the Asian I and II genotype, a property that has perhaps rendered its success globally and resulted in its namesake – cosmopolitan.

Another striking feature of the dengue epidemiology in Singapore is the diversity of DENV strains or lineages that are co-circulating within the island. In the last 3 years, there appeared to be an increase in genotypes and lineages co-circulating on the island state. Not only

does it substantiate the impact of globalization on dengue transmission, it also reflects the increased global activity of dengue in recent years. Our molecular epidemiological surveillance indicates that while some of the newly introduced dengue viruses spread within the country and established into localized outbreaks, most of them were short-lived. Our data suggest that importations of dengue viruses contributed a large part to the diversity of dengue viruses. The geo- and phylogenetic clustering of samples from patients with recent travel history and subsequent local cases further provides evidence on the role of imported virus strains on local transmission (Lee and Ng, unpublished data). A similar scenario has also been reported in Taiwan, where multiple dengue epidemics were initiated by different importations of dengue virus (Huang et al., 2007; King et al., 2000). A report from China also demonstrated the role of importation of DENV, including those closely related to Singaporean viruses, on the epidemiology of dengue in China (Chen, 2011). Together with *in situ* evolution, virus importations play an important role in shaping the epidemiology of dengue in Singapore. Generally, we see a random distribution of different lineages around the island. This is likely due to an efficient transport system that spans all over the island. For example, the initial emergence of clade III and IV DENV-2 was associated with an increased number of cases in several localities in Singapore. However, these viruses had spread across the island ever since and currently, there is no clear spatial differentiation among the different clades of DENV-2 within Singapore.

The high diversity of dengue viruses in Singapore appears to contrast other reports. Puerto Rico reported that among 91 DENV-2 isolates collected over a period of 18 years, the majority of the viruses belonged to 2 genotypes, the American and Asian/American genotypes, and only 1 fell into the Asian I genotype (Ben-nett et al., 2006). The contrast could be due to the increased globalization in recent decades and Singapore's position as a regional and global commercial and travel hub in a dengue hyper-endemic region – factors that could lead to frequent exchanges and importation of dengue viruses through human movement. The high frequency of overseas travel by Singapore residents is reflected by the fact that half of the population above 15 years old made at least 1 trip overseas in 2005 (Department of Statistics, Singapore, 2005). Singapore also received 11.6 million visitors in 2010 on its 700 km² island (Department of Statistics, Singapore, 2011). However, we also do not rule out the possibility that the high level of diversity among circulating dengue viruses in Singapore could be a result of intense sampling, which may have contributed to the higher detection level of various genotypes.

Our virologic surveillance effort has been part of an integrated vector control program in Singapore. Through the years, this has contributed significantly to ground operations in the effort to suppress the spread and minimizing the magnitude of dengue outbreaks through early warning of outbreaks and guided decisions in control strategy (Lee et al., 2010). Virological surveillance also has a direct impact on the evaluation of control effort. For instance, our genotyping revealed that a large dengue cluster of DENV-3 in 2009 in the western part of Singapore was different from those detected subsequently in the other parts of the island. This provided the assurance that the containment of DENV-3 in the western part of Singapore was successful and thus allowed the stepping down of vector control operations in that area.

5. Conclusions

In summary, *in situ* evolution of viruses and importations play a major role in shaping the molecular epidemiology of dengue in Singapore. The implication is significant, as a highly diverse virus population provides ample opportunity for selection and emergence of

more epidemic viral strains, thus increasing the risk of severe disease outbreaks. Currently, there is very little understanding on the characteristics, such as virulence and epidemic potential, of various genotypes and strains of dengue viruses. Thorough regional and global virus surveillance, coupled with the associated epidemiological and clinical knowledge, is urgently needed to enhance our understanding of the disease and the causative virus. Regional and global virus surveillance also has great potential in delivering early warning of outbreaks and situational awareness, thus allowing the early implementation of enhanced vector control.

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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.meegid.2011.10.012](https://doi.org/10.1016/j.meegid.2011.10.012).

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