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Genomic analysis and growth characteristic of dengue viruses from Makassar, Indonesia

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ABSTRACT

Dengue fever is currently the most important mosquito-borne viral disease in Indonesia. In South Sulawesi province, most regions report dengue cases including the capital city, Makassar. Currently, no information is available on the serotypes and genotypes of the viruses circulating in the area. To understand the dynamic of dengue disease in Makassar, we carried out dengue fever surveillance study during 2007–2010. A total of 455 patients were recruited, in which antigen and serological detection revealed the confirmed dengue cases in 43.3% of patients. Molecular detection confirmed the dengue cases in 27.7% of patients, demonstrating that dengue places a significant disease burden on the community. Serotyping revealed that dengue virus serotype 1 (DENV-1) was the most predominant serotype, followed by DENV-2, -3, and -4. To determine the molecular evolution of the viruses, we conducted whole-genome sequencing of 80 isolates. Phylogenetic analysis grouped DENV-2, -3 and -4 to the Cosmopolitan genotype, Genotype I and Genotype II, respectively. Intriguingly, each serotype paints a different picture of evolution and transmission. DENV-1 appears to be undergoing a clade replacement with Genotype IV being supplanted by Genotype I. The Cosmopolitan DENV-2 isolates were found to be regionally endemic and is frequently being exchanged between countries in the region. By contrast, DENV-3 and DENV-4 isolates were related to strains with a long history in Indonesia although the DENV-3 strains appear to have been following a distinct evolutionary path since approximately 1998. To assess whether the various DENV serotypes/genotypes possess different growth characteristics, we performed growth kinetic assays on selected viruses. We observed the relatively higher rate of replication for DENV-1 and -2 compared to DENV-3 and -4. Within the DENV-1, viruses from Genotype I grow faster than that of Genotype IV. This higher replication rate may underlie their ability to replace the circulation of Genotype IV in the community.

1. Introduction

Dengue fever is an acute febrile disease caused by dengue virus (DENV) infection, transmitted through the mosquito vector *Aedes aegypti*. The symptoms of the DENV infection vary from classic Dengue Fever (DF) to severe forms such as Dengue Hemorrhagic Fever (DHF) and the potentially fatal Dengue Shock Syndrome (DSS). DENV is an enveloped flavivirus with a 10.7 kb single-stranded positive-sense RNA genome. There are four antigenically distinct serotypes of the virus (DENV-1, -2, -3, and -4), with around 65% genome similarity, circulating throughout the tropical and sub-tropical regions of the world (Gubler, 1998; Guzman et al.,

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2010). The disease caused by the four serotypes is symptomatically identical.

Indonesia is a dengue endemic country that experiences periodic major outbreaks of the disease, such as those in 1998 (Corwin et al., 2001) and 2004 (Suwandono et al., 2006). In addition, annual and sporadic occurrences regularly afflict all the 33 provinces of the vast Indonesian archipelago (Sumarmo, 1987). While there have been studies describing the molecular genetics of dengue viruses isolated from Western part of the Indonesian archipelago (Sumatra and Java Islands) (Ong et al., 2008; Raekiansyah et al., 2005), no data is available for other major islands in the Eastern part of Indonesia, such as Sulawesi island. Data from the South Sulawesi provincial health authority (www.dinkes-sulsel.go.id) described the increasing number of dengue incidents in South Sulawesi province in 2007, in which 5333 patients were admitted to hospitals. In the capital city, Makassar, the dengue incidence was recorded at 425 cases with 5 fatalities. Dengue diagnosis in Makassar is commonly performed based on clinical symptoms only as the lack of resources and relatively high cost of laboratory tests prohibit serological confirmation. Because of this, the reported incidence may not represent the true disease burden.

Comparative analysis of dengue genomes has been used widely to study the genetic diversity of the dengue viruses (Holmes and Burch, 2000; Holmes and Twiddy, 2003) and previous studies have also suggested a correlation between viral pathogenicity with its genetic structures in which mutations in viral genome enhances the viral pathogenesis (Leitmeyer et al., 1999; Pandey and Igarashi, 2000). To understand the dengue disease dynamic in Makassar city, Indonesia, we studied the molecular evolution of circulating dengue viruses by analyzing the genomes of the viruses isolated in the area. We observed the diversity of serotypes and genotypes of dengue viruses and hypothesized that spatial isolation may contribute to the diversity of dengue viruses circulating in Makassar, South Sulawesi, Indonesia. To complement the viral genetic data, we performed viral growth characterization of various serotypes/genotypes. This study provides information in the genetic and biological characteristics of the diverse dengue virus strains circulating in Makassar, Indonesia.

2. Materials and methods

2.1. Study site and patient recruitment

We conducted surveillance of febrile patients in Makassar, the capital city of South Sulawesi province, Indonesia. The city resides in an area of about 175.77 km^2, inhabited by about 1.3 million people (http://makassarkota.go.id). Makassar city is located about 1400 km east of the Indonesian capital of Jakarta. Dengue-suspected febrile patients admitted to Dr. Wahidin Sudirohusodo, Labuang Baji and Daya Hospitals and primary health care centers in Makassar were enrolled in the study upon obtaining written consent. For the minors/children participants, written consents were obtained from parents/legal guardians. We obtained ethical clearances for this study from the Hasanuddin University Medical Research Ethics Committee and the Eijkman Institute Research Ethics Committee. All patients aged between 5 and 100 years old with fever >38.0 °C for less than 72 h and without concurrent signs of upper respiratory infection or obvious alternate diagnoses to dengue were invited to participate in the study. In total, we obtained 455 serum samples during the patient recruitment period (May 2007–August 2010).

Initial screening on dengue-suspected patients was determined serologically using anti-dengue IgM and IgG (Panbio Dengue Duo IgM/IgG enzyme-linked immunosorbent assay (ELISA) kit (Panbio, Brisbane, Australia)). Dengue confirmation by detection of NS1 antigen was done using NS1 rapid test kit (Standard Diagnostics, Korea). Primary versus secondary dengue infection was determined using the ELISA results according to manufacturer's protocol. Briefly, the positive IgM (>11 Panbio Units) and negative IgG (<22 Panbio Units) indicated primary infection while positive IgG (>22 Panbio Units), which may be accompanied by elevated IgM levels, indicated secondary infection. The presence of viral RNA in NS1-positive samples was further confirmed by reverse transcription-polymerase chain reaction (RT-PCR) using method described by Lanciotti et al. (1992), with modification according to Harris et al. (1998). All dengue positive cases were categorized either dengue fever (DF) or Dengue Hemorrhagic Fever (DHF) according to criteria described by the WHO (WHO, 1997).

2.2. Virus propagation, cell lines, RNA extraction and serotyping

Viruses were isolated from RT-PCR positive samples, either directly from patients' sera or after one passage (or maximum of two passages for low titer isolates) of virus propagation in C6/36 *Aedes albopictus* gut cell lines. Baby hamster kidney (BHK-21) and C6/36 cell lines were maintained in RPMI 1640 medium (Gibco, Carlsbad, CA). African green monkey kidney (Vero76) cell line was maintained in Minimum Essential Medium (MEM) with Earle's salts (Gibco). Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) for cell maintenance. BHK-21 and Vero76 cell culture was maintained in 37 °C incubator (ESCO) supplemented with 5% CO_2. C6/36 cell culture was maintained in 28 °C incubator. Virus stock titer was measured using modified standard plaque assay method (Fink et al., 2007) and recorded as plaque forming unit (PFU) equivalent/ml.

QIAamp Viral RNA Mini Kits (Qiagen, Hilden, Germany) were used to extract viral genomic RNAs from patients' sera or cell culture supernatant according to the manufacturer's instructions. Dengue viral RNA was reverse-transcribed into cDNA using Superscript III reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) and DENV-specific primers (Lanciotti et al., 1992). Subsequently, cDNA was amplified using *Taq* DNA polymerase (Roche, Mannheim, Germany). The four dengue serotypes were distinguished by PCR product size.

2.3. Whole genome sequencing

Five overlapping PCR fragments covering the whole dengue genome were amplified using methods described previously (Ong et al., 2008; Schreiber et al., 2009) with slight modifications. Briefly, genomic RNAs were reverse-transcribed into cDNA using primers specific for each serotype. The primers used for DENV-4 sequencing were as listed in Supplementary Table 1. Fragment amplification was performed using high-fidelity *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). Typical PCR conditions were as follows: templates were denatured for 2 min at 95 °C, followed by 45 cycles of 30 s denaturation at 95 °C, 1 min annealing at 55 °C, and 4.5 min extension at 72 °C. Templates were allowed a 10 min final extension step at 72 °C followed by storage at 4 °C. PCR products were purified from 0.8% agarose gel using QIAquick gel extraction kit (Qiagen) and used as template for cycle sequencing. DNA sequencing was performed using BigDye Dideoxy Terminator sequencing kits v3.1 (Applied Biosystems, Foster City, CA) using the method described in the kit. In addition to the sequencing and amplification primers previously described by Ong et al. (2008), a new set of primers for DENV-4 were used. Primer sequences and predicted genome binding positions of RT-PCR primers for DENV-4 are listed in Supplementary Tables 2 and 3, respectively. Purified sequencing reactions were then run on

ABI3130×l (Applied Biosystems) sequencer at the Eijkman Institute sequencing facility. Contig assembly was performed using SeqScape 2.5 (Applied Biosystem) with additional manual adjustment performed when manual inspection of the assembly showed some discrepancies. All genome sequences obtained in this study have been deposited in GenBank (Table 1).

2.4. Sequence, phylogenetic and evolutionary analysis

We downloaded all DNA sequences for the DENV-1, -2, -3 and -4 from NCBI GenBank database as of 13 April 2012. All sequences from respected dengue serotype which had 90% identities with any of the samples for 90% coverage of their sequences were searched

Table 1
Characteristics of the Makassar DENV samples with whole-genomes sequenced.

