Virus Research 143 (2009) 1–5

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Importation and co-circulation of multiple serotypes of dengue virus in Sarawak, Malaysia

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ARTICLE INFO

Article history:
Received 25 January 2009
Received in revised form 23 February 2009
Accepted 25 February 2009
Available online 28 March 2009

Keywords:
Dengue
Sarawak
Evolution
Epidemiology
Phylogeny
South-East Asia

ABSTRACT

Although dengue is a common disease in South-East Asia, there is a marked absence of virological data from the Malaysian state of Sarawak located on the island of Borneo. From 1997 to 2002 we noted the co-circulation of DENV-2, DENV-3 and DENV-4 in Sarawak. To determine the origins of these Sarawak viruses we obtained the complete E gene sequences of 21 isolates. A phylogenetic analysis revealed multiple entries of DENV-2 and DENV-4 into Sarawak, such that multiple lineages co-circulate, yet with little exportation from Sarawak. Notably, all viral isolates were most closely related to those circulating in different localities in South-East Asia. In sum, our analysis reveals a frequent traffic of DENV in South-East Asia, with Sarawak representing a local sink population.

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1. Introduction

Dengue is one of the most important emerging diseases of humans, causing considerable mortality and morbidity in tropical and sub-tropical regions on a global scale. The causative single-strand positive-sense RNA virus (DENV, family *Flaviviridae*) is also notable for its genetic diversity, which is manifest as four serotypes (DENV-1 to DENV-4), that likely interact in a variety of complex ways (Adams et al., 2006; Ferguson et al., 1999; Wearing and Rohani, 2006), and which are themselves structured into phylogenetically discrete genotypes (reviewed in Holmes and Twiddy, 2003).

Although a hindrance to future vaccination, the extensive genetic diversity of DENV has allowed important insights into the evolution and epidemiology of this virus, with phylogenetic studies of the envelope (E) protein the main analytical tool to date (for example, Chungue et al., 1995; Goncalvez et al., 2002; Lanciotti et al., 1994; Sittisombut et al., 1997; Zhang et al., 2005). Indeed, molecular epidemiological studies of DENV have revealed a number of important generalities, including that genotype distributions have a clear geographical component, with some restricted to specific geographic localities while others are more widespread, causing infections on multiple continents (Holmes and Twiddy, 2003). Importantly, the importation of 'foreign' DENV lineages into new geographic regions is sometimes associated with the appearance of more severe manifestations of dengue disease, most notably dengue hemorrhagic fever (DHF), and the displacement of indigenous viral lineages, in turn suggesting that these lineages also differ in fitness (Messer et al., 2002, 2003; Rico-Hesse et al., 1997). Describing the global genetic diversity of DENV, particularly patterns of viral migration, is therefore of great importance.

Although dengue is at highest prevalence in South-East Asia, where it is one of the leading causes of pediatric disease, there are still some Asian localities where the genetic diversity and origins of DENV remain opaque. One such locality is Sarawak, a Malaysian state that spans western Borneo, and which shares a long border with Kalimantan, Indonesia to the east. Sarawak is a sparsely populated state of only two million inhabitants, with half the population located in the three major towns of Kuching, Sibu and Miri, and the rest spread over much more rural and remote towns and villages. The principle dengue mosquito vector, *Aedes aegypti*, is found the in towns, while *Aedes albopictus* is the more important vector in the villages. There is little data on DENV in Sarawak. Although the work of Chang and colleagues in the 1970s and 1980s suggest that the virus is endemic in Sarawak and occasionally causes outbreaks (Chang and Jute, 1986; Chang et al., 1981), there is cur-

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doi:10.1016/j.virusres.2009.02.020

Table 1
Dengue virus isolates from Sarawak, Malaysia isolated in this study.

