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Short Communications

Molecular Epidemiology of Dengue Viruses Co-circulating in Upper Myanmar in 2006

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Abstract: To understand the molecular epidemiology of circulating dengue viruses (DENV) in Upper Myanmar, DENV isolation was attempted by inoculating the sera of a panel of 110 serum samples onto a C6/36 mosquito cell line. The samples were collected from dengue (DEN) patients admitted at Mandalay Children's Hospital in 2006. Infected culture fluids were subjected to a RT-PCR to detect the DENV genome. Three DENV strains were isolated. This was the first DENV isolation performed either in Mandalay or in Upper Myanmar. One strain belonged to DENV serotype-3 (DENV-3), and two other strains belonged to DENV serotype-4 (DEN-4). The sequence data for the envelope gene of these strains were used in a phylogenetic comparison of DENV-3 and DENV-4 from various countries. Phylogenetic analyses revealed that this DENV-3 strain was clustered within genotype II, and the two DENV-4 strains were clustered within genotype I in each serotype. The Myanmar strains were closely related to strains from the neighboring countries of Thailand and Bangladesh. These results are important for elucidating the trends of recent and future DEN outbreaks in Myanmar.
Key words: Dengue virus, Molecular epidemiology, Upper Myanmar

Introduction

The dengue virus (DENV) belongs to the genus *Flavivirus* of the family *Flaviviridae*, which exists as four serotypes (DENV-1,-2,-3, and -4) [1]. DENV infection is the most important of the mosquito-borne viral diseases, and it affects mainly tropical and subtropical countries [2]. It is well documented that all four serotypes of DENV co-circulate in Asian countries including Myanmar [3, 4]. The first major epidemic of dengue hemorrhagic fever (DHF) occurred in Myanmar in 1970 [5]. Currently, DHF occurs throughout the country, with the notable exception of the Chin State. Almost 80% of cases are reported from three divisions (Yangon, Bago and Mandalay) and one state (Mon), with more than 50% of cases recorded exclusively from the Yangon Division [5]. DEN outbreaks have been recorded in Upper Myanmar, especially in Mandalay, the largest city in the region. However, an extensive study has never been accomplished due to insufficient laboratory facilities. The present study focused on highlighting current DENV infections in Upper Myanmar with a special emphasis on molecular epidemiology.

Methods

Patients

In total, 110 serum samples were obtained from 110 patients (≤ 12 years old) who were clinically suspected for DEN according to World Health Organization [6] criteria and who were admitted to the 550-bed Mandalay Children's Hospital (MCH), Mandalay City, Upper Myanmar in 2006 with the informed consent of parents or legal

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guardians. The study protocol was reviewed and approved by the Ethical Committee on Medical Research Involving Human Subjects, Department of Medical Research (Upper Myanmar), Pyin Oo Lwin, Myanmar. The sera were stored at −70°C until further use.

Methods

Both IgM- and IgG-capture ELISAs were performed using Dengue Duo IgM-capture and IgG-capture ELISA Kits (PANBIO, Brisbane, Australia) to determine primary and secondary DENV infections. All the commercial kits in the present study were used following the manufacturer's instructions.

The frozen sera were transferred to Japan, and the virus culture was conducted in the Department of Virology, Institute of Tropical Medicine, Nagasaki University, Japan. Each serum sample was inoculated onto *Aedes albopictus* clone C6/36 mosquito cells and incubated at 28°C for 7 days [7]. The presence of DENV in the infected culture fluid (ICF) was verified by in-house Flavivirus antigen detection ELISA (Ag-ELISA) [8] and RT-PCR. RNA extraction from ICF was performed using a viral RNA Mini Kit (QIAGEN, Hilden, Germany). DENV serotyping was done using 4 sets of serotype-specific primers [9–11] employing the PrimeScript™ One Step RT-PCR Kit (Takara Bio Inc., Shiga, Japan).