No	Sample ID	Accession no.	Sex	Isolation time	Serotype	Genotype	Infection status
1.	D1/ID/MKS-0055/2007	KC762649	F	Jul 2007	DENV-1	I	Primary
2.	D1/ID/MKS-0056/2007	KC762625	F	Jun 2007	DENV-1	I	Primary
3.	D1/ID/MKS-0077/2007	KC762654	F	Jul 2007	DENV-1	I	Primary
4.	D1/ID/MKS-0080/2007	KC762636	F	Jul 2007	DENV-1	I	Primary
5.	D1/ID/MKS-0088/2007	KC762635	M	Oct 2007	DENV-1	I	Primary
6.	D1/ID/MKS-0109/2007	KC762650	F	Jun 2007	DENV-1	I	Primary
7.	D1/ID/MKS-0206/2007	KC762623	F	Jul 2007	DENV-1	I	Primary
8.	D1/ID/MKS-0248/2007	KC762632	M	Aug 2007	DENV-1	I	Primary
9.	D1/ID/MKS-0344/2007	KC762646	M	Aug 2007	DENV-1	I	Primary
10.	D1/ID/MKS-0352/2007	KC762637	F	Dec 2007	DENV-1	I	Primary
11.	D1/ID/MKS-0390/2007	KC762620	F	Dec 2007	DENV-1	IV	Primary
12.	D1/ID/MKS-0395/2008	KC762631	M	Feb 2008	DENV-1	I	Primary
13.	D1/ID/MKS-0397/2008	KC762621	F	Feb 2008	DENV-1	I	Primary
14.	D1/ID/MKS-0398/2008	KC762643	M	Feb 2008	DENV-1	I	Primary
15.	D1/ID/MKS-0483/2007	KC762651	M	Dec 2007	DENV-1	IV	Primary
16.	D1/ID/MKS-0486/2008	KC762653	M	Feb 2008	DENV-1	IV	Primary
17.	D1/ID/MKS-2029/2008	KC762640	M	Mar 2008	DENV-1	IV	Primary
18.	D1/ID/MKS-2040/2008	KC762645	F	Feb 2008	DENV-1	I	Primary
19.	D1/ID/MKS-2058/2008	KC762628	F	Feb 2008	DENV-1	IV	Primary
20.	D1/ID/MKS-2082/2008	KC762624	M	Feb 2008	DENV-1	I	Primary
21.	D1/ID/MKS-2094/2008	KC762644	M	Feb 2008	DENV-1	I	Primary
22.	D1/ID/MKS-2097/2008	KC762630	F	Feb 2008	DENV-1	IV	Primary
23.	D1/ID/MKS-2138/2008	KC762626	F	Feb 2008	DENV-1	I	Primary
24.	D1/ID/MKS-2147/2008	KC762641	F	Feb 2008	DENV-1	I	Primary
25.	D1/ID/MKS-2194/2008	KC762648	M	Apr 2008	DENV-1	I	Primary
26.	D1/ID/MKS-2200/2008	KC762622	M	Apr 2008	DENV-1	I	Primary
27.	D1/ID/MKS-2201/2008	KC762634	F	Apr 2008	DENV-1	I	Primary
28.	D1/ID/MKS-2216/2008	KC762633	M	Apr 2008	DENV-1	I	Primary
29.	D1/ID/MKS-1001/2008	KC762652	F	Apr 2008	DENV-1	I	Primary
30.	D1/ID/MKS-2004/2008	KC762638	F	Feb 2008	DENV-1	I	Primary
31.	D1/ID/MKS-IF039/2008	KC762629	F	Mar 2008	DENV-1	I	Primary
32.	D1/ID/MKS-IF062/2008	KC762627	F	Apr 2008	DENV-1	I	Secondary
33.	D1/ID/MKS-WS72/2010	KC762647	M	Mar 2010	DENV-1	I	Primary
34.	D1/ID/MKS-WS81/2010	KC762639	M	Apr 2010	DENV-1	I	Primary
35.	D1/ID/MKS-WS88/2010	KC762642	M	Jul 2010	DENV-1	I	Primary
36.	D2/ID/MKS-0068/2007	KC762665	M	Jul 2007	DENV-2	Cosmopolitan	Primary
37.	D2/ID/MKS-0071/2007	KC762669	M	Jul 2007	DENV-2	Cosmopolitan	Primary
38.	D2/ID/MKS-0082/2007	KC762658	M	Aug 2007	DENV-2	Cosmopolitan	Secondary
39.	D2/ID/MKS-0084/2007	KC762660	M	Nov 2007	DENV-2	Cosmopolitan	Primary
40.	D2/ID/MKS-0091/2007	KC762670	M	Dec 2007	DENV-2	Cosmopolitan	Primary
41.	D2/ID/MKS-0099/2007	KC762676	F	Jul 2007	DENV-2	Cosmopolitan	Primary
42.	D2/ID/MKS-0297/2007	KC762662	M	Nov 2007	DENV-2	Cosmopolitan	Primary
43.	D2/ID/MKS-0412/2007	KC762661	F	Sep 2007	DENV-2	Cosmopolitan	Secondary
44.	D2/ID/MKS-0417/2007	KC762655	F	Sep 2007	DENV-2	Cosmopolitan	Primary
45.	D2/ID/MKS-0502/2007	KC762656	M	Dec 2007	DENV-2	Cosmopolitan	Primary
46.	D2/ID/MKS-2032/2008	KC762675	M	Mar 2008	DENV-2	Cosmopolitan	Primary
47.	D2/ID/MKS-2108/2008	KC762671	F	Feb 2008	DENV-2	Cosmopolitan	Primary
48.	D2/ID/MKS-2145/2008	KC762664	F	Mar 2008	DENV-2	Cosmopolitan	Primary
49.	D2/ID/MKS-2167/2008	KC762674	F	Mar 2008	DENV-2	Cosmopolitan	Primary
50.	D2/ID/MKS-2198/2008	KC762672	F	Apr 2008	DENV-2	Cosmopolitan	Primary
51.	D2/ID/MKS-2204/2008	KC762667	M	Apr 2008	DENV-2	Cosmopolitan	Primary
52.	D2/ID/MKS-2210/2008	KC762659	F	Apr 2008	DENV-2	Cosmopolitan	Secondary
53.	D2/ID/MKS-2234/2008	KC762657	M	Apr 2008	DENV-2	Cosmopolitan	Primary
54.	D2/ID/MKS-3007/2008	KC762666	F	Feb 2008	DENV-2	Cosmopolitan	Primary
55.	D2/ID/MKS-2024/2008	KC762663	M	Feb 2008	DENV-2	Cosmopolitan	Primary
56.	D2/ID/MKS-2018/2008	KC762673	M	Mar 2008	DENV-2	Cosmopolitan	Primary
57.	D2/ID/MKS-IF011/2008	KC762677	M	Feb 2008	DENV-2	Cosmopolitan	Primary
58.	D2/ID/MKS-IF014/2008	KC762668	F	Mar 2008	DENV-2	Cosmopolitan	Primary
59.	D2/ID/MKS-WS73/2010	KC762678	M	Mar 2010	DENV-2	Cosmopolitan	Primary
60.	D2/ID/MKS-WS79a/2010	KC762679	F	Mar 2010	DENV-2	Cosmopolitan	Primary
61.	D2/ID/MKS-WS80/2010	KC762680	M	Apr 2010	DENV-2	Cosmopolitan	Primary
62.	D3/ID/MKS-0057/2007	KC762681	F	Jun 2007	DENV-3	I	Primary
63.	D3/ID/MKS-0076/2007	KC762682	F	Jul 2007	DENV-3	I	Primary

(*continued on next page*)

Table 1 (*continued*)

No	Sample ID	Accession no.	Sex	Isolation time	Serotype	Genotype	Infection status
64.	D3/ID/MKS-0079/2007	KC762683	F	Jul 2007	DENV-3	I	Primary
65.	D3/ID/MKS-0098/2007	KC762684	M	Jul 2007	DENV-3	I	Secondary
66.	D3/ID/MKS-0172/2007	KC762686	M	Jul 2007	DENV-3	I	Secondary
67.	D3/ID/MKS-0388/2008	KC762685	F	Feb 2008	DENV-3	I	Primary
68.	D3/ID/MKS-0396/2008	KC762687	M	Feb 2008	DENV-3	I	Primary
69.	D3/ID/MKS-2065/2008	KC762689	F	Feb 2008	DENV-3	I	Primary
70.	D3/ID/MKS-3000/2008	KC762690	M	Aug 2008	DENV-3	I	Primary
71.	D3/ID/MKS-2006/2008	KC762688	M	Apr 2008	DENV-3	I	Primary
72.	D3/ID/MKS-IF058/2008	KC762691	M	Apr 2008	DENV-3	I	Primary
73.	D3/ID/MKS-WS78/2010	KC762692	F	Mar 2010	DENV-3	I	Secondary
74.	D3/ID/MKS-WS79b/2010	KC762693	F	Mar 2010	DENV-3	I	Primary
75.	D4/ID/MKS-0033/2007	KC762694	M	May 2007	DENV-4	II	Primary
76.	D4/ID/MKS-0070/2007	KC762695	M	Jul 2007	DENV-4	II	Primary
77.	D4/ID/MKS-0252/2008	KC762696	M	Aug 2007	DENV-4	II	Primary
78.	D4/ID/MKS-0706/2008	KC762697	-	Feb 2008	DENV-4	II	Primary
79.	D4/ID/MKS-2139/2008	KC762699	M	Mar 2008	DENV-4	II	Primary
80.	D4/ID/MKS-2007/2008	KC762698	M	Apr 2008	DENV-4	II	Primary

using USEARCH (Edgar, 2010). The obtained sequences together with the samples were then aligned using MUSCLE (Edgar, 2004). The alignments were trimmed for their 5′-UTR and 3′-UTR leaving only the coding regions. A maximum likelihood phylogenetic tree using GTR substitution model and gamma distribution for varying rate for each serotype was constructed by FastTree (Price et al., 2010). The phylogenetic trees were used to collect 60 most-closely related published sequences from each of the samples as reference sequences by calculating the patristic distance as implemented in DendroPy (Sukumaran and Holder, 2010).