Isolate	Serotype	Place isolated	Settlement	Year	Comments
CS85-3	DENV-2	Serian	Small town	1997	From same patient, serial specimens; sporadic case
CS85-4	DENV-2	Serian	Small town	1997	As above
LF5	DENV-2	Lundu	Village	1999	Isolated from a single febrile case during a DENV-4 outbreak
SB8540	DENV-2	Sibu	Large town	2002	Outbreak in Sibu town
SB8553	DENV-2	Sibu	Large town	2002	As above
SB8571	DENV-2	Sibu	Large town	2002	As above
SB8586	DENV-2	Sibu	Large town	2002	As above
SB8699	DENV-2	Sibu	Large town	2002	As above
MY18-3	DENV-3	Sibu	Large town	1997	Fatal case, isolate from CSF; adenovirus type 21 also isolated from CSF
CS81-1	DENV-3	Serian	Small town	1997	Sporadic case
LF11	DENV-4	Lundu	Village	1999	Outbreak in small rural village in southwestern Sarawak
LF32	DENV-4	Lundu	Village	1999	As above
LF34	DENV-4	Lundu	Village	1999	As above
LF54	DENV-4	Lundu	Village	1999	As above
LF67	DENV-4	Lundu	Village	1999	As above
LF68	DENV-4	Lundu	Village	1999	As above
LF76	DENV-4	Lundu	Village	1999	As above
L115	DENV-4	Lundu	Village	1999	Case contact, asymptomatic, Lundu outbreak
CS101-1	DENV-4	Serian	Small town	1997	From same patient, serial specimens; sporadic case
CS101-2	DENV-4	Serian	Small town	1997	As above