The desired DNA bands were excised from the agarose gel, and were extracted and purified using QIAEX® II Gel Extraction Kit (QIAGEN, Hilden, Germany). The primer extension dideoxy chain termination method was used for direct sequencing of the PCR product. DNA sequencing analysis was performed with BigDye® Terminator version 3.1 Cycle Sequencing Ready Reaction Mixture (Applied Biosystems, Foster City, USA) following the thermal cycle sequencing parameters described previously [12]. The reaction mixture was then purified using an AGENCOURT® CLEANSEQ® Sequencing Reaction Clean-up system (Agencourt Bioscience Corp., Massachusetts, USA). The final product was loaded on an ABI Prism™ Capillary Sequencer 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, USA). Nucleotide sequences were edited and homology searches and comparisons of the sequences done using DNASIS (Mac version 3.6 Software system; Hitachi, Tokyo, Japan). Nucleotide sequence alignments were carried out using CLUSTAL X, version 2.0 [13], and the phylogenetic analysis was performed using either the heuristic or the branch and bound algorithm of PAUP version 4.0b10 (Altivec) software [14]. The neighbor-joining method was used to construct the phylogenetic tree with a bootstrap analysis of 1,000 replicates [15].

The Genbank accession numbers, EU478408, EU478409 and EU478410, for the three Myanmar isolates used in the present study, and the accession numbers of all the other strains used for the phylogenetic analysis, are listed in Tables 1 and 2 with the geographic origin and the year of isolation.

Results

Among 110 clinically diagnosed DEN patients, 70 (64%) were positive for both IgM and IgG by DEN IgM capture and DEN IgG capture ELISA and were confirmed as secondary DENV infections. Primary DENV infection was confirmed in 26 (24%) patients who were positive only for IgM. The remaining 14 (13%) patients were not confirmed to have DENV infections. Among the 96 dengue-confirmed patients, dengue virus strains were sucessfully

Table 1. Clinical information of the 110 patients suspected with dengue virus infection and from whom blood samples were collected

Parameters	Confirmed dengue cases[a] (%)	Non dengue cases[b] (%)
number of cases	96 (87)	14 (13)
Mean age in years (± SD)	5.5 (± 3.2)	5.6 (± 4.0)
Male/Female	49/47	6/8
DHF I	26 (27)	5 (36)
DHF II	32 (33)	5 (36)
DHF III	21 (22)	2 (14)
DHF IV	1 (1)	0 (0)
DSS	16 (17)	2 (14)

Confirmed dengue cases were positive for dengue IgM capture ELISA. Non-dengue cases were negative for dengue IgM capture ELISA.

DHF I, dengue hemorrhagic fever grade I; DHF II, dengue hemorrhagic fever grade II; DHF III, dengue hemorrhagic fever grade III; DHF IV, dengue hemorrhagic fever grade IV; DSS, dengue shock syndrome. DHF grading were classified according to WHO criteria (WHO, 1997).