MUSCLE (Edgar, 2004) was utilized to create the multiple sequence alignment of each dengue virus serotype for the untrimmed sample and the collected reference sequences. We partitioned the alignments into 5-UTR, coding and 3-UTR regions accordingly. We determined the best model of evolution for each region by sampling across substitution model space in Bayesian MCMC analysis as implemented in MrBayes (Huelsenbeck and Ronquist, 2001). For all serotypes, we selected the GTR with invariable sites and 4 classes of gamma-distribution (GTR + I + Γ_4) with codon model and TrN model with invariable sites and 4 classes of gamma-distribution (TrN + I + Γ_4) for the coding region and 3′-UTR, respectively, as these were the models implemented in BEAST that were close enough to the models suggested by MrBayes. Bayesian Monte Carlo Markov Chain (MCMC) simulations with the selected evolution models as partitioned models and relaxed molecular clock were generated by BEAST (Drummond and Rambaut, 2007) and used to estimate the evolutionary parameters of each serotype. A Bayesian skyline plot was utilized as a coalescent prior as described in previous publications (Ong et al., 2008; Schreiber et al., 2009). For each type of dengue virus, we conducted a run of 100,000,000 replications with sampling of every 1000th, resulting in 100,000 chains. MCC (Maximum Clade Credibility) phylogenetics trees and evolutionary parameters were summarized with 25% burn-in. Tree visualization and sample labeling was performed using FigTree software (http://tree.bio.ed.ac.uk/software/figtree).

2.5. Viral growth kinetic assay

Vero76 cells were seeded at a density of 2×10^5 cells and allowed to attach overnight in 24 well plates (Nunc). Cells were then infected with viruses in duplicate using a multiplicity of infection (moi) of 0.1 in 1× MEM supplemented with 2% FBS and incubated at 37 °C, 5% CO_2 for 1 h. Three to five different virus isolates for each serotype were assayed. For comparison between Genotypes of DENV-1, four different isolates were used for each genotype. Inoculation media were aspirated and replenished with fresh medium following incubation. Culture supernatants were collected at 12 h intervals (i.e., 12, 24, 36, 48, 60, and 72 h post inoculation) and stored directly in −80 °C freezer. Viral titers from each time point were quantified using a standard plaque assay on BHK-21 cell lines as described previously (Fink et al., 2007) and plotted as kinetic curves showing the mean titer and standard deviation. Statistical analysis was done using SPSS Statistics software version 17.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was used to analyze and compare means of viral titer among DENV serotypes. Comparison of viral growth kinetic between two genotypes of DENV-1 was done using independent-samples Student's *t*-test. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Dengue incidence in Makassar city

Four hundred and fifty-five febrile patients were recruited during May 2007–August 2010. Of these, 197 (43.3%) patients were confirmed dengue as determined by NS1 rapid test kit. RT-PCR detections were performed on NS1-positive samples and a total of 126 sera (27.7% of all dengue-suspected patients) were detected as dengue positive. The male to female ratio was 1.14, with an average age of 12.5 ± 10.2 years old. Cases occurred predominantly in those aged between 11 and 20 years old (40%). Most dengue positive cases ($n = 165$ or 83.8%) were secondary infections, as determined by IgM and IgG ELISA ratio.

3.2. Dengue virus serotype distribution

We performed serotyping on RT-PCR-confirmed dengue cases using a standard RT-PCR serotyping method (Lanciotti et al., 1992) and obtained results in 126 cases, that demonstrated the circulation of all four dengue serotypes in Makassar. The predominant serotype was DENV-1 with 51 isolates (41%), followed by DENV-2 with 39 isolates (31%). Twenty-five isolates (20%) were DENV-3; and the remaining nine isolates (7%) were DENV-4. Two samples were mixed-infection of DENV-1 and -2 and DENV-2 and -3. We compared previous publications on serotype distributions in other parts on Indonesia (Corwin et al., 2001; Graham et al., 1999; Porter et al., 2005; Sukri et al., 2003; Suwandono et al., 2006; Yamanaka et al., 2011) with our results. We observed a distinct serotype distribution of DENV in Makassar compared to other cities throughout the Indonesian archipelago although we cannot be certain that

these historical reports reflect current serotype distributions in those cities.

3.3. Phylogenetic analysis of dengue viruses

To determine the predominant genotypes in Makassar, we performed full-length genome sequencing of the isolated viruses. Virus genomic RNAs were extracted either from patient sera or after one passage of virus propagation in tissue culture. We obtained the complete genome sequences of 80 viruses covering all four serotypes. Table 1 describes the characteristics of the sequenced isolates including the length of the sequenced genomes.

Using a fast maximum-likelihood method as implemented in FastTree, we performed an approximate phylogenetic analysis of all dengue serotypes to select 60 most-closely related sequences of each dengue sample for reference sequences for consecutive analysis.

The MCC (Maximum Clade Credibility) phylogenetic tree of DENV-1 isolates and their closest reference sequences was shown in Fig. 1. Based on the DENV-1 genotype classification by Goncalvez et al. (2002), we observed the circulation of two genotypes of DENV-1 in Makassar. The majority of the viruses, including all recent 2010 samples (29 isolates) belong to Genotype I. The 28 isolates of Genotype I were from single ancestor and had the time to the most recent common ancestor (TMRCA) around 2005. One of the recent samples (WS88) probably followed an independent route of introduction as it had similar lineage with samples from Singapore. All of Genotype I samples were clustered together with viruses from China, Thailand, and Singapore, including the Singapore dengue outbreak isolates of 2005. The rest of the Makassar's DENV-1 viruses (6 isolates) were grouped into Genotype IV. Three of the isolates were clustered together with samples from Brunei (in 2006), Hawaii (in 2001), Philippines (in 2004) and China (in 1991), and their ancestor was likely to be the same as the West Pacific sample of 1974 isolate. The other three isolates were clustered with China isolates of 2002/2003 and Indonesian city of Palembang in 1998. It should be noted that DENV-1 isolates from other Indonesian region were not included in the analysis as they are more likely to distantly related to Makassar samples and were not selected during the FastTree/patristic distance filtering process. This observation contributes to new information on the DENV-1 genotype distribution in Indonesia.

We determined 26 DENV-2 genome sequences from Makassar isolates and constructed an MCC phylogenetic analysis of those viruses together with previously published DENV-2 genomes. Based on the DENV-2 genotype classification reported by Twiddy et al. (2002), all of our Makassar isolates were grouped into the Cosmopolitan genotype (Fig. 2). This genotype is widely disseminated from India to South East Asia, Africa, the Middle East, and Australia. Within the Cosmopolitan genotype, the Makassar isolates grouped together with two isolates from Jakarta in 2004 and many isolates from Singapore (in 2004, 2005 and 2008). The phylogenetic trees suggested the presence of spatial isolation among DENV-2 strains from cities in the western and eastern parts of Indonesia, but the absence of spatial isolation between Makassar and Singapore. The tree also indicated that DENV-2 viruses currently circulating in Makassar were of multiple entries.

Phylogenetic analysis of 13 DENV-3 genome sequences from Makassar identified them as belonging to Genotype I, based on the classification described by Lanciotti et al. (1994). This genotype consists of viruses from Indonesia, Malaysia, the Philippines and the South Pacific islands (Lanciotti et al., 1994). Indonesian viruses collected in 1973, 1978 and 1985 also grouped into this genotype (Lanciotti et al., 1994), as did recent isolates from Jakarta in 2004 and Palembang in 1998 (Ong et al., 2008), as shown in Fig. 3. Nine Makassar isolates were clustered with Indonesian isolates of 1998 and 1988 from Jakarta and were from single common ancestor with TMRCA of 1990. Four other Makassar isolates, including the recent 2010 samples, were clustered with the rest of Indonesian isolates from 1998 and 2004, as well as from Timor Leste in 2005 and 2005/2009 from Singapore. This may indicate that all the Makassar samples were originated from and spread in Indonesia and neighboring countries such as Singapore and Timor Leste. TMRCA of all Makassar isolates together with other Indonesian isolates was 1965. Together this suggests the presence of strong temporal structure and that the Makassar's DENV-3 viruses appeared to be part of an older lineage which was indeed endemic in the Indonesian region and had been circulating for more than four decades.

We performed phylogenetic analysis on the genomes of 6 DENV-4 viruses isolated from Makassar and compared them with available DENV-4 genomes in the GenBank. As shown in Fig. 4, the Makassar isolates fell into Genotype II based on Lanciotti classification (Lanciotti et al., 1997). This genotype is commonly found in South-east Asia and America (Lanciotti et al., 1997). The Makassar isolates grouped closely with other Indonesian isolates from Jakarta that were isolated in 2004. Four of the Makassar samples in the same lineage with the Jakarta samples were originated from a lineage which has higher mutation rate relative to other samples. The TMRCA of these 4 Makassar samples and the related Jakarta samples was around year 1990. This clustering suggests the endemicity of this DENV-4 genotype in Indonesia, and the presence of strong temporal link among DENV-4 viruses in Indonesia.

3.4. Evolutionary dynamics and amino acid comparison of Makassar dengue viruses

The estimated evolutionary rates for substitution dynamics of all serotypes, as calculated by BEAST, were 1.1×10^{-3} substitution/site/year (95% highest probability density (HPD), 0.6×10^{-3}–1.8×10^{-3} substitution/site/year) and 1.5×10^{-3} substitution/site/year (95% HPD, 0.7×10^{-3}–1.7×10^{-3} substitution/site/year) for DENV-1 and DENV-2, respectively. Similar estimates, 1.1×10^{-3} substitution/site/year (95% HPD, 6.2×10^{-4}–1.5×10^{-3} substitution/site/year) and 0.2×10^{-3} substitution/site/year (95% HPD, 1.5×10^{-4}–0.8×10^{-3} substitution/site/year), were observed for DENV-3 and DENV-4, respectively.

The estimated ages of the most recent common ancestors (TMRCA) for all serotypes were 62 months (95% HPD, 53–91 months), 58 months (95% HPD, 51–86 months), 56 months (95% HPD, 49–82 months) and 55 months (95% HPD, 45–78 months) for DENV-1, DENV-2, DENV-3 and DENV-4, respectively.

