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Emergence of dengue virus type 4 genotype IIA in Malaysia

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Phylogenetic analyses of the envelope (E) gene sequence of five recently isolated dengue virus type 4 (DENV-4) suggested the emergence of a distinct geographical and temporal DENV-4 subgenotype IIA in Malaysia. Four of the isolates had direct ancestral lineage with DENV-4 Indonesia 1973 and showed evidence of intra-serotypic recombination with the other recently isolated DENV-4, MY01-22713. The E gene of isolate MY01-22713 had strong evidence of an earlier recombination involving DENV-4 genotype II Indonesia 1976 and genotype I Malaysia 1969. These results suggest that intra-serotypic recombination amongst DENV-4 from independent ancestral lineages may have contributed to the emergence of DENV-4 subgenotype IIA in Malaysia.

Dengue is a mosquito-borne disease affecting at least 50 million people around the world annually (WHO, 1998). In Malaysia, dengue has been endemic since its first description by Skae in 1902 (Rudnick, 1986). The annual incidence of dengue in Malaysia is about 367 cases in a 'quiet' year to about 6628 cases in a 'busy' year. Similar to other countries within the region, all four dengue virus serotypes have been associated with dengue fever (DF) and dengue haemorrhagic fever in Malaysia. Dengue virus type 4 (DENV-4), the once predominant (40 to ~64%) serotype isolated from DF patients in Malaysia during the period 1967 to 1969 (Rudnick, 1986) has, however, been isolated in less than 5% of DF cases for almost a decade with no reported isolation in the last 5 years (WHO, 2000). Nonetheless, against the background of a very 'quiet' year in 2001, six DENV-4 were isolated within 5 months from patients attending the University of Malaya Medical Center, Kuala Lumpur. We report here the characterization of the envelope (E) gene of five of the DENV-4 isolates in an effort to trace the potential origin of the virus.

Dengue virus was isolated from the serum of DF patients using C6/36 cells cultured in EMEM supplemented with 10% heat-inactivated foetal bovine serum (FBS). After adsorption for an hour, the infected cell culture was incubated at 28 °C in EMEM supplemented with 2% FBS. After cytopathic effects had been observed in infected C6/36 cell cultures, virus RNA was extracted from the supernatant. The virus was typed initially using specific monoclonal antibodies; this was confirmed by performing multiplex RT–PCR using a forward primer, DV1, and sets of four serotype-specific reverse primers DSP1, DSP2, DSP3 and DSP4 to amplify a portion of NS3 region from the different dengue virus serotypes (Seah *et al.*, 1995). The DENV-2 positive control generated an expected band size of approximately 362 bp while the DENV-4 control generated a band of about 426 bp. RT–PCR of all five DENV-4 isolates resulted in bands of 426 bp in size indicating that all five dengue virus isolates used in this study were DENV-4 (data not shown).

The potential phylogenetic relationships of the DENV-4 isolates were examined by determining the complete E gene sequence following the methods described by Wang *et al.* (2000). Reverse-transcription was performed at 42 °C for 1 h; denaturation at 95 °C for 2 min; and 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final elongation at 72 °C for 5 min. The amplified fragments were purified and sequenced using Applied Biosystems Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits and Applied Biosystems model 377 Sequencer (USA). The sequences (accession nos AJ428556, AJ428557, AJ428558, AJ428559 and AJ428560) were aligned together with other previously described DENV-4 isolates identified by geographical location and year of isolation (Wang *et al.*, 2000) and the sylvatic DENV-4 (Malaysia 75-P75-215, 73-P73-1120, 75-P75-514) that were isolated in the 1960s from *Aedes niveus* group mosquitoes living in Malaysian forests (Rudnick, 1984). Phylogenetic analyses were performed using both the distance matrix and character state methods. For distance matrix analyses, multiple alignments of the nucleotide sequences and the deduced amino acids were performed using CLUSTAL X version 1.81 (Thompson *et al.*, 1997) and the resulting alignment was optimized manually using GENEDOC version 2.5 (Nicholas & Nicholas, 1997). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using DENV-2 virus Jamaica strain as the outgroup. The strength of the phylogenetic trees was estimated by bootstrap analyses using 1000 replicates. All trees were displayed using TREEVIEW version