Table 2. DENV-3 strains used for phylogenetic analysis

Strain	Code in tree	Geographic origin	Year of isolation	Accession number
BDH 02-01	Bdesh 0201	Bangladesh	2002	AY 496871
BDH 02-07	Bdesh 0207	Bangladesh	2002	AY 496877
114	Bdesh 00114	Bangladesh	2000	AY656669
165	Bdesh 00165	Bangladesh	2000	AY656671
058	Bdesh 00058	Bangladesh	2000	AY656674
Jacob	Bdesh 2001	Bangladesh	2001	AY656673
68784	Brazil 2000	Brazil	2000	AY038605
80-2	China 80	China	1980	AF317645
Cuba-21/02	Cuba 02	Cuba	2002	AY702031
29472	Fiji 92	Fiji	1992	L11422
1416	India 84	India	1984	L11424
228761	Indon 73	Indonesia	1973	L11425
1280	Indon 78	Indonesia	1978	L11426
85-159	Indon 85	Indonesia	1985	L11428
1300	Malay 74	Malaysia	1974	L11429
29586	Malay 81	Malaysia	1981	L11427
LN 5547	Malay 92	Malaysia	1992	AF147457
LN 1746	Malay 93	Malaysia	1993	AF147458
LN 6083	Malay 94	Malaysia	1994	AF147460
D3/H/IMTSSA-MART/2001/2012	Martiniq 01	Martinique	2001	AY099340
MEX6097	Mexico 95	Mexico	1995	AY146763
1559	Mozambiq 85	Mozambique	1985	L11430
31985 KLA	Myan 98	Myanmar	1998	AY145712
DV3/Myanmar/0508aTw/ 2005	Myan 05	Myanmar	2005	DQ518666
DV3/Mandalay.MYA/ H58/2006*	Myan 06	Myanmar	2006	EU478409
24/94	Nicaragu 94	Nicaragua	1994	AY702033
D3 PY/A59/03	Paraguay 03	Paraguay	2003	DQ 118885
H 87	Philip 56	Philippines	1956	L 11423
168-AP-2	Philip 83	Philippines	1983	L11432
PhMH-J1-97	Philip 97	Philippines	1997	AY 496879
PR6	PRico 63	Puerto Rico	1963	L11433
1340	PRico 77	Puerto Rico	1977	L11434
1696	Samoa 86	Samoa	1986	L11435
1326	SLanka 81	Sri Lanka	1981	L11431
1594	SLanka 85	Sri Lanka	1985	L11436
260698	SLanka 89	Sri Lanka	1989	L11437
2783	SLanka 91	Sri Lanka	1991	L11438
D3/Srilanka 9912aTw/1999	SLanka 99	Sri Lanka	1999	DQ 518679
2167	Tahiti 89	Tahiti	1989	L11619
D3/Taiwan/813KH9408a/1994	Taiwan 94	Taiwan	1994	DQ 518667
D3/Taiwan/701TN9811a/1998	Taiwan 98	Taiwan	1998	DQ 518662
D3/Taiwan/807KH0509a/2005	Taiwan 05	Taiwan	2005	DQ 518659
5987	Thai 62	Thailand	1962	L11440
CH3489D73-1	Thai 73	Thailand	1973	L11620
D86-007	Thai 86	Thailand	1986	L11441
MK315	Thai 87	Thailand	1987	L11442
D88-303	Thai 88	Thailand	1988	AY145714
D89-273	Thai 89	Thailand	1989	AY145715
D91-393	Thai 91	Thailand	1991	AY145716
D92-431	Thai 92	Thailand	1992	AY145719
D92-423	Thai 92	Thailand	1992	AY145718
D93-044	Thai 93	Thailand	1993	AY145720
D94-283	Thai 94	Thailand	1994	AY145723
D95-0400	Thai 95	Thailand	1995	AY145725
D 96-313	Thai 96	Thailand	1996	AY145726
D 97-0291	Thai 97	Thailand	1997	AY145730
00-27-1 Hu NIID	NIID 2000	Thailand/Bangladesh	2000	AB111080
LARD 5990	Venezu 2000	Venezuela	2000	AY146764
LARD 6668	Venezu 2001	Venezuela	2001	AY146774
D3/Vietnam/9609aTw/1996	Vietnam 96	Vietnam	1996	DQ518655
D3/Vietnam/0409aTw/2004	Vietnam 04	Vietnam	2004	DQ518656
D3/Vietnam/0507aTw/2005	Vietnam 05	Vietnam	2005	DQ518658

* New strain from Myanmar presented in this study

isolated from three patients whose sera were collected within 7 days from the onset of fever. One isolate was DEN-3 from a patient having a primary DENV infection with DHF grade (I), and two isolates were DEN-4 from a patient having primary DENV infection with DSS. The other patient had secondary DENV infection with DHF grade (I). The clinical information of the 110 patients is shown in Table 1.

The nucleotide sequence of the E gene of the newly isolated DENV-3 strain from Upper Myanmar, designated as the DV3/Mandalay.MYA/H58/2006 strain (Myan 06, code in tree), was compared with other published sequences of 61 DENV-3 strains originating from various geographic regions (Table 2). The phylogenetic tree constructed for the 62 DENV-3 strains, employing the DENV-2 New Guinea C strain as an out-group strain, is shown in Fig. 1. The tree reveals that the newly isolated DENV-3 strain from Upper Myanmar was grouped together with previously published strains from Lower Myanmar, as well as the strains from Thailand, Bangladesh, Malaysia, Vietnam and Taiwan in a well-defined genotype II.