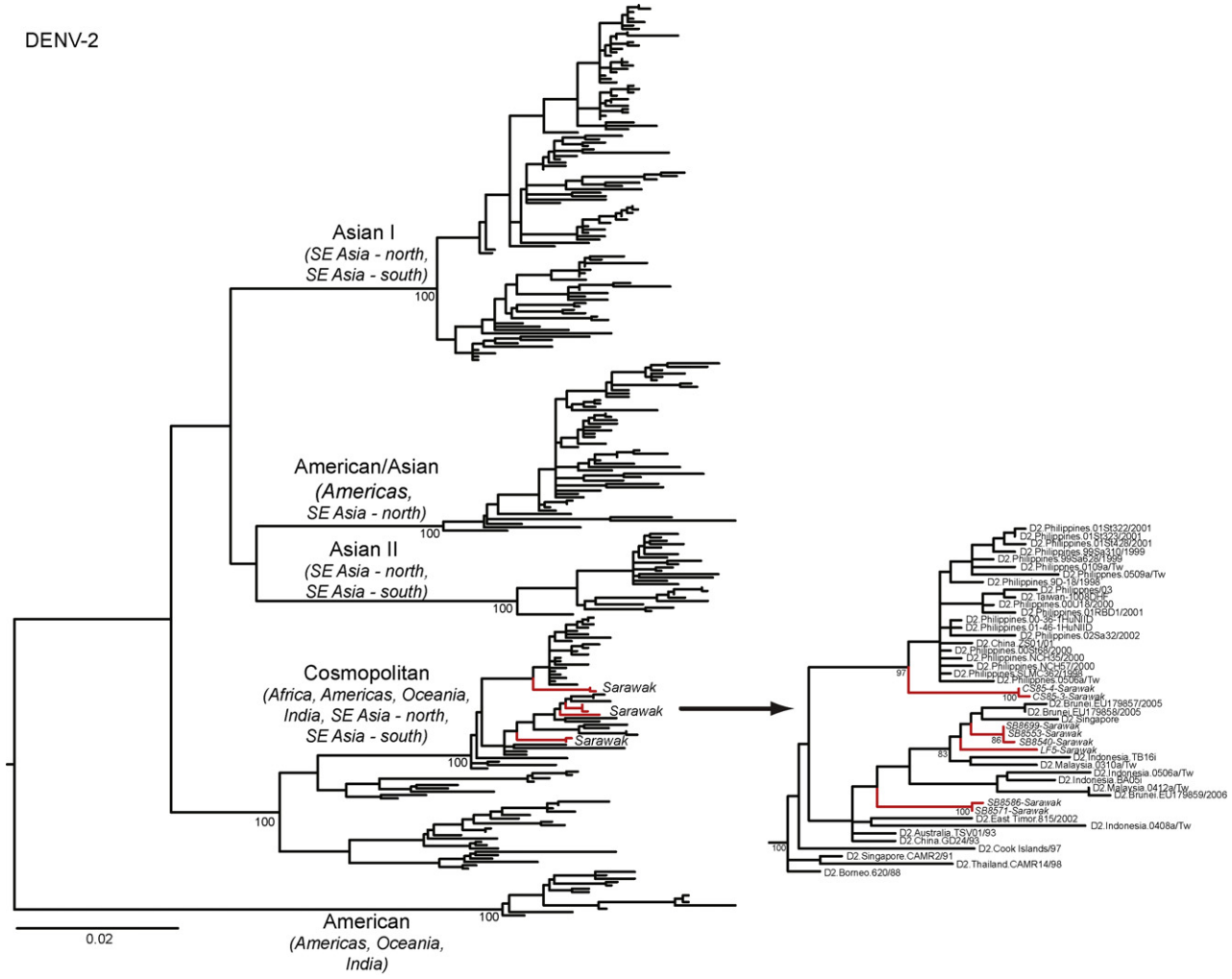

Fig. 1. Maximum likelihood phylogenetic tree of 273 isolates of the complete E gene of DENV-2. The global genotypes of DENV-2 are marked and those viruses from Sarawak are shown in red. The insert represents a magnification of the portion of the phylogenetic tree that contains the Sarawak viruses. The tree is mid-point root for clarity only, all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site, and bootstrap values are shown for key nodes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

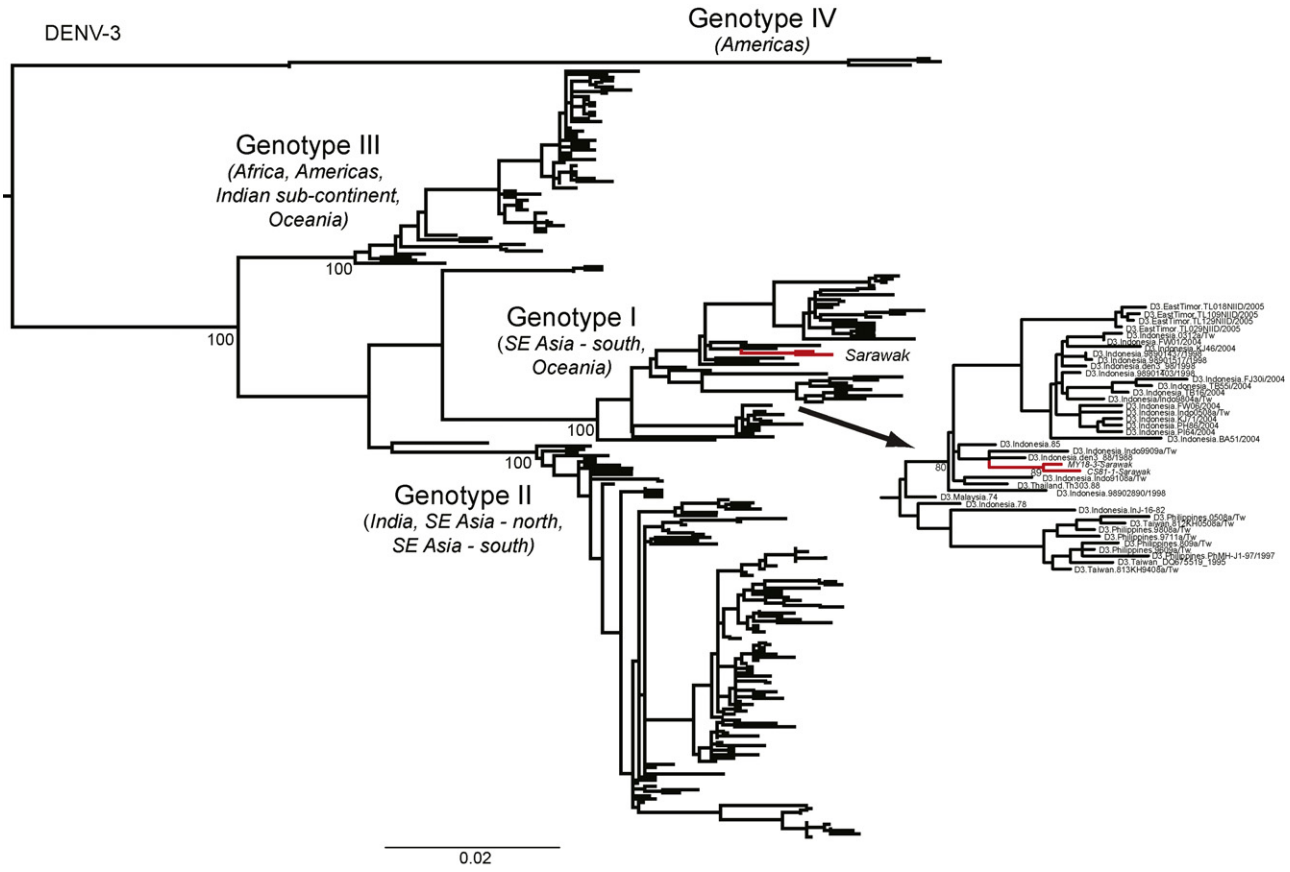

Fig. 2. Maximum likelihood phylogenetic tree of 248 isolates of the complete E gene of DENV-3. The global genotypes of DENV-3 are marked and those viruses from Sarawak are shown in red. The insert represents a magnification of the portion of the phylogenetic tree that contains the Sarawak viruses. The tree is mid-point root for clarity only, all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site, and bootstrap values are shown for key nodes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

rently no data on the phylogenetic relationship of the Sarawak viruses to DENV found in South-East Asia and more distant locations.