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Table 1. Summary of amino acid differences within the E gene of the recently isolated Malaysian DENV-4 in comparison to other known DENV-4

DENV-4 isolate	Amino acid position																							
	19	46	108	120	132	148	154	162	203	265	329	335	340	342	355	364	382	384	429	455	461	478	494
Dominica 1981	A	T	F	S	I	T	D	A	K	A	A	V	R	V	T	V	V	N	F	I	F	T	Q
Malaysia 75-P75-215	T	I	.	.	V	A	S	T	G	T	T	I	K	M	I	I	A	D	.	.	I	S	.
Malaysia 73-P73-1120	T	I	.	.	V	A	S	T	G	T	T	I	K	M	I	I	A	D	.	.	I	S	.
Malaysia 75-P75-514	T	I	.	.	V	A	S	T	G	T	T	I	K	M	I	I	A	D	.	.	I	S	.
Thailand 1963	.	I	L	.	.	.	.	.	.	T	.	I	.	.	.	.	.	D	S	V	L	.	H
Thailand 1978	.	I	L	.	.	.	.	.	.	T	.	.	.	.	.	.	.	D	L	V	.	S	H
Thailand 1984	.	I	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	D	L	V	.	S	H
Malaysia 1969	.	I	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	D	L	V	.	S	H
Sri Lanka 1978	.	I	L	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	L	V	.	S	H
Philippines 1956	.	I	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	D	L	V	.	.	.
Philippines 1964	.	I	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	D	L	V	.	.	H
Philippines 1984	.	I	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	D	L	V	.	.	H
Indonesia 1973	.	.	L	L	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	V	.	.	.
Malaysia 2001-22713	.	.	V	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	L	.	.
Malaysia 2001-23314	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	V	.	.	.
Malaysia 2001-23264	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	V	.	.	.
Malaysia 2001-23096	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	V	.	.	.
Malaysia 2001-23298	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	V	.	.	.
Indonesia 1976	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	L	.	.	.	.
El Salvador 1994	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Tahiti 1979	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Puerto Rico 1986	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Tahiti 1985	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
New Caledonia 1984	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
El Salvador 1983	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mexico 1984	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Brazil 1982	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Thailand 1994	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

1.6.6 (Page, 1996). Maximum-parsimony and maximum-likelihood analyses performed using PAUP (PAUPSearch, SeqLab, GCG Wisconsin Package, Accelrys Inc., USA) from multiple alignments made with PILEUP (SeqLab, GCG Wisconsin Package, Accelrys Inc., USA) yielded results similar to those obtained from the distance matrix analyses, differing only within the sylvatic isolates genotype. Hence, only results obtained from the distance matrix method were presented. Potential recombinant sequences within the E gene were examined using SIMPLOT version 3.2 (Lole *et al*., 1999). Putative recombinant sequence was queried against two potential parental sequences after all gaps were stripped with a distant sequence as the outgroup. A sliding window of 180 nucleotides was moved in steps of 10 nucleotides at a time and the resulting similarity values were plotted along the E gene sequence. Recombination was identified when conflicting E gene sequence profiles appeared, suggesting acquisition of sequences from a different parental genotype. Bootscanning analyses which utilized the bootstrapping procedures of Salminen *et al*. (1995) and Worobey & Holmes (1999) were performed using the maximum-likelihood method with 100 resamplings. Bootstrap values of 70% were used to indicate robust support for the topologies.

Pairwise comparisons of the sequences showed that the recently isolated Malaysian DENV-4 isolates had nucleotide sequence similarity of at least 92% to the previously reported epidemic/endemic strains and 86% to the sylvatic strains. The nucleotide changes were distributed throughout the E gene with most of them located at the third nucleotide of a codon resulting in no amino acid changes. The amino acid similarity was ~ 95% to the sylvatic strains and ranged from 96 to 98% to other epidemic/endemic strains (data not shown). These findings were comparable to those previously reported for all other DENV-4 isolates (Lanciotti *et al*., 1997; Wang *et al*., 2000). Furthermore, alignment of the deduced amino acid sequences showed conservation of the 12 cysteine amino acids involved in disulphide bond formation and the putative *N*-linked glycosylation sites at amino acids 67 and 153 among all the DENV-4 isolates. Only a single amino acid difference (Phe → Val) at amino acid 108, however, was noted within the glycine-rich putative fusion domain (amino acids 98–111) in isolate Malaysia 2001-22713 (MY01-22713) in comparison to the remaining four Malaysian DENV-4 isolates (Table 1). This single amino acid difference was not surprising, however, since a number of other DENV-4 isolates had different amino acids at the same position. Amino acids that were characteristic of