Similarly, the nucleotide sequences of the E gene of two newly isolated DENV-4 strains from Upper Myanmar, designated as the DV4/Sagaing.MYA/H27/2006 strain (Myan 06 Sgg, code in tree) and the DV4/Mandalay.MYA/H64/2006 strain (Myan 06 Mdy, code in tree), were compared with other published sequences of 59 DENV-4 strains originating from various geographic regions (Table 3). The phylogenetic tree constructed for a total of 61 DENV-4 strains is shown in Fig. 2. The tree reveals that the two new strains from Upper Myanmar were grouped together with those from Thailand, Cambodia, Malaysia, India, Sri Lanka, China and the Philippines in the Asian genotype I.

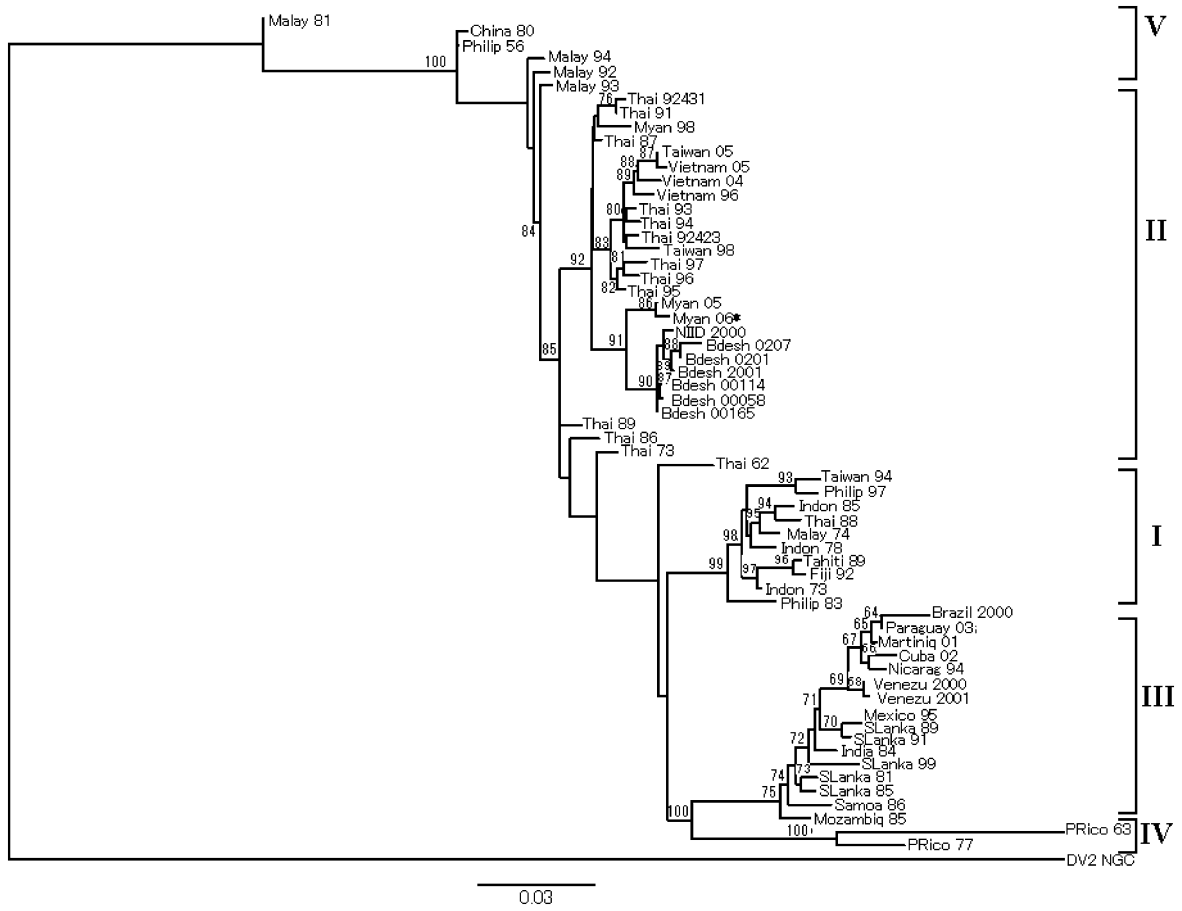

Fig. 1. Phylogenetic tree of DENV-3 strains (n = 62). The tree is rooted by the DENV-2-NGC (New Guinea C, Accession No. M29095) strain (Table 2). All horizontal branch lengths are drawn to a scale of nucleotide substitutions per site. Bootstrap support values are shown and the genotypes of DENV-3 are indicated. For simplicity, each strain name was replaced by a code that consists of the country and the year of isolation. The DENV-3 isolate presented in the present study is indicated by a asterisk (*).

Table 3. DENV-4 strains used for phylogenetic analysis

Strain	Code in tree	Geographic origin	Year of isolation	Accession number
Bahamas A/98	Bahamas 98A	Bahamas	1998	AY 152364
Barbados B/93	Barbados 93B	Barbados	1993	AY 152376
Barbados/99	Barbados 99	Barbados	1999	AY 152368
1385/82	Brazil 82	Brazil	1982	U 18425
02-21-1 Hu NIID	Cambodia 02	Cambodia	2002	AB 111089
China.GuangzhoB5	China B5	China	NA	AF 289029
814669/81	Dominica 81	Dominica	1981	AF 326573
M 44/81	Dominica M44	Dominica	1981	AY 152360
1411/83	El Salva 83	El Salvador	1983	U 18426
BC 6494/94	El Salva 94	El Salvador	1994	U 18427
Honduras/91	Honduras 91	Honduras	1991	AY 152379
96-33-1 Hu NIID	India 96	India	1996	AB 111086
30153/73	Indon 73	Indonesia	1973	U 18428
1036/76	Indon 76	Indonesia	1976	U 18429
1132/77	Indon 77	Indonesia	1977	U 18430
02-12-1 Hu NIID	Indon 02	Indonesia	2002	AB 111088