The aim of this study was to provide information on the origins and genetic diversity of DENV circulating in Sarawak, again utilizing a phylogenetic analysis of E gene sequences. We included viral isolates from sporadic cases as well as from outbreaks from years 1997 to 2002. During this period DENV was isolated from specimens submitted for investigation from Serian (population 88,000) and Lundu (population 30,000), located in the southwestern part of Sarawak and close to the Indonesian border, and from Sibu (population 215,000), a major gateway town to the Rejang River which runs through the Bornean rainforest.

2. Methods

2.1. Specimens

The virology laboratory of Universiti Malaysia Sarawak has been supporting outbreak investigations of the Sarawak Health Department (SHD) in the state of Sarawak, Malaysia since 1997. Serum specimens from suspected dengue patients have been submitted by the SHD for dengue serological and virological investigations during routine vector control operations since 1997. The DENV isolated from 1997 to 2002, as well as some background epidemiological information, are listed in Table 1.

2.2. Isolation of dengue virus

Temperature adapted C6/36 mosquito cells grown in Leibovitz 15 media supplemented with 10% tryptose phosphate broth and 1% heat inactivated foetal calf serum were seeded into 24 well plates and incubated at 30 °C overnight. Five μl of serum were inoculated onto the monolayers and incubated at 30 °C for 2 weeks. Media was changed after 5–6 days in culture and replenished regularly throughout the 2-week incubation. A cytopathic effect (CPE) was observed in the C6/38 monolayer following media change and characterized by large syncytia. When a CPE was observed, media was changed again and the contents of the well were harvested with no further change of media when the CPE was extensive, usually one to two days later. A second blind passage was always performed with day 14 harvests when no CPE was observed in the first passage. Virus isolates were aliquoted and stored at −70 °C until used. All putative isolates were subjected to a second passage in C6/36 cells for use in identification of virus.

2.3. Identification of dengue virus

The presence of dengue virus was determined by an antigen capture ELISA and by RT-PCR. Clarified culture supernatant from passage two harvests were loaded into duplicate wells of a microtitre plate which had been coated overnight with a rabbit anti-mouse IgG (Dako, Glostrup, Denmark) followed by a dengue reactive mouse monoclonal antibody, MF4/5/A5 (Venture Technologies, Penang, Malaysia). If there were dengue virus anti-