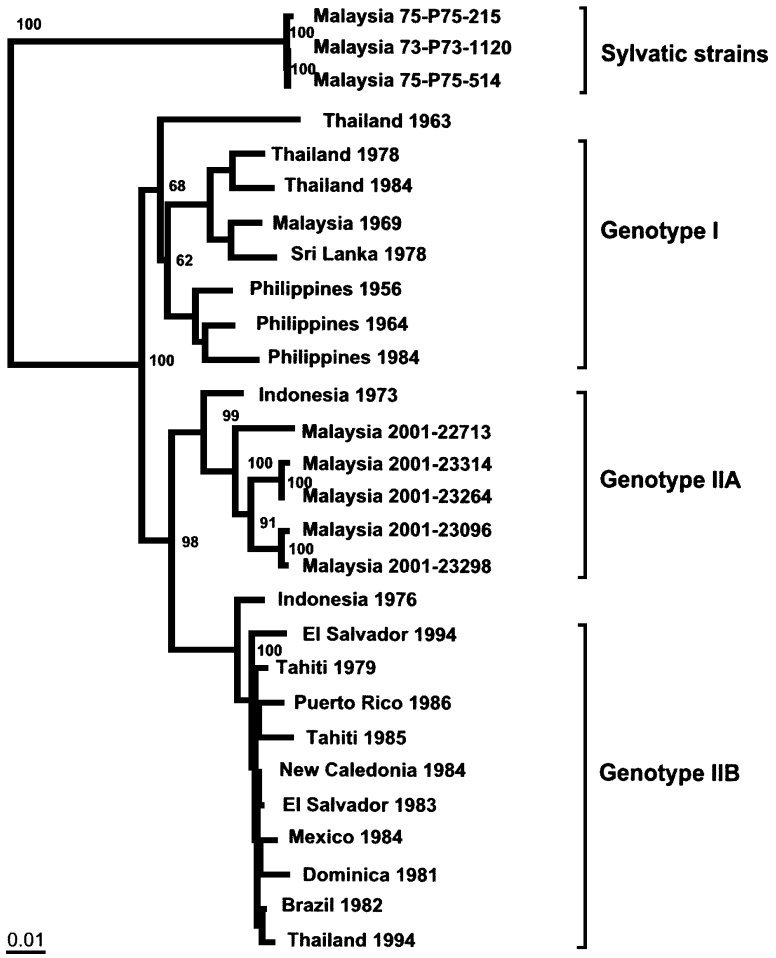

Fig. 1. Phylogenetic tree depicting the relationships of DENV-4 genotype IIa with other known DENV-4. The tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using nucleic acid sequences of the E gene. Bootstrap values are shown as percentages derived from 1000 samplings. The scale reflects the number of nucleotide substitutions per site along the branches. DENV-2 Jamaica strain used as outgroup is not shown.