Jamaica/81	Jamaica 81	Jamaica	1981	AY 152389
Jamaica/83	Jamaica 83	Jamaica	1983	AY 152384
P7-1006	Malay 69	Malaysia	1969	AF 231722
P73-1120	Malay 73	Malaysia	1973	AF 231724
P75-514	Malay 75	Malaysia	1975	AF 231723
MY 01-23096	Malay 2001	Malaysia	2001	AJ 428557
1492/84	Mexico 84	Mexico	1984	U 18431
Mexico/91	Mexico 91	Mexico	1991	AY 152378
Montserrat-A/94	Montser 94	Montserrat	1994	AY 152369
DV4/Sagaing.MYA/H27/2006*	Myan 06 Sgg	Myanmar	2006	EU 478410
DV4/Mandalay.MYA/H64/2006*	Myan 06 Mdy	Myanmar	2006	EU 478408
5489/84	New Cale 84	New Caledonia	1984	U 18432
H241/56	Philip 56	Philippines	1956	U 18433
16589/64	Philip 64	Philippines	1964	U 18434
12123/84	Philip 84	Philippines	1984	U 18435
M5/82	PRico 82	Puerto Rico	1982	AY 152336
M32/85	PRico 85 M32	Puerto Rico	1985	AY 152856
M33/85	PRico 85 M33	Puerto Rico	1985	AY 152857
1650/86	PRico 86	Puerto Rico	1986	U 18436
69/87	PRico 87	Puerto Rico	1987	AY 152252
96/90	PRico 90	Puerto Rico	1990	AY 152855
28/92	PRico 92	Puerto Rico	1992	AY 152196
84/94	PRico 94	Puerto Rico	1994	AY 152084
17/98	PRico 98	Puerto Rico	1998	AY 152056
S-44750/78	SLanka 78	Sri Lanka	1978	U 18437
B/82	Surinam 82	Surinam	1982	AY 152386
A/94	Surinam 94	Surinam	1994	AY 152372
S-44754/79	Tahiti 79	Tahiti	1979	U 18438
114-094-85/85	Tahiti 85	Tahiti	1985	U 18439
TC 2443/63	Thai 63	Thailand	1963	U 18440
Thai D4-0087/77	Thai 770087	Thailand	1977	AY 618991
Thailand/78	Thai 78	Thailand	1978	U 18441
Thai D4-0348/91	Thai 910348	Thailand	1991	AY 618990
703-4/94	Thai 94	Thailand	1994	AF 231726
Thai D4-0017/97	Thai 970017	Thailand	1997	AY 618978
Thai D4-0476/97	Thai 970476	Thailand	1997	AY 618979
99-10-1 Hu NIID	Thai 99	Thailand	1999	AB 111087
Thai D4-0734/00	Thai 000734	Thailand	2000	AY 618993
Thai D4-0759/00	Thai 000759	Thailand	2000	AY 618938
Thai D4-0439/01	Thai 010439	Thailand	2001	AY 618940
Thai D4-0485/01	Thai 010485	Thailand	2001	AY 618992
Thai D4-0352/02	Thai 020352	Thailand	2002	AY 618945
Trinidad A/82	Trinidad 82	Trinidad	1982	AY 152382
Trinidad A/84	Trinidad 84	Trinidad	1984	AY 152380
Trinidad/94	Trinidad 94	Trinidad	1994	AY 152377