A relatively low ratio of non-synonymous to synonymous substitution per site (dN/dS) in all genes indicated that there was no evidence for positive selection in any gene and no evidence for site-specific positive selection of the Makassar viruses (Table 2). Several genes had ratio of 1.0 for dN/dS in DENV-4, however due to small number of DENV-4 isolates, we feel that the dN/dS for DENV-4 cannot be determined confidently.

Comparative analysis at the amino acid levels were illustrated in Fig. 5 for DENV-1 Genotype I (A), DENV-1 Genotype IV (B), DENV-2 (C), DENV-3 (D) and DENV-4 (E). Although DENV-1 Genotype I had the most sample number, the differences were the smallest among the sample set, and substantially much smaller than the DENV-1 genotype IV. In DENV-1 Genotype I residue 123 of E gene was observed to be either Serine (S) or Threonin (T) in many 2007 samples, but only observed to be T in all 2008 samples. Likewise, residue 222 of NS1 gene in some 2007 samples were observed to be S or T, while the rest of 2008 samples were observed to be T. However, residue 829 of NS5 gene which were observed to be Isoleucine (I) for all 2007 samples, started to have Valine (V) in some of 2008 samples. The rest of changes in the

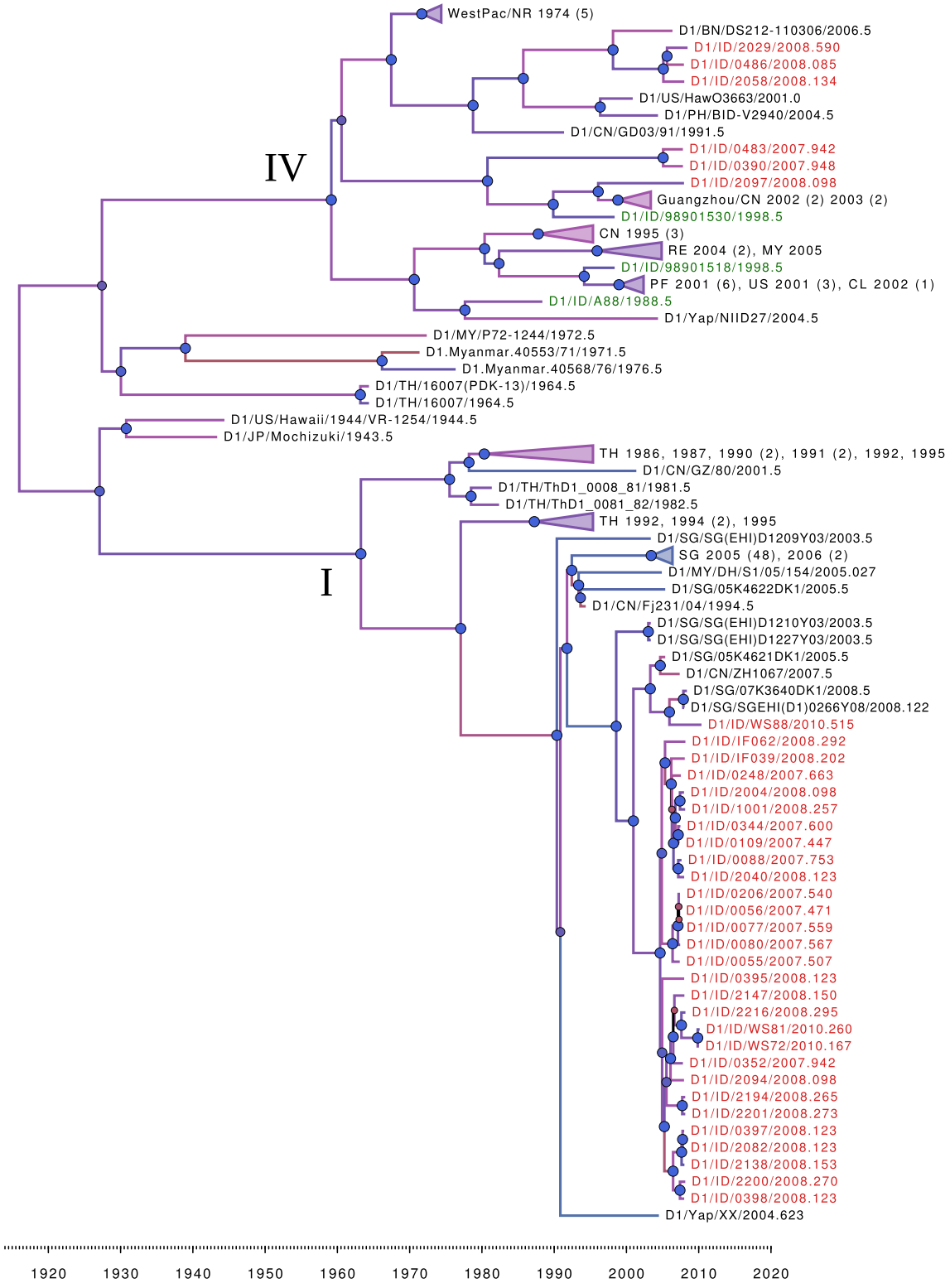

Fig. 1. MCC (Maximum Clade Credibility) phylogenetic tree of DENV-1 Genotype I and Genotype IV. The tree consisted of 35 complete genomes of DENV-1 viruses isolated from Makassar, Indonesia together with their close-distanced genomes available in the GenBank database and was constructed using Bayesian inference as implemented in BEAST. All Makassar isolates were denoted with red labels, while samples from Indonesian cities of Palembang and Jakarta were denoted with green labels. Posterior probabilities of all major branches had values of more than 0.75, indicating the significant of the tree topology. The red line for a branch indicated that the rate of mutation of that particular branch was relatively faster compared to the overall rate of mutation, while the blue line indicated slower rate of mutation. The MCC tree branched into 2 main branches, the genotype IV (upper branch) and I (lower branch). Six of the Makassar isolates grouped into genotype IV, while the rest of Makassar isolates grouped into genotype I. Labels indicating the origin of isolates are as follow: BN (Brunei), CL (Chile), CN (China), ID (Indonesia), JP (Japan), MY (Malaysia), NR (Nauru Island), PF (Polynesian French), PH (Philippines), RE (Reunion Island), SG (Singapore), SC (Seychelles Island), TH (Thailand), and US (United States). Dots in the nodes represent the posterior probability, with large blue dots, medium purple dots and small red dots indicate posterior probability >0.75, 0.75–0.5, and <0.5, respectively.

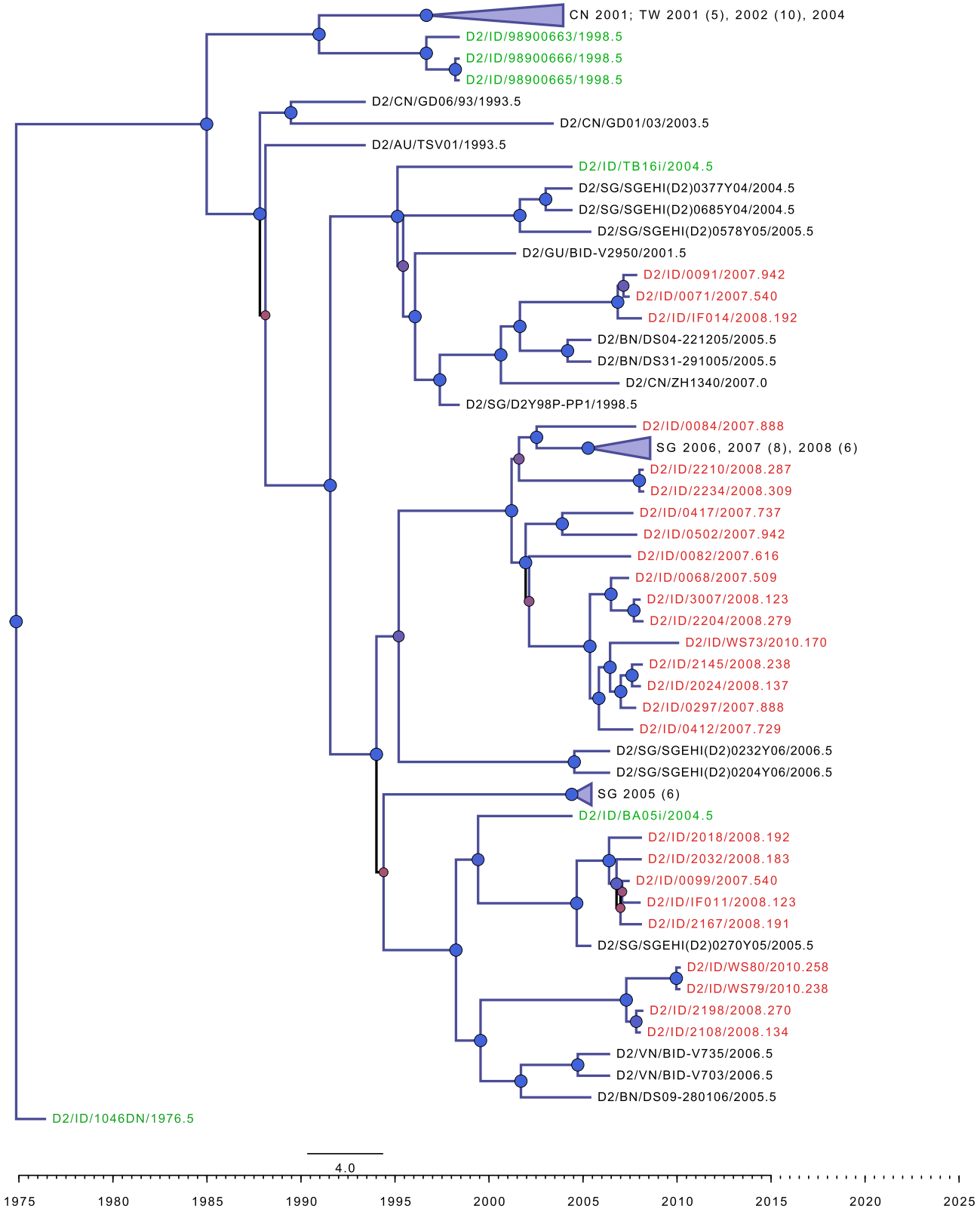

Fig. 2. MCC phylogenetic tree of DENV-2 genotype Cosmopolitan. The tree consisted of 26 complete genomes of DENV-2 viruses isolated from Makassar, Indonesia, together with their close-distanced genomes available in the GenBank database and was constructed using Bayesian inference as implemented in BEAST. All Makassar isolates were denoted with red labels, while samples from Indonesian cities of Palembang and Jakarta were denoted with green labels. Posterior probabilities of all major branches had values of more than 0.75, indicating the significant of the tree topology. The red line for a branch indicated that the rate of mutation of that particular branch was relatively faster compared to the overall rate of mutation, while the blue line indicated slower rate of mutation. Labels indicating the origin of isolates are as follow: AU (Australia), BN (Brunei), CN (China), GU (Guam), ID (Indonesia), SG (Singapore), TW (Taiwan) and VN (Vietnam). Dots in the nodes represent the posterior probability, with large blue dots, medium purple dots and small red dots indicate posterior probability > 0.75, 0.75–0.5, and < 0.5, respectively.