the sylvatic isolates (amino acids 19, 132, 148, 154, 162, 203, 329, 335, 340, 342, 355, 364, 382, 461 and 478), on the other hand, were not found in any of the recently isolated Malaysian DENV-4, suggesting that the isolates could not have evolved recently from sylvatic origin. Examination of the amino acid sequences also revealed four distinct amino acids at positions 46, 265, 429 and 494 (Thr, Ala, Phe and Gln) that could be used to differentiate all the DENV-4 into at least two genogroups (Table 1). The remaining two amino acids at positions 384 and 455 (Asp and Val) identified by Lanciotti *et al*. (1997) as characteristic for DENV-4 genotype I were found also in the recently isolated Malaysian DENV-4. In addition, only the recently isolated MY01-23314, -23264, -23096 and -23298 and Indonesia 1973 (ID73) DENV-4 had leucine at position 120 when compared to all other DENV-4 (Table 1), suggesting that this single amino acid change could be unique to the recently isolated Malaysian DENV-4 and ID73. A phylogenetic tree drawn using the E gene nucleotide sequences showed three well-supported DENV-4 clusters (bootstrap values of 100%) (Fig. 1). These clusters were similar to that previously identified as genotype I consisting of viruses from Thailand (TH), Malaysia 1969 (MY69), Sri Lanka (SE) and Philippines (PH); genotype II comprises mainly isolates from South America and the Pacific Islands (Lanciotti *et al*., 1997) and the sylvatic isolates form a distinctly different genotype (Wang *et al*., 2000). The recently isolated Malaysian DENV-4 isolates subclustered together with ID73 into a separate and well-supported (98%) subcluster within genogroup II, hence denoted as genotype IIA in the present study (Fig. 1). A phylogenetic tree drawn using the deduced amino acid sequence further supported separation of all but one of the isolates (MY01-22713) into a different subgenogroup (data not shown). Except for isolate MY01-22713, all other recently isolated Malaysian DENV-4 had aspartic acid at position 384, similar to ID73 virus. MY01-22713, on the other hand, had asparagine, similar to all other DENV-4 of genotype IIB (Table 1). The presence of aspartic acid has been suggested to be the reason that DENV-4 ID73 could be effectively neutralized by DENV-4 genotype I-specific serum and not with genotype II-specific serum (Lanciotti *et al*., 1997). This suggested the possibility that the E gene of DENV-4 ID73 together with the recent Malaysian isolates were mosaics of DENV-4 genotype I and II. Evidence of recombination ($>$ 70% bootstrap support) between DENV-4 genotype I (MY69) and genotype II (ID76)