* New strains from Myanmar presented in this study

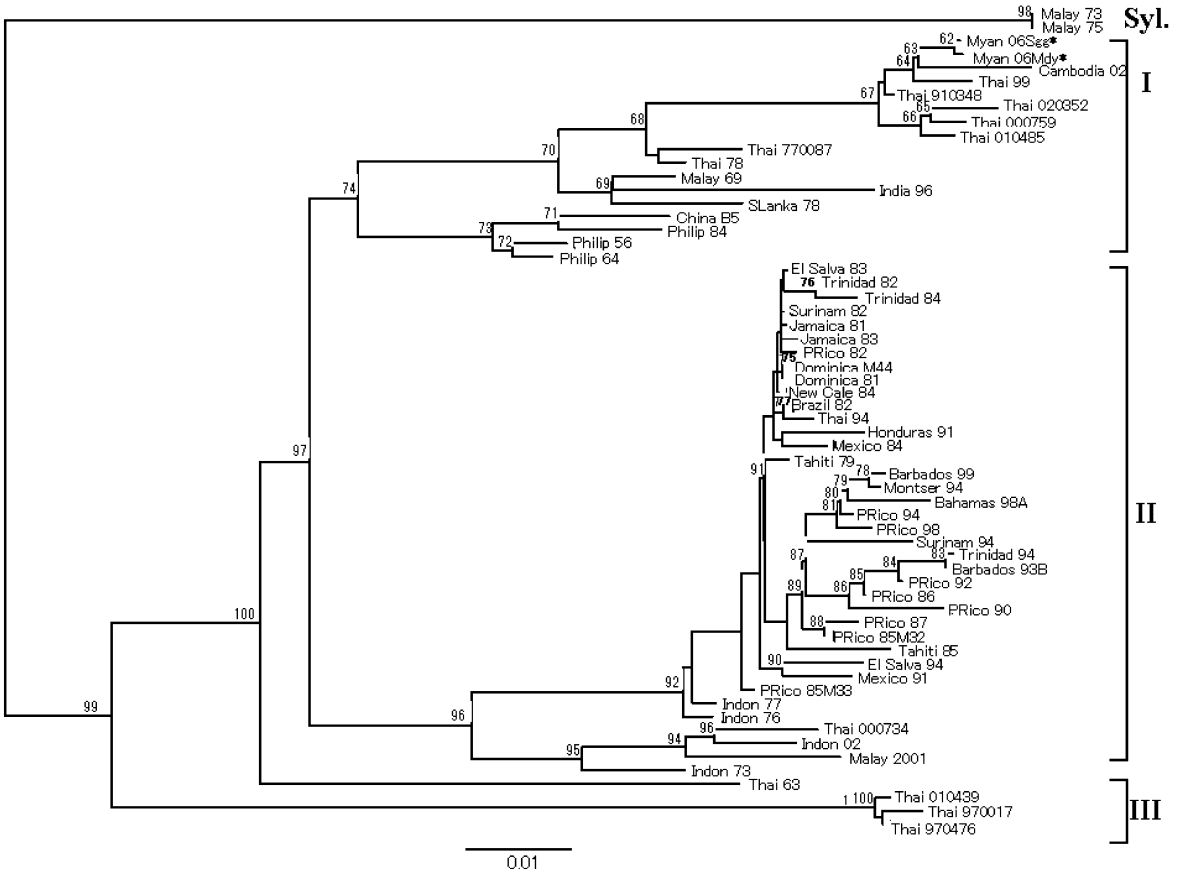

Fig. 2. Phylogenetic tree of DENV-4 strains (n = 61). The tree is rooted by the two sylvatic strains, Malay73 and Malay75 (Table 3). All horizontal branch lengths are drawn to a scale reflecting nucleotide substitutions per site. Bootstrap support values are shown and the genotypes of DENV-4 are indicated. For simplicity, each strain name was replaced by a code that consists of the country and the year of isolation. The DENV-4 isolates presented in the present study are indicated by a asterisk (*).

Discussion

The DENV-3 isolate from Upper Myanmar in the present study belonged to genotype II, like two previously published Lower Myanmar strains: the 31985 KLA strain (Myan 98, code in tree) and the DV3/Myanmar/0508aTw/2005 strain (Myan 05, code in tree). It clustered together with strains from Bangladesh in a well-defined sub-cluster. Further support came from the fact that three unique aminoacid (aa) changes, I140T, S447G and A489T, were found in this strain and were shared by Myan 05 and the Bangladesh strains. To examine the introduction of the DENV-3 genotype II to the country (although DENV-3 isolates from Myanmar are very few), we compared an older strain, Myan 98, to the two most recent ones: Myan 05 and Myan 06 [16]. In the phylogenetic tree, Myan 98 was clustered in a separate sub-cluster of genotype II together with earlier Thai strains. This clustering is supported by four aa changes, I140T, S447G, A489T and A479V, which are present in the two most recent Myanmar isolates, Myan 05 and Myan 06, but are not present in either the Myan 98 strain or in the Thai isolates that were clustered together with the latter strain. These results indicate that the genotype II of DENV-3 reached Myanmar most likely through independent entries from Thailand, a supposition supported by the appearance of the more recent lineage