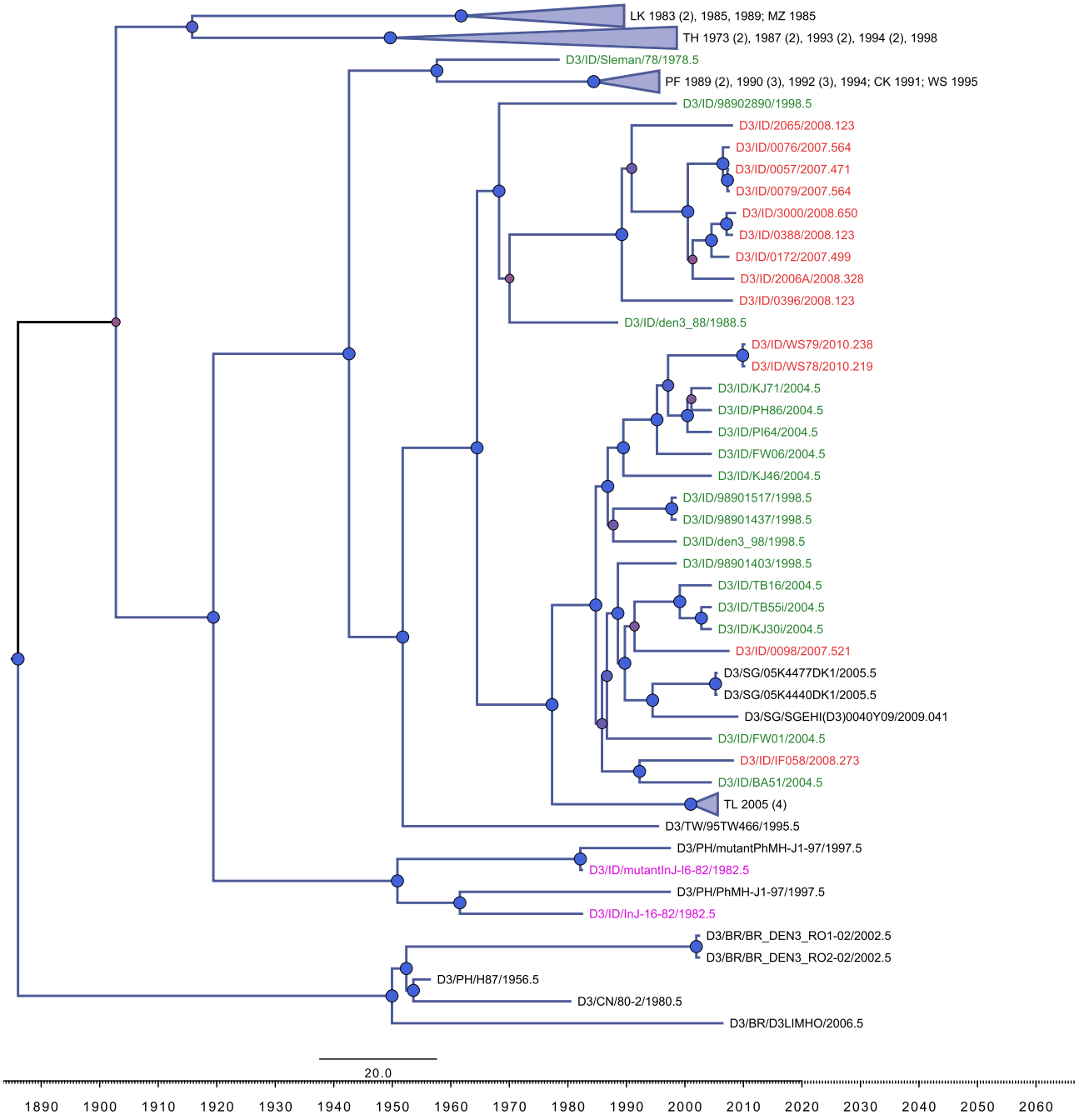

Fig. 3. MCC phylogenetic tree of DENV-3 Genotype I. The tree consisted of 13 complete genomes of DENV-3 viruses isolated from Makassar, Indonesia, together with their close-distanced genomes available in the GenBank database and was constructed using Bayesian inference as implemented in BEAST. All Makassar isolates were denoted with red labels, while samples from other Indonesian regions were denoted with green labels. Magenta labels indicated Indonesian wild-type samples that have undergone serial passages in Vero cells. Posterior probabilities of all major branches had values of more than 0.75, indicating the significant of the tree topology. The red line for a branch indicated that the rate of mutation of that particular branch was relatively faster compared to the overall rate of mutation, while the blue line indicated slower rate of mutation. Labels indicating the origin of isolates are as follow: BR (Brazil), CK (Cook Islands), CN (China), ID (Indonesia), LK (Sri Lanka), MZ (Mozambique), PF (Polynesian French), PH (Philippines), SG (Singapore), TH (Thailand), TL (East Timor), TW (Taiwan), and WS (Western Samoa). Dots in the nodes represent the posterior probability, with large blue dots, medium purple dots and small red dots indicate posterior probability > 0.75, 0.75–0.5, and < 0.5, respectively.

amino acids of the Makassar samples were unlikely to be significant.

In DENV-1 Genotype IV, the most diverse samples, we observed more significant differences in the amino acid changes. Some sites, notably residue 217 of E, 115 of NS2B, 119 of NS3, 426 of NS3, 123 of NS4A genes, contained amino acid Proline (P) which structurally different than that of other amino acids as it had cyclic group in its backbone chain, which might bend the backbone slightly (Fig. 5B).

In DENV-2, similar differences also occurred in the amino acid changes. Residue 217, 332 and 384 of E, 320 of NS1, and 837 of NS5 contained a mix of amino acid Proline (P) and Leucine (L). Changes that were likely to be significant occurred in M (residue 57 and 71), E (residue 346), and NS5 (residue 563 and 570) as they involved changes of amino acids with different physicochemical properties (Fig. 5C).

Both DENV-3 and DENV-4 comparative analysis showed that most differences came from 1 or 2 amino acid changes in most of