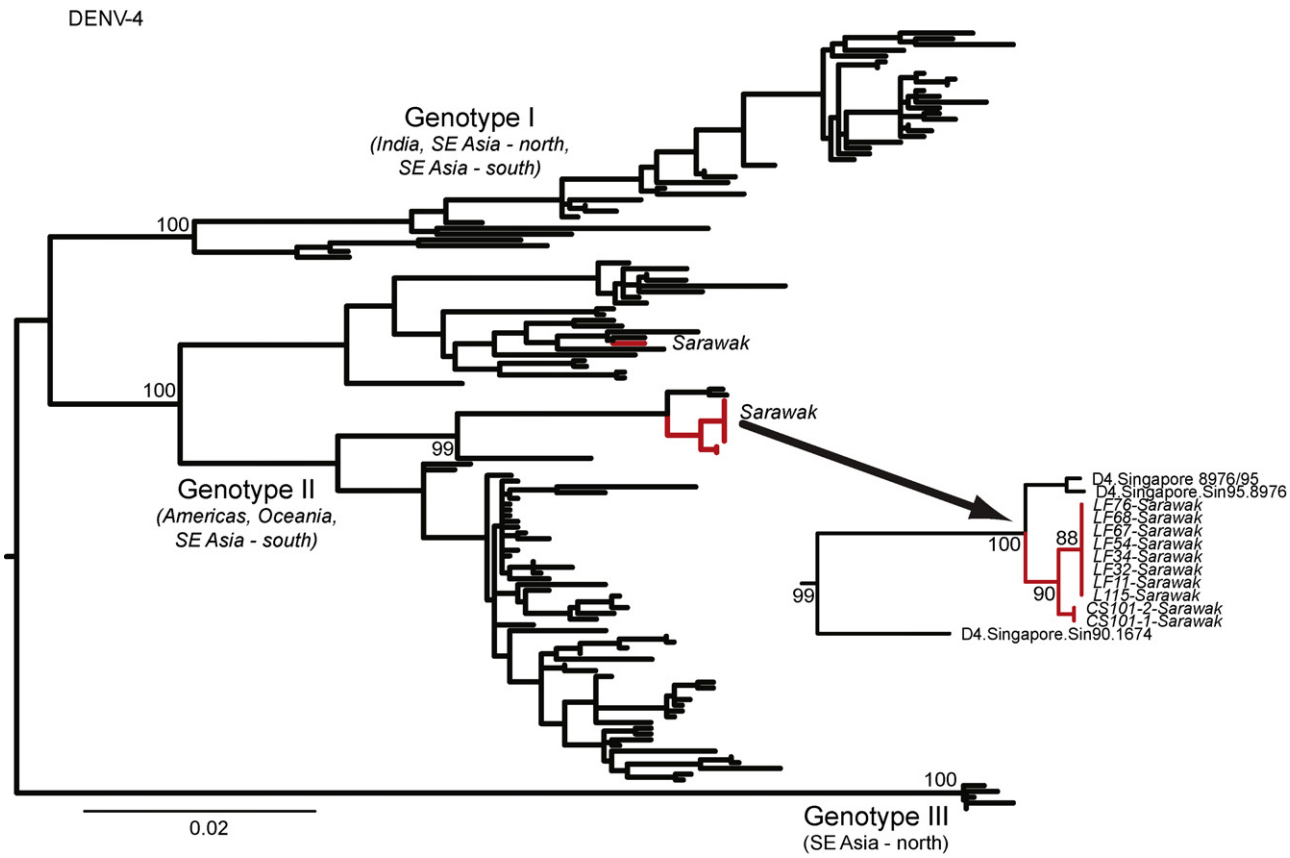

Fig. 3. Maximum likelihood phylogenetic tree of 136 isolates of the complete E gene of DENV-4. The global genotypes of DENV-4 are marked and those viruses from Sarawak are shown in red. The insert shows a magnification of the portion of the phylogenetic tree that contains the Sarawak viruses. The tree is mid-point root for clarity only, all horizontal branch lengths are drawn to a scale of nucleotide substitutions per site, and bootstrap values are shown for key nodes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

gens in the harvest, these would be captured in the wells and the antigens were detected with a purified pooled convalescent dengue serum followed by a rabbit anti-human IgG conjugated with HRP, colour generation achieved by incubation with OPD and hydrogen peroxide, and stopped with 2N sulphuric acid after 30 min. The OD was read at 492 nm in a standard microplate reader.

RNA was extracted from cell culture supernatants by using Tri Reagent (Sigma, St Louis, MO, USA) following the manufacturer's instructions and RT-PCR was performed using primers and methods described by Tanaka (1993) to confirm the presence of DENV and to provide serotype identification.

2.4. Sequencing of the complete E gene

Primers for RT-PCR and sequencing of the E gene of DENV-2, DENV-3 and DENV-4 isolates were designed based on alignments of complete genome sequences of each serotype obtained from GenBank (Supplementary Table 1). The complete E gene of each DENV serotype was amplified in a single RT-PCR reaction using primer sets flanking the E gene. Briefly, for each DENV serotype, cDNA was transcribed from extracted viral RNA using the PCR antisense PCR primer followed by PCR under the following cycling conditions: 94 °C (5 mins), 5 cycles of 94 °C (60 s), 45 °C (60 s) and 72 °C (90 s), and 35 cycles of 94 °C (60 s), 55 °C (60 s) and 72 °C (90 s) followed by a final extension at 72 °C (5 min). Amplified products were excised and purified from agarose gels using the GENECLEAN III kit (BIO101, Vista, CA, USA). Each amplified product was sequenced from both strands using overlapping sequencing primers. Sequencing was performed using BigDye v3.1 (Applied Biosystems, Foster City, CA, USA) and run on the ABI377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Complete DENV E gene sequences generated in this study were deposited into GenBank and assigned accession numbers FM986654 to FM986674.