including the isolates from 2005 and 2006 (Fig. 1). The fact that the Bangladesh strains isolated from 2000 to 2002 showed little evidence of independent evolution suggests that genotype II was also introduced recently from neighboring countries. Our results support Podder et al.'s (2006) suggestion that recent DEN outbreaks in Bangladesh (2000 and 2001) were associated with the introduction of DENV-3 from eastern countries, rather than the evolution of a virulent strain *in situ* [17]. In addition, Islam et al. (2006) speculated that DENV-3 circulating in Bangladesh in 2002 might have entered from neighboring countries [12]. Recently, it was reported that seven DENV-3 strains isolated from Yangon (Lower Myanmar) in 2007 belonged to genotype III [18]. Therefore, it ap-

pears that more than one DENV-3 genotype is circulating in the country. It would be interesting to analyze the time and route of introduction.

The two newly isolated DENV-4 strains from Upper Myanmar in the present study were clustered together with other Asian strains in genotype I being the closest related strains from Thailand and Cambodia [9], but V238M and L489P aa changes were unique to Myan 06 Mdy and Myan 06 Sgg strains, respectively. Although the existence of DENV-1 and DENV-2 among the circulating viruses in Upper Myanmar could not be ruled out, the present study demonstrated that DENV-3 and DENV-4 were co-circulating in the area in 2006. This is the first report on the molecular analysis of DENV-4 strains in Myanmar, particularly those circulating in the upper part of the country. Therefore, if DENV-3 is currently regarded as the prevailing serotype for recent outbreaks, then DENV-4 might be in the pipeline to take the lead in future outbreaks in Myanmar.

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Potential Conflict of Interest

We declare no conflicts of interest.

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