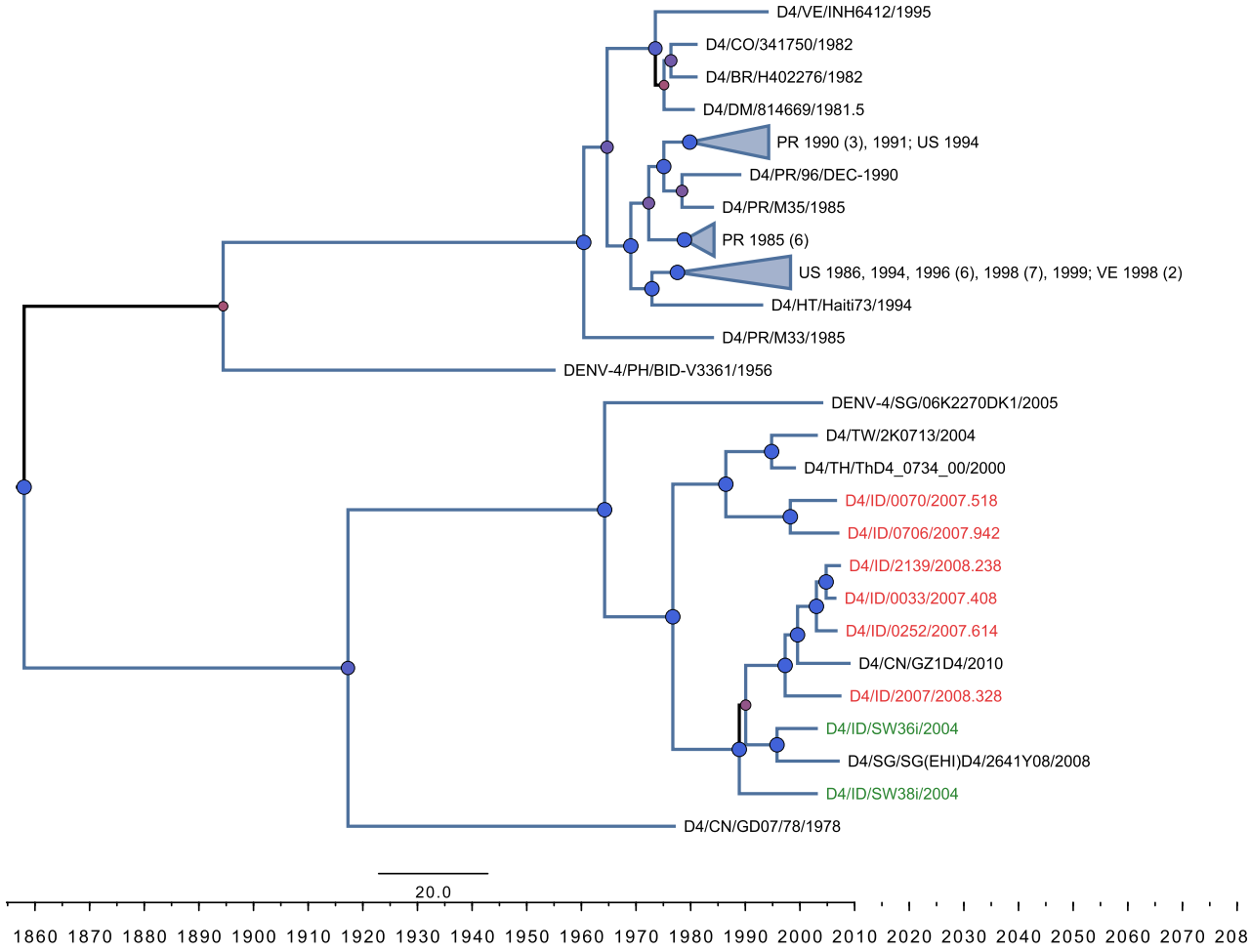

Fig. 4. MCC phylogenetic tree of DENV-4 Genotype II. The tree consisted of 6 complete genomes of DENV-4 viruses isolated from Makassar, Indonesia, together with their close-distanced genomes available in the GenBank database and was constructed using Bayesian inference as implemented in BEAST. All Makassar isolates were denoted with red labels, while samples from other Indonesian regions were denoted with green labels. Posterior probabilities of all major branches had values of more than 0.75, indicating the significant of the tree topology. The red line for a branch indicated that the rate of mutation of that particular branch was relatively faster compared to the overall rate of mutation, while the blue line indicated slower rate of mutation. Labels indicating the origin of isolates are as follow: BR (Brazil), CO (Colombia), CN (China), DM (Dominica), ID (Indonesia), HT (Haiti), PF (Polynesian French), PH (Philippines), PR (Puerto Rico), SG (Singapore), TH (Thailand), TW (Taiwan), US (United States), and VE (Venezuela). Dots in the nodes represent the posterior probability, with large blue dots, medium purple dots and small red dots indicate posterior probability >0.75, 0.75–0.5, and <0.5, respectively.

Table 2
Evolutionary patterns among the proteins of DENV-1, -2, -3 and -4 sampled from Makassar, Indonesia during 2007–2010.

Protein	Length (bp)	DENV-1		DENV-2		DENV-3		DENV-4	
		IS	dN/dS	IS	dN/dS	IS	dN/dS	IS	dN/dS
Capsid (C)	342	24	0.25	12	0.17	1	0	1	1.0
Membrane (M)	498	52	0.13	19	0.11	2	0.5	0	0
Envelope (E)	1479	167	0.11	53	0.10	11	0.1	0	0
NS1	1056	102	0.18	40	0.17	5	0.2	1	1.0
NS2A	654	75	0.19	27	0.19	7	0.29	3	0
NS2B	390	36	0.19	13	0.08	3	0	3	0.67
NS3	1857	187	0.11	67	0.03	15	0.2	2	1.0
NS4A	450	49	0.10	14	0	0	0	0	0
2K	68	8	0	4	0	0	0	1	0
NS4B	744	73	0.04	27	0.07	4	0	2	0.5
NS5	2679	230	0.10	103	0.18	10	0	8	0
Genome*	10170	1003	NA	379	NA	58	NA	21	NA

* Coding region only; IS, number of informative sites; NA, not applicable.

the sites. Unfortunately, the sample sizes of both DENV-3 and DENV-4 are too small to confidently detect any significant changes or evidence of positive selection within these clades (Fig. 5D and E).

3.5. Growth characteristics of Makassar DENV

Our study observed the circulation of all four DENV serotypes in Makassar. Phylogenetic analyses revealed the genotypes of viruses

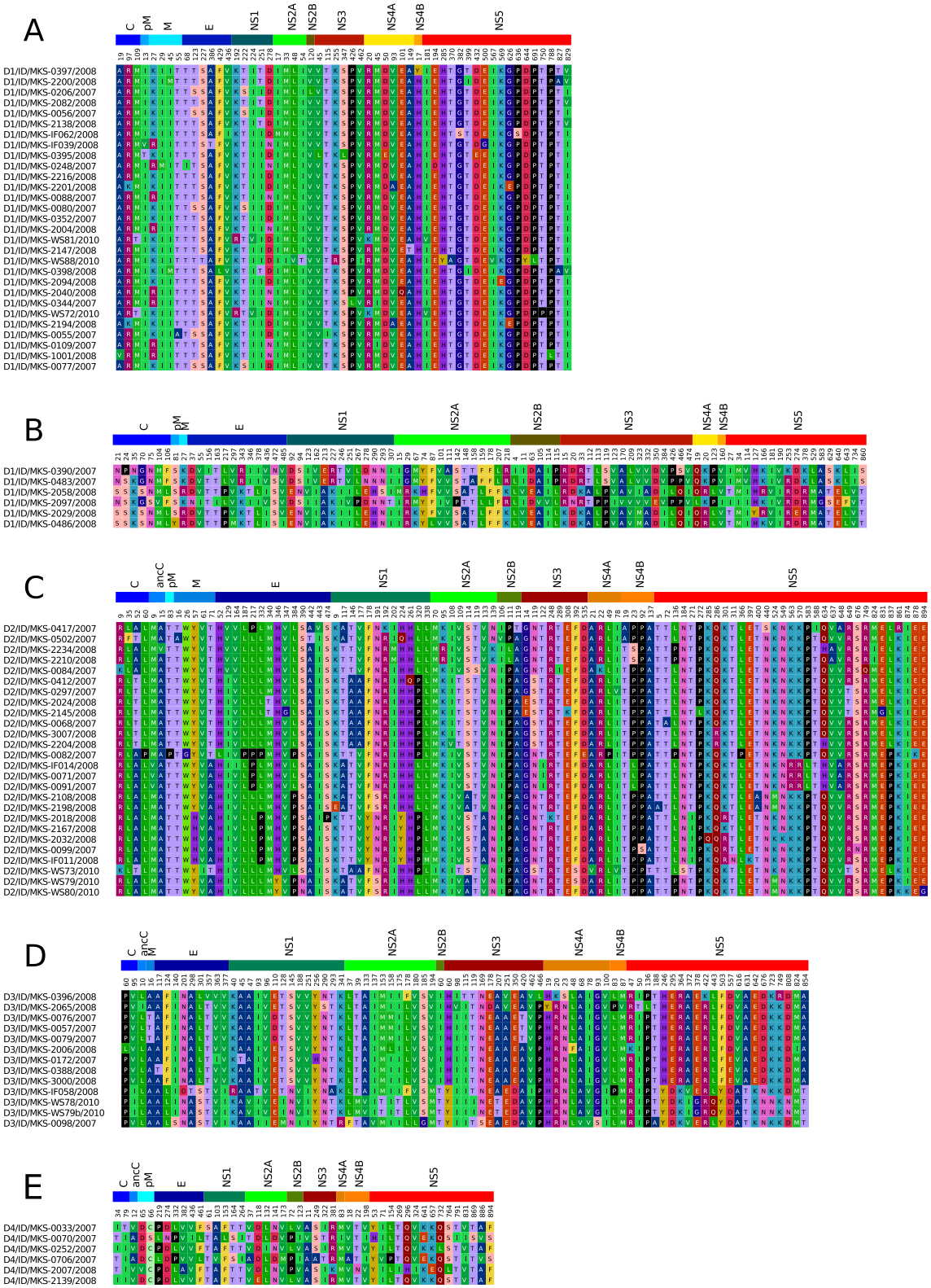

Fig. 5. Comparative analyses of amino acid substitutions among Makassar's DENV-1 viruses genotype I (A) and IV (B), DENV-2 viruses (C), DENV-3 viruses (D), and DENV-4 (E). Genes in which the substitutions present are shown above the amino-acid numbers. Amino acid coloring scheme is according to AESNN3 (Lin et al., 2002).

isolated in this study and grouped the Makassar virus isolates into several clades within each genotype (Figs. 1–4). It has been suggested that particular genotypes of DENV possess different rates of transmission and that some genotypes have a tendency to cause more severe forms of dengue disease and are thus more virulent than others (Rico-Hesse, 2010). One way to estimate the virulence is by assessing the replication kinetics of the virus (Rico-Hesse, 2010).

To determine the growth characteristics of Makassar's DENV, we measured the replication kinetics of all four serotypes and

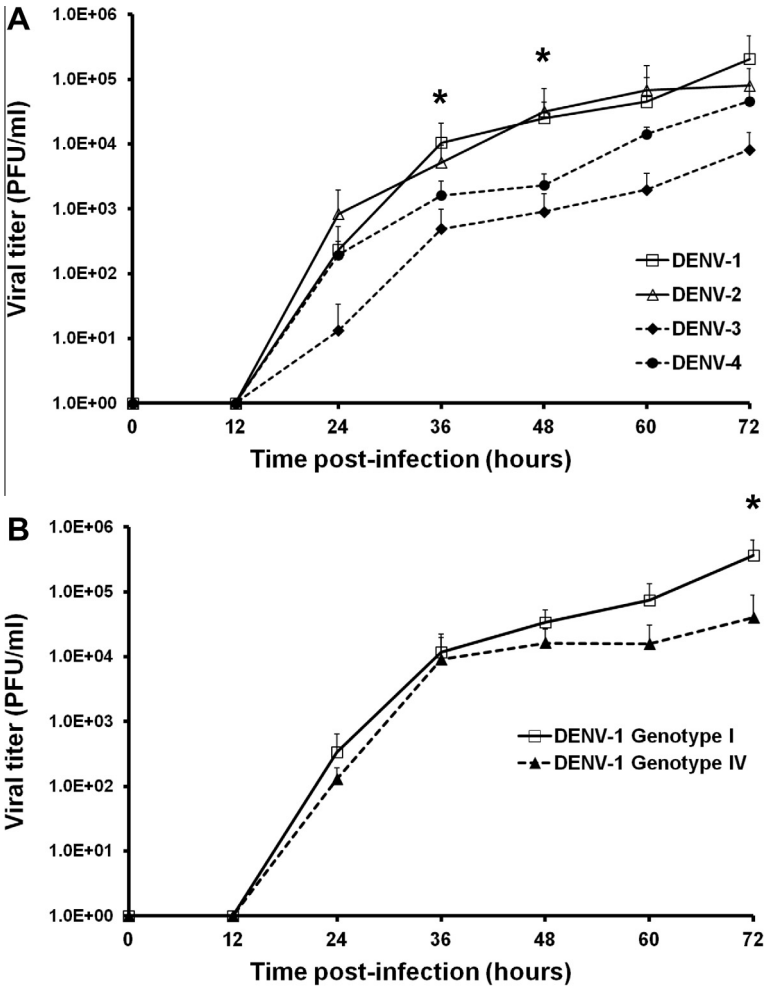

Fig. 6. Replication kinetic assays of selected DENV from Makassar. (A) Replication kinetic comparison of four DENV serotypes. (B) Replication kinetic comparison of DENV-1 Genotype I and Genotype IV strains. Data were the mean ± SD of three to five virus isolates representing each serotype/genotype performed in duplicate.

genotypes of DENV grown in mammalian Vero76 cell line. Three to four viruses representing each serotype/genotype were assayed for their replication kinetics. Growth kinetic comparison of DENV-1, -2, -3, and -4 is shown in Fig. 6. We observed serotype-specific differences in replication at 36 and 48 h post-infection, with DENV-1 and -2 replicating to higher levels compared with DENV-3 and -4 (Fig. 6A).