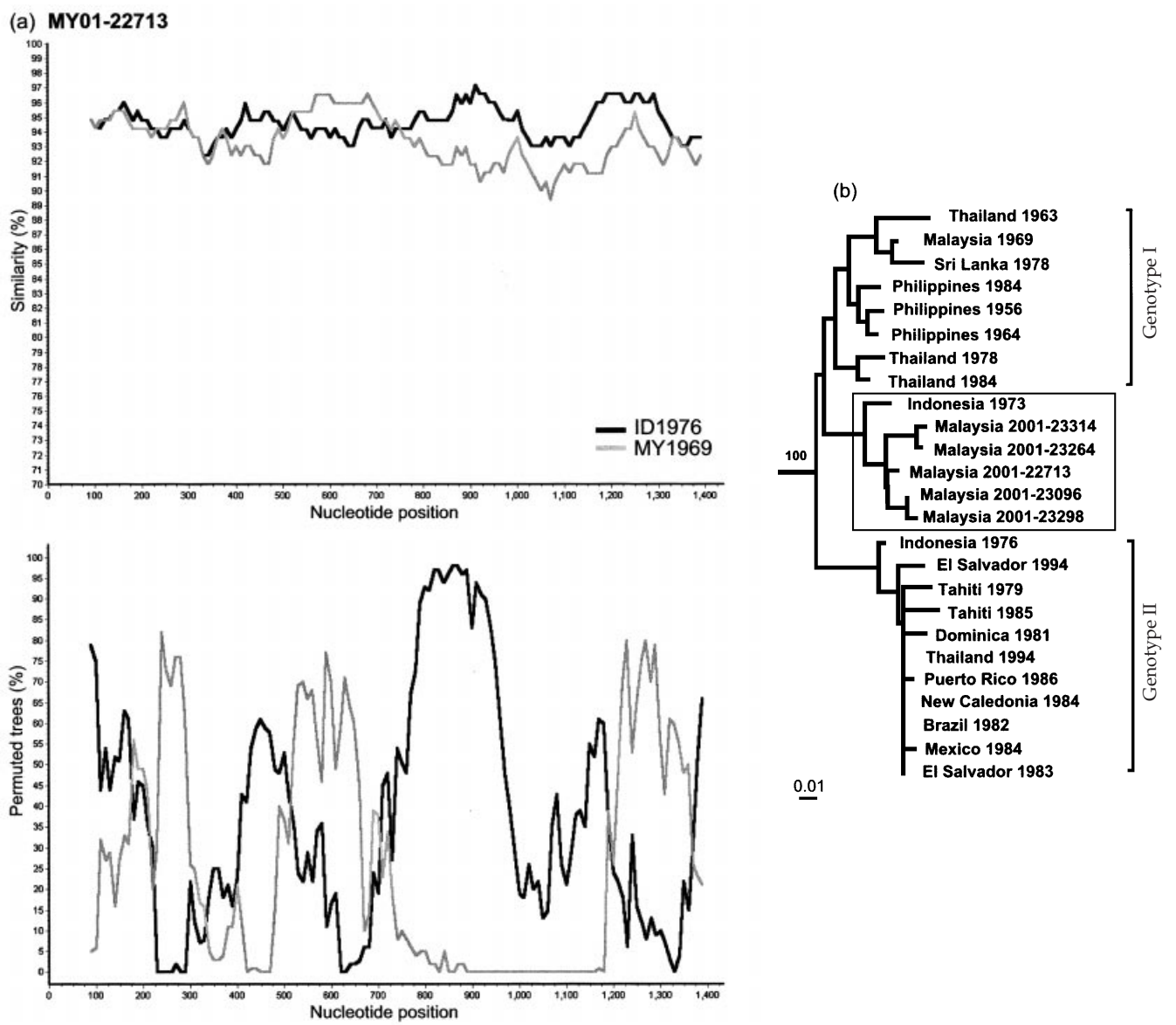

Fig. 2. For legend see facing page.

was obtained from similarity plot and bootscanning analyses performed on the recently isolated Malaysian DENV-4 MY01–22713 (Fig. 2a). However, only weak evidence supporting recombination between genotype I and II in DENV-4 ID73 and the remaining recently isolated Malaysian DENV-4 was obtained. This finding was similar to that reported by Worobey *et al*. (1999) when the E gene of DENV-4 ID73 was queried against DENV-4 ID77 (genotype II) and PH73 (genotype I) as possible parental lineages. Despite weak statistical support, it was argued in that study that ID73 is indeed a genuine recombinant. However, a phylogenetic tree, drawn using nucleotides at positions 561–800 identified from the breakpoint analyses, placed (100% bootstrap support) the recently isolated Malaysian DENV-4 and ID73 into DENV-4 genotype I (Fig. 2b), thus lending support to the earlier assertion that the E gene of DENV-4 ID73 and the recently isolated Malaysian DENV-4 are mosaics of DENV-4 genotype I and II.

A phylogenetic tree constructed earlier (Fig. 1) using the entire E gene suggested that DENV-4 genogroup IIA had originated from DENV-4 ID73. However, amongst the recently isolated DENV-4, isolate MY01-22713 varied significantly from the rest by having the strongest evidence of mosaicism and a possible neutralization site that is similar to genotype IIB at amino acid 384, unlike the rest of the isolates which resembled genotype I. Since DENV-4 MY01-22713 was isolated at least 4 months earlier than the remaining isolates, it raised the possibility that perhaps the later isolates had

