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Molecular characterization of dengue viruses isolated from patients in Central Java, Indonesia

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ABSTRACT

Background: Dengue is hyper-endemic in Indonesia. Purwokerto city in Central Java province is routinely ravaged by the disease. Despite the endemicity of dengue in this city, there is still no data on the virological aspects of dengue in the city. We conducted a molecular surveillance study of the circulating dengue viruses (DENV) in Purwokerto city to gain information on the virus origin, serotype and genotype distribution, and phylogenetic characteristics of DENV.
Methods: A cross-sectional dengue molecular surveillance study was conducted in Purwokerto. Sera were collected from dengue-suspected patients attending three hospitals in the city. Diagnosis was performed using dengue NS1 antigen and IgG/IgM antibodies detection. DENV serotyping was performed using Simplexa Dengue real-time RT-PCR. Sequencing was conducted to obtain full-length DENV Envelope (E) gene sequences, which were then used in phylogenetic and genotypic analyses. Patients' clinical and demographic data were collected and analyzed.
Results: A total of 105 dengue-suspected patients' sera were collected, in which 80 (76.2%) were positive for IgM and/or IgG, and 57 (54.2%) were confirmed as dengue by NS1 antigen and/or DENV RNA detection using RT-PCR. Serotyping was successful for 47 isolates. All four serotypes circulated in the area with DENV-3 as the predominant serotype. Phylogenetic analyses grouped the isolates into Genotype I for DENV-1, Cosmopolitan genotype for DENV-2, and Genotype I and II for DENV-3 and -4, respectively. The analyses also revealed the close relatedness of Purwokerto isolates to other DENV strains from Indonesia and neighboring countries.
Conclusion: We reveal the molecular and virological characteristics of DENV in Purwokerto, Banyumas regency, Central Java. The genotype and phylogenetic analyses indicate the endemicity of the circulating DENV in the city. Our serotype and genotype data provide references for future dengue molecular epidemiology studies and disease management in the region.

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Introduction

Dengue is considered as the most prevalent arthropod-borne viral disease in the world with significant burden to people in tropical and subtropical regions [1]. The clinical manifestations of the disease range from the mild dengue fever (DF) to the more severe cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. The disease is caused by dengue virus (DENV), a positive-sense single stranded RNA virus member of Flaviviridae family [3]. The 10.7 kb RNA genome encodes three structural (C, prM/M, E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins [3,4]. The diverse characteristics of the virus is depicted by the presence of four serotypes (DENV-1, -2, -3, and -4), in which each serotype can be further divided into phylogenetically distinct clusters termed genotypes [5,6]. These genotypes vary in their geographical distributions, epidemic potential, fitness, and virulence [6,7].

Indonesia has been experiencing epidemic cycles of dengue since its first introduction in the country in 1968 in Jakarta and Surabaya [8]. The country suffered the highest economic burden of dengue in Southeast Asia region [9]. The frequent dengue cases is often followed by increasing numbers of infections and sever-

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ity which affected all 34 provinces in the country [8,10]. As most countries in Southeast Asia except Singapore and Malaysia, dengue surveillance in Indonesia remains largely passive [11]. Therefore, local surveillance is important to gain comprehensive information on dengue epidemiology which can be used to improve control strategies and management of the disease.

Banyumas regency in Central Java, Indonesia is affected by dengue in an annual basis [12]. Purwokerto, the capital city of Banyumas has experienced frequent dengue outbreaks with higher risks of dengue infection compared to other towns in surrounding area in Banyumas regency [13]. Apart of information on the socio-economic, epidemiology, and vector data, to the best of our knowledge, there is still no report on the virological aspects of dengue in this city where no data on circulating serotypes and diversity of the genotypes are available. Previously, we have reported dengue molecular and virological characteristics in Semarang in 2012, a city in Central Java which resided approximately 188 km from Purwokerto [14]. In this study, we conducted a prospective cross-sectional molecular surveillance study in Purwokerto to gain information on DENV molecular characteristics, in particular the serotype distribution and genotype diversity of DENV circulating in the city.

Materials and methods

Study design, study site and patient recruitment

We conducted a cross-sectional dengue molecular surveillance study in Purwokerto city, Banyumas regency, Central Java, Indonesia. This city is located at the southern part of Central Java, at the altitude of 75 m above sea level with 7°26′ south latitude and 109°14′ east longitude. Dengue-suspected patients were recruited from inpatient wards of three hospitals in the city, namely RSUD Prof. Dr. Margono Soekardjo central hospital, RSU Sinar Kasih, and RSU St. Elisabeth during period of June through August 2015. Inclusion criteria included patients with fever >38 °C during the first five days of fever, accompanied with at least one clinical signs of dengue such as rash, arthralgia, malaise, retro-orbital pain, signs of DHF or DSS. We excluded fever patients with clear symptoms of upper respiratory tract infections and/or obviously diagnosed as non-dengue and unwilling to participate in this study. The hematology data were obtained from the routine blood tests performed by the hospitals. All dengue positive cases were categorized clinically either as DF, DHF, DSS, or expanded dengue syndrome according to WHO-SEARO [15].

Peripheral venous blood was collected in serum separator tubes and taken to the laboratory for serum separation. Serum was kept frozen at −80 °C until analysis. Ethical approval for the involvement of human participants was granted by the Research Ethics Commission of the Faculty of Medicine, Universitas Jenderal Soedirman, approval No. 065/KEPK/IV/2015.