2.5. Evolutionary analysis

The E gene sequences of 8 DENV-2, 2 DENV-3 and 11 DENV-4 were obtained in this study (Table 1). For one case each from DENV-2 and DENV-4 two sequences from the same patient were obtained. These sequences were combined with expansive and comparative data sets for each serotype available on GenBank. This resulted in final data sets of the following size: DENV-2 = 273 sequences, 1485 nt; DENV-3 = 248 sequences, 1479 nt; DENV-4 = 136 sequences, 1485 nt. Sequence alignment was trivial in each case. For each serotype a maximum likelihood (ML) phylogenetic tree was estimated using the method implemented in the PAUP* package (Swofford, 2003), utilizing the best-fit model of nucleotide substitution as determined by MODELTEST (Posada and Crandall, 1998), which in all cases was the most general GTR+I+Γ_4 model (parameter values available from the authors on request). A neighbor-joining bootstrap re-sampling analysis (1000 replications) was performed to assess the support for specific nodes, again utilizing the ML substitution model.

3. Results and discussion

Despite the relative geographic isolation of Sarawak, as well as its sparse population, it is notable that three of the four DENV serotypes co-circulated in this region during the study period (no

DENV-1 was detected). Dengue outbreaks occur occasionally in Sarawak and are not usually associated with the large disease outbreaks that frequently plague peninsular Malaysia. Often the outbreaks in Sarawak are confined to specific schools, housing estates and villages (Table 1), and are contained relatively quickly through vector control.

The maximum likelihood trees for the E gene data sets of DENV-2, DENV-3 and DENV-4 are presented in Figs. 1–3, respectively (the viruses from Sarawak are depicted in red in each case). To increase clarity, inserts are included which show a magnification of the parts of the phylogenetic tree that contain the Sarawak viruses. In the case of DENV-2 all 8 isolates from Sarawak fell within the geographically disperse 'Cosmopolitan' genotype that has previously been detected in Africa, the Americas, India, Oceania, and in diverse localities within South-East Asia (Fig. 1). Similarly, the two DENV-3 isolates both fell within genotype I that is found in the southern part of South-East Asia and Oceania (Fig. 2). Finally, the 11 isolates of DENV-4 from Sarawak sequenced here all clustered within genotype II that has previously been sampled in the Americas, Oceania, as well as the southern part of South-East Asia (Fig. 3). Closer inspection of the phylogenetic trees (the inserts) reveals that those viruses in Sarawak are always most closely related to DENV that circulate in neighboring geographic regions, such as peninsular Malaysia, Indonesia, The Philippines, Singapore, and Thailand. Such strong phylogenetic links to DENV that already circulate in South-East Asia, as well as the mix of Asian localities on the phylogenetic tree, indicates that there is a frequent traffic of dengue viruses in this geographical region.

Although local gene flow appears to be commonplace, and despite the sparseness of sampling, our phylogenetic analysis also suggests that Sarawak is a 'sink' population for DENV, with the local importation of viruses occurring relatively frequently, but with no evidence on these data that DENV are ever exported from Sarawak. In particular, not only do multiple serotypes co-circulate in Sarawak, but in the case of DENV-2 and DENV-4 multiple and phylogenetically distinct lineages also co-circulate in this locality, strongly suggesting that there have been a number of independent importation events. This effect is particularly notable in the case of DENV-2 where the E gene phylogeny suggests there have been at least three, and perhaps four, independent entries of DENV into this region, manifest as distinct clusters of Sarawak viruses that are separated by high bootstrap values (see insert in Fig. 1). This is further supported by the observation that those DENV-2 viruses sampled from Serian and Sibu form phylogenetically distinct groups, again indicative of independent entry. In addition, three DENV-2 sequences from the neighboring country of Brunei (Osman et al., 2008) form separate lineages on the phylogenetic tree of this virus, indicating that they have also been introduced independently, even though Sarawak and Brunei share a border.

Finally, it is also interesting to note that both independent entries of DENV-4 into Sarawak, which are relatively phylogenetically distinct, were most closely related to viruses sampled from Singapore. These distinct lineages have different geographical associations within Sarawak, with one lineage associated with Lundu and the other with Serian. Singapore is a major destination for Sarawak youth seeking work, education and training opportunities, and leads to significant traffic between the two locations. That Singapore is clearly a major economic and travel hub in South-East Asia may also mean that it plays an important role in the local dissemination of DENV, although this will need to be explored using a far larger sample of viruses.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2009.02.020.

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