Next, we compared the replication kinetics of viruses belong to two different genotypes of DENV-1, i.e., Genotype I and IV (Fig. 6B). Genotype I apparently replicated at higher level compared to Genotype IV (p value < 0.05). This suggests the relatively better fitness of Genotype I which may underlie the ability of this Genotype to replace the Genotype IV in the community. Similar kinetic patterns were observed when other cell line (C6/36) was used in growth kinetic experiment (data not shown). Replication kinetic assays on four viruses representing four clades of DENV-2 did not show any significant difference (data not shown).

4. Discussion

We conducted the first molecular-typing study of dengue in Makassar-Indonesia to investigate dengue disease dynamics in the area from 2007 to 2010. As confirmed by NS1 antigen detection, we observed dengue viruses infected a remarkable 43.3% of febrile patients presenting to hospital. This data reflects the importance of the disease in Makassar and the considerable burden it must place on the local economy and health infrastructure. In terms of infection status, based on IgM and IgG ELISA detection, our data showed a high number of secondary dengue infections in hospitalized patients who participated in this study (83.8%), indicating a sustained disease intensity over a number of years. The age distribution of the hospitalized patients was found to be highest in young patients (10–20 years old), similar to other parts of Indonesia (Corwin et al., 2001). It is likely that this predominant age group may correlate with the higher proportion of secondary infections observed.

To date, no serotype distribution data has been available for Makassar. Our study demonstrated the circulation of all four dengue serotypes. We observed the predominance of DENV-1 (41%) followed by DENV-2, -3, -4. This distribution is distinct from historical reports in other parts of Indonesia, especially in the Western regions of the archipelago, e.g., in the cities of Jakarta and Palembang in 2004, in which DENV-3 was the predominant serotype in the area and correlated with severe symptoms. It would be interesting to know if the current serotype distributions in Jakarta and Palembang match their historical values or if they are now closer to what we have found in Makassar.

To study the evolutionary dynamics and phylogeny of Makassar's dengue viruses, we performed whole-genome sequencing of 80 virus isolates. Most of the genomes were sequenced from primary infection samples due to difficulties in virus isolation from

secondary infection samples, as previously reported (Jarman et al., 2011). Based on the published classification of dengue virus's genotypes, we identified the co-circulation of Genotypes I and IV of the DENV-1 serotype. The nearest geographical areas that have been reported as harboring Genotype I are Singapore (Schreiber et al., 2009), Thailand (Ong et al., 2008) and Surabaya (Yamanaka et al., 2011). The clustering of all Genotype I samples together with viruses from China, Thailand, and Singapore may suggest that the Genotype I isolates were originated from China, migrated to Thailand and passed Singapore before entering Makassar (Fig. 1). Genotype IV was predominant in Jakarta in 2004 (Schreiber et al., 2009) and was also present in Makassar in smaller numbers. The close genetic relationship between the Makassar and Singapore Genotype I isolates suggests a recent importation has occurred and that the incumbent clade is being replaced. While the Genotype IV strains from Makassar are quite diverse the Genotype I strains appear to be relatively uniform further suggesting a recent introduction. We will continue to survey viruses from the region to see if Genotype I succeed in displacing Genotype IV.

Makassar's DENV-2 viruses grouped into the Cosmopolitan genotype. This genotype circulates widely in India, South East Asia, Africa, the Middle East, and Australia (Twiddy et al., 2002). This genotype was also circulating in the cities of Jakarta in 2004 and Palembang in 1998. Phylogenetic analysis showed that closely related Cosmopolitan genotype strains were isolated in Jakarta in 2004, Brunei and Singapore in 2005, Vietnam in 2006 and Makassar in 2007 and 2008. It appears that the Cosmopolitan genotype is very successful in spreading, regionally endemic and continually re-distributing in South East Asia. The phylogenetic trees suggested the presence of spatial isolation among DENV-2 strains from cities in the western and eastern parts of Indonesia, but the absence of spatial isolation between Makassar and Singapore (Fig. 2). The tree also indicated that DENV-2 viruses currently circulating in Makassar were of multiple entries. Presumably, frequent direct air and sea travel between Makassar and Singapore is rapidly distributing the viruses between the two cities. Another explanation of the possible spatial isolation among Indonesian cities is that there is no other whole genome sequence data that has been reported and submitted to GenBank.

Our phylogenetic analysis revealed that Makassar's DENV-3 viruses grouped into Genotype I, similar to isolates from Jakarta in 2004 and Palembang in 1998, and same genotype as Indonesian strains from 1978, 1988, 1996 and 1998 isolates. This genotype has been described as causing four epidemics in the region in the past (Ong et al., 2008), and is most likely a local, Indonesian endemic strain that has circulated for more than three decades. It is interesting that the isolates we found in Makassar appear to be more closely related to strains from 1996 and 1998 than the strains more recently found in Timor-Leste, Jakarta, Singapore and Palembang suggesting DENV-3 in Makassar has been following a separate evolutionary history since 1988.

The DENV-4 viruses we isolated clustered into Genotype II (SE Asia and America), the same genotype as those isolated in Jakarta in 2004. Four of the Makassar samples were originated from a lineage with higher mutation rates relative to other samples, and were direct descendant from ancestor of those Jakarta samples with TMRCA around year 1990. Higher mutation rates might indicate that this particular lineage was spreading rather quickly, as in outbreak cases. As such, those particular Makassar isolates were assumed to be descendant of Jakarta outbreak in 1998. Partial sequences of viruses from 1973, 1976, 1977 isolates also cluster into this clade (not shown). This genotype appears to be endemic in Indonesia and has been circulating for over 30 years.

A Bayesian coalescent approach was used to infer the evolutionary dynamics for all serotypes of isolates within 2007–2010 in Makassar (Drummond and Rambaut, 2007). We observed the mean of evolution rates for DENV-1, DENV-2 and DENV-3 were similar and roughly equivalent to those estimated previously for all DENV serotypes (Twiddy et al., 2003). The mean age of the common ancestor for DENV-1, DENV-2 and DENV-3 indicated that each of these diverse lineages had common ancestors already present in Makassar since approximately 2003. Since the number of samples for DENV-4 was small, we could not confidently estimate the mean age of the common ancestor of these isolates. That we did not see any convincing evidence for positive selection in any of the serotypes indicates that these strains are already well adapted to their environment.

The analysis based on the amino acid sequences of the Indonesian samples showed that some positions were undergoing amino acid substitutions from 2007 to 2008. That we did not see any convincing evidence for positive selection in any of the serotypes indicates that these strains are already well adapted to their environment, and that the amino acid substitutions might not have affected the characteristics of viruses substantially.

Genetic variations in dengue viruses have been described as being risk factors for severe disease (Balmaseda et al., 2006; Messer et al., 2003; Rico-Hesse et al., 1997) and particular serotypes/genotypes are suggested to have a tendency to cause more severe forms of dengue (i.e., DHF and DSS) and larger outbreaks. For example, DENV-2 and DENV-3 have been associated with severe disease and DENV-4 is claimed to lead to a milder illness (Nisalak et al., 2003; Vaughn et al., 2000). One of the ways to estimate the virulence or fitness of a virus *in vitro* is by looking at the virus replication rate (Rico-Hesse, 2010). In this study, we compared the replication kinetics of various serotype/genotypes of DENV to determine whether particular serotype/genotypes replicate better than others do.

The co-circulation of two DENV-1 genotypes (i.e., Genotype I and IV) prompted us to compare their replication rates. Isolates grouped into Genotype I apparently replicated at higher level compared to Genotype IV. This suggests the relatively better fitness of Genotype I which may underlie the ability of this genotype to replace the Genotype IV in the community. Thus, this observation suggests that different genotypes of DENV may exhibit different phenotypic characteristics, as in the case of American and South East Asian genotypes of DENV-2 (Leitmeyer et al., 1999; Rico-Hesse et al., 1997). Continuous surveillance is currently underway to confirm the predominant circulation of Genotype I.

The DENV-2 American and South East Asian genotypes are known to have differences in their replication ability. American genotypes replicated less well than the South East Asian viruses in *in vitro* experiments (Pryor et al., 2001). As all of our DENV-2 viruses were grouped into Cosmopolitan genotype (Fig. 3), we could not perform replication comparisons against other genotypes. However, within the Cosmopolitan genotype, we observed the grouping of viruses into clades. To determine whether there are differences in growth, four viruses representing each clade were assayed for their replication kinetics. We observed no significant differences among those four viruses (data not shown), suggesting that members of the genotype are quite homogenous in their growth rates. Similar observation is also present in DENV-3 and -4 (data not shown). A typical DENV growth kinetic profile was also observed in other virus isolates from other cities in Indonesia (data not shown).

In summary, we have revealed the dengue serotype and genotype distribution in Makassar and observed that although these viruses have very similar evolutionary dynamics they have remarkably different phylogeographic histories. We observed the different replication rates of DENV-1 Genotype I and Genotype IV, which may underlie the ability of Genotype I in replacing the Genotype IV in Makassar, Indonesia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.03.006.