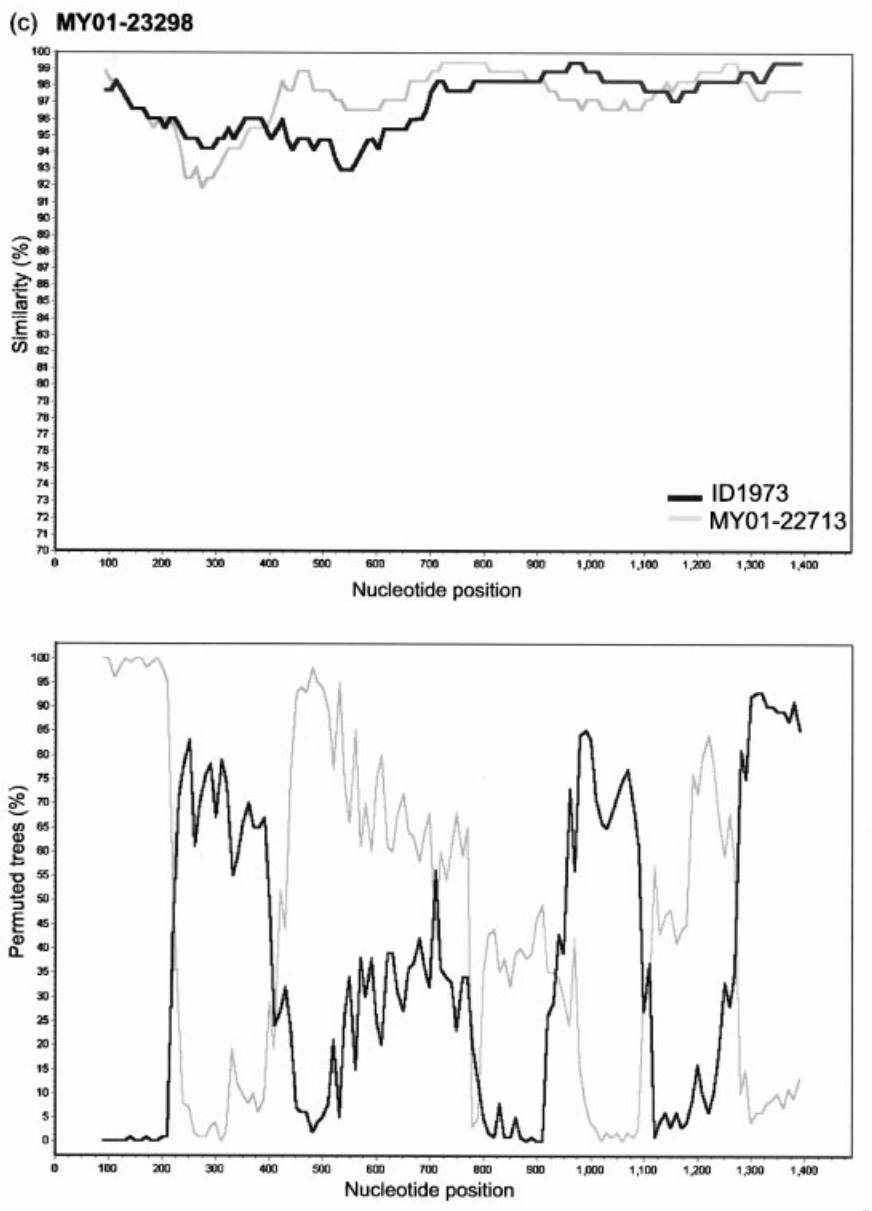

Fig. 2. Similarity plot analyses for determination of potential recombinant sequences in the E gene of DENV-4 genotype IIA. The similarity plots were generated using SIMPLOT version 3.2 (Lole *et al.*, 1999). The upper panel shows results of pairwise comparisons between the query isolates, MY01-22713 (a) and MY01-23298 (c), shown in bold above the plot, and their potential parents, MY1969 and ID76 and MY01-22713 and ID1973, respectively. The vertical axis is the percent similarity between the query sequence and each parental sequence plotted at the midpoint of each sliding window of 180 nt at 10 nt per increment after all gaps were stripped. The horizontal axis is the nucleotide numbers counted from the 5′-terminal of E gene. The lower panel is the result from bootscan analyses illustrating the likelihood of clustering of the putative recombinants with respect to the parental isolates.(b) A neighbour-joining tree (Saitou & Nei, 1987) constructed from alignment of the putative recombinant sequences (nucleotides 561–800) places the recently isolated Malaysian DENV-4 genotype IIA (boxed) into DENV-4 genotype I. The tree was rooted using the sylvatic DENV-4 E gene sequence (not shown) and the bootstrap value shown was derived from 1000 replicates.

diverged recently as a result of intra-typic recombination. Evidence of recombination (> 70% bootstrap support) within the E gene of the four isolates, MY01-23096, -23298, -23264 and –23314, was obtained when the sequences were queried against ID73 and MY01-22713 as the parental isolates (Fig. 2c). These findings suggest that DENV-4 ID73 is potentially a bona fide ancestor of the recently emerged DENV-4 genotype IIA, whereas isolate MY01-22713 may have emerged independently from different ancestral lineages, perhaps following an earlier intra-serotypic recombination between

DENV-4 genotype I (MY69) and genotype II (ID76) as indicated above (Fig. 2a).

In summary, evidence supporting the emergence of DENV-4 genotype IIA in Malaysia from different ancestral lineages following inter-typic recombination is presented. Whether DENV-4 genotype IIA remained localized in Malaysia, however, remains to be seen. Nonetheless, these findings, along with others (Worobey *et al.*, 1999; Tolou *et al.*, 2001; Uzcategui *et al.*, 2001), strongly suggest that recombination amongst specific DENV serotypes has occurred in a natural population and new genotypes could emerge especially in a population where multiple strains of the virus are co-circulating.

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