References

Balmaseda, A., Hammond, S.N., Perez, L., Tellez, Y., Saborio, S.I., Mercado, J.C., Cuadra, R., Rocha, J., Perez, M.A., Silva, S., Rocha, C., Harris, E., 2006. Serotype-specific differences in clinical manifestations of dengue. Am. J. Trop. Med. Hyg. 74, 449–456.

Corwin, A.L., Larasati, R.P., Bangs, M.J., Wuryadi, S., Arjoso, S., Sukri, N., Listyaningsih, E., Hartati, S., Namursa, R., Anwar, Z., Chandra, S., Loho, B., Ahmad, H., Campbell, J.R., Porter, K.R., 2001. Epidemic dengue transmission in southern Sumatra, Indonesia. Trans. R. Soc. Trop. Med. Hyg. 95, 257–265.

Drummond, A.J., Rambaut, A., 2007. BEAST: bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. http://dx.doi.org/10.1093/bioinformatics/btq461.

Fink, J., Gu, F., Ling, L., Tolfvenstam, T., Olfat, F., Chin, K.C., Aw, P., George, J., Kuznetsov, V.A., Schreiber, M., Vasudevan, S.G., Hibberd, M.L., 2007. Host gene expression profiling of dengue virus infection in cell lines and patients. PLoS Negl. Trop. Dis. 1, e86.

Goncalvez, A.P., Escalante, A.A., Pujol, F.H., Ludert, J.E., Tovar, D., Salas, R.A., Liprandi, F., 2002. Diversity and evolution of the envelope gene of dengue virus type 1. Virology 303, 110–119.

Graham, R.R., Juffrie, M., Tan, R., Hayes, C.G., Laksono, I., Ma'roef, C., Erlin, Sutaryo., Porter, K.R., Halstead, S.B., 1999. A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia I. Studies in 1995–1996. Am. J. Trop. Med. Hyg. 61, 412–419.

Gubler, D.J., 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. 11, 480–496.

Guzman, M.G., Halstead, S.B., Artsob, H., Buchy, P., Farrar, J., Gubler, D.J., Hunsperger, E., Kroeger, A., Margolis, H.S., Martínez, E., Nathan, M.B., Pelegrino, J.L., Simmons, C., Yoksan, S., Peeling, R.W., 2010. Dengue: a continuing global threat. Nat. Rev. Microbiol., S7–16.

Harris, E., Roberts, T.G., Smith, L., Selle, J., Kramer, L.D., Valle, S., Sandoval, E., Balmaseda, A., 1998. Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. J. Clin. Microbiol. 36, 2634–2639.

Holmes, E.C., Burch, S.S., 2000. The causes and consequences of genetic variation in dengue virus. Trends Microbiol. 8, 74–77.

Holmes, E.C., Twiddy, S.S., 2003. The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol 3, 19–28.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Jarman, R.G., Nisalak, A., Anderson, K.B., Klungthong, C., Thaisomboonsuk, B., Kaneechit, W., Kalayanarooj, S., Gibbons, R.V., 2011. Factors influencing dengue virus isolation by C6/36 cell culture and mosquito inoculation of nested PCR-positive clinical samples. Am. J. Trop. Med. Hyg. 84, 218–223. http://dx.doi.org/10.4269/ajtmh.2011.09-0798.

Lanciotti, R.S., Calisher, C.H., Gubler, D.J., Chang, G.J., Vorndam, A.V., 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbiol. 30, 545–551.

Lanciotti, R.S., Gubler, D.J., Trent, D.W., 1997. Molecular evolution and phylogeny of dengue-4 viruses. J. Gen. Virol. 78 (Pt 9), 2279–2284.

Lanciotti, R.S., Lewis, J.G., Gubler, D.J., Trent, D.W., 1994. Molecular evolution and epidemiology of dengue-3 viruses. J. Gen. Virol. 75 (Pt 1), 65–75.

Leitmeyer, K.C., Vaughn, D.W., Watts, D.M., Salas, R., Villalobos, I., de, C., Ramos, C., Rico-Hesse, R., 1999. Dengue virus structural differences that correlate with pathogenesis. J. Virol. 73, 4738–4747.

Lin, K., May, A.C., Taylor, W.R., 2002. Amino acid encoding schemes from protein structure alignments: multi-dimensional vectors to describe residue types. J. Theor. Biol. 216, 361–365.

Messer, W.B., Gubler, D.J., Harris, E., Sivananthan, K., de Silva, A.M., 2003. Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg. Infect. Dis. 9, 800–809.

Nisalak, A., Endy, T.P., Nimmannitya, S., Kalayanarooj, S., Thisayakorn, U., Scott, R.M., Burke, D.S., Hoke, C.H., Innis, B.L., Vaughn, D.W., 2003. Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am. J. Trop. Med. Hyg. 68, 191–202.

Ong, S.H., Yip, J.T., Chen, Y.L., Liu, W., Harun, S., Lystiyaningsih, E., Heriyanto, B., Beckett, C.G., Mitchell, W.P., Hibberd, M.L., Suwandono, A., Vasudevan, S.G., Schreiber, M.J., 2008. Periodic re-emergence of endemic strains with strong epidemic potential-a proposed explanation for the 2004 Indonesian dengue epidemic. Infect. Genet. Evol. 8, 191–204.

Pandey, B.D., Igarashi, A., 2000. Severity-related molecular differences among nineteen strains of dengue type 2 viruses. Microbiol. Immunol. 44, 179–188.

Porter, K.R., Beckett, C.G., Kosasih, H., Tan, R.I., Alisjahbana, B., Rudiman, P.I., Widjaja, S., Listiyaningsih, E., Ma'Roef, C.N., McArdle, J.L., Parwati, I., Sudjana, P., Jusuf, H., Yuwono, D., Wuryadi, S., 2005. Epidemiology of dengue and dengue hemorrhagic fever in a cohort of adults living in Bandung, West Java, Indonesia. Am J Trop Med Hyg 72, 60–66.

Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. PLoS One 5, e9490. http://dx.doi.org/10.1371/journal.pone.0009490.

Pryor, M.J., Carr, J.M., Hocking, H., Davidson, A.D., Li, P., Wright, P.J., 2001. Replication of dengue virus type 2 in human monocyte-derived macrophages: comparisons of isolates and recombinant viruses with substitutions at amino acid 390 in the envelope glycoprotein. Am. J. Trop. Med. Hyg. 65, 427–434.

Raekiansyah, M., Pramesyanti, A., Bela, B., Kosasih, H., Ma'roef, C.N., Tobing, S.Y., Rudiman, P.I., Alisjahbana, B., Endi, T.P., Green, S., Kalayanarooj, S., Rothman, A.L., Sudiro, T.M., 2005. Genetic variations and relationship among dengue virus type 3 strains isolated from patients with mild or severe form of dengue disease in Indonesia and Thailand. Southeast Asian J. Trop. Med. Public Health 36, 1187–1197.

Rico-Hesse, R., 2010. Dengue virus virulence and transmission determinants. Curr. Top. Microbiol. Immunol. 338, 45–55.

Rico-Hesse, R., Harrison, L.M., Salas, R.A., Tovar, D., Nisalak, A., Ramos, C., Boshell, J., de Mesa, M.T., Nogueira, R.M., da Rosa, A.T., 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230, 244–251.

Schreiber, M.J., Holmes, E.C., Ong, S.H., Soh, H.S., Liu, W., Tanner, L., Aw, P.P., Tan, H.C., Ng, L.C., Leo, Y.S., Low, J.G., Ong, A., Ooi, E.E., Vasudevan, S.G., Hibberd, M.L., 2009. Genomic epidemiology of a dengue virus epidemic in urban Singapore. J. Virol. 2009.

Sukri, N.C., Laras, K., Wandra, T., Didi, S., Larasati, R.P., Rachdyatmaka, J.R., Osok, S., Tjia, P., Saragih, J.M., Hartati, S., Listyaningsih, E., Porter, K.R., Beckett, C.G., Prawira, I.S., Punjabi, N., Suparmanto, S.A., Beecham, H.J., Bangs, M.J., Corwin, A.L., 2003. Transmission of epidemic dengue hemorrhagic fever in easternmost Indonesia. Am. J. Trop. Med. Hyg. 68, 529–535.

Sukumaran, J., Holder, M.T., 2010. DendroPy: a Python library for phylogenetic computing. Bioinformatics 26, 1569–1571. http://dx.doi.org/10.1093/bioinformatics/btq228.

Sumarmo, 1987. Dengue haemorrhagic fever in Indonesia. Southeast Asian. J. Trop. Med. Public Health 18, 269–274.

Suwandono, A., Kosasih, H., Nurhayati, Kusriastuti, R., Harun, S., Ma'roef, C., Wuryadi, S., Herianto, B., Yuwono, D., Porter, K.R., Beckett, C.G., Blair, P.J., 2006. Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004. Trans. R. Soc. Trop. Med. Hyg. 100, 855–862.

Twiddy, S.S., Farrar, J.J., Vinh Chau, N., Wills, B., Gould, E.A., Gritsun, T., Lloyd, G., Holmes, E.C., 2002. Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. Virology 298, 63–72.

Twiddy, S.S., Holmes, E.C., Rambaut, A., 2003. Inferring the rate and time-scale of dengue virus evolution. Mol. Biol. Evol. 20, 122–129.

Vaughn, D.W., Green, S., Kalayanarooj, S., Innis, B.L., Nimmannitya, S., Suntayakorn, S., Endy, T.P., Raengsakulrach, B., Rothman, A.L., Ennis, F.A., Nisalak, A., 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J. Infect. Dis. 181, 2–9.

WHO, 1997. Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control. World Health Organization, Geneva.

Yamanaka, A., Mulyatno, K.C., Susilowati, H., Hendrianto, E., Ginting, A.P., Sary, D.D., Rantam, F.A., Soegijanto, S., Konishi, E., 2011. Displacement of the predominant dengue virus from type 2 to type 1 with a subsequent genotype shift from IV to I in Surabaya, Indonesia 2008–2010. PLoS One 6, e27322. http://dx.doi.org/10.1371/journal.pone.0027322.