Dengue diagnosis, nucleic acid extraction and serotyping

The SD BIOLINE Dengue Duo rapid test (Dengue NS1 Ag + IgG/IgM) (Standard Diagnostics, Alere, Korea) was used to detect the presence of NS1 antigen and IgG/IgM antibodies to DENV. Further serological diagnosis was performed using Panbio Dengue Duo ELISA (Alere), in which the IgG/IgM scores were used to determine the primary versus secondary infection, performed according to the kit's instructions. The DENV RNA was extracted using MagNA Pure Total Nucleic Acid extraction kit (Roche, Mannheim, Germany) and performed using automated MagNA Pure LC 2.0 extraction system (Roche), according to protocol described by the manufacturer. The resulting RNA was then assayed using Simplexa Dengue real-time RT-PCR (DiaSorin, Saluggia, Italy) assay which simultaneously detect the presence of DENV and its serotype [16]. Strict controls were applied on RNA extraction and RT-PCR procedures to prevent cross-contamination.

DENV E gene sequencing

The sequencing of DENV E genes were performed at Eijkman Institute's sequencing facility, using RNA directly extracted from patients' sera. Superscript III Reverse Transcriptase (Invitrogen-Thermo Scientific, Carlsbad, CA, USA) was used to reverse-transcribed the DENV RNA into cDNA which was then PCR-amplified using *Pfu* Turbo DNA Polymerase (Stratagene-Agilent Technologies, La Jolla, CA, USA). PCR-amplified DNA was purified from 0.8% gel using QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified amplicon was used in cycle sequencing reactions using eight overlapping primers for each serotype from both strands and BigDyeDideoxy Terminator sequencing kits v.3.1 (Applied Biosystems-Thermo Scientific), as described elsewhere [17]. Purified DNA was subjected to capillary sequencing performed on 3130xl Genetic Analyzer (Applied Biosystems). Sequence reads were assembled using SeqScape v.2.5 software (Applied Biosystems) with manual inspection performed where ambiguities observed.

Phylogenetic analysis

Phylogenetic analysis was conducted by aligning full-length E gene sequences of Purwokerto isolates with other publicly available DENV sequences worldwide retrieved from GenBank as of March, 2016. All nonrelated sequences were removed and finally 60 taxa which are the most closely related to Purwokerto isolates were selected per serotype to clarify the tree view. These sequences were subjected to robust phylogenetic analyses. Dataset for each serotype was prepared using BEAUti v.1.8.3 graphical interface with the tip of each isolate calibrated using the year of isolation. Bayesian Markov Chain Monte Carlo (MCMC) method was implemented for phylogenetic reconstruction and molecular clock analyses as implemented in BEAST v.1.8.3 using General Time Reversible (GTR) model with four Gamma parameters (G_4) and invariant (I) sites, relaxed uncorrelated lognormal molecular clock and Bayesian skyline prior, with 100 million generations and sampled for every 1000th iteration and 10% burn-in employed. The initial estimated evolutionary rate was set at 7.6×10^{-4} substitutions per site per year, as previously described [18]. MCMC trace was analyzed using Tracer v.1.5.0 to monitor adequate Effective Sampling Size (ESS) for all parameters. TreeAnnotator v.1.8.3 was used to create Maximum clade credibility (MCC) tree which visualized in FigTree v.1.4.0.

The classification of genotypes in each serotype was based on classifications by Goncalvez et al. [19], Twiddy et al. [20], Lanciotti et al. [21], and Lanciotti et al. [22], for DENV-1, -2, -3 and -4, respectively.

Statistical analysis

Statistical analysis was performed using SPSS Statistics software, version 17.0 (SPSS Inc., Chicago, IL) and R statistical software (http://www.r-project.org). Pearson chi-squared test was used to correlate the clinical manifestations and DENV serotypes. One-way ANOVA test was used to compare groups of hematology data and DENV serotypes. A probability value of $p < 0.05$ was considered statistically significant.

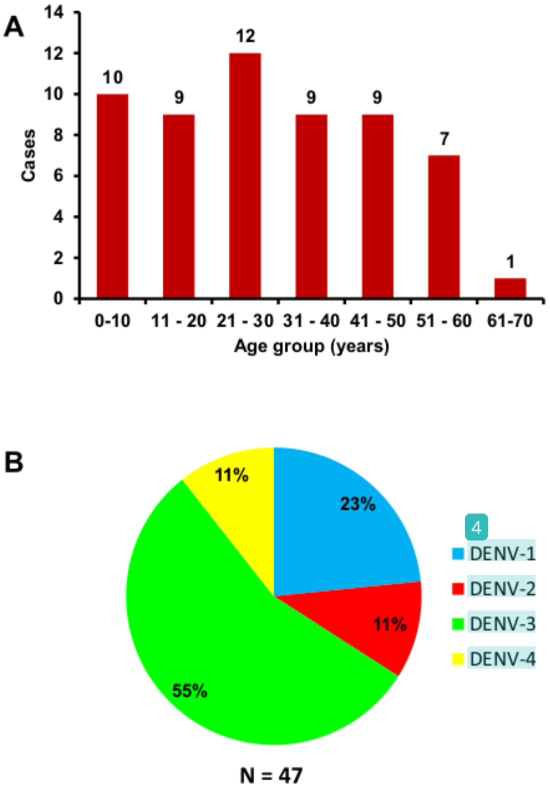

Fig. 1. The distribution of dengue cases in Purwokerto in 2015 determined by the age of dengue-confirmed patients (A) and the percentage of circulating DENV serotypes (B).

Results

Patients characteristics, clinical manifestation, and DENV serotypes

A total of 105 serum samples from dengue-suspected patients were collected during the study. The age of the patients ranging from 3 to 65 years (mean ± SD = 29.5 ± 15.6 years). Among them, 80 (76%) were positive for IgM and/or IgG, and 57 (54.2%) were confirmed to have dengue infection by NS1 antigen and/or DENV RNA detection using RT-PCR (82% positivity by RT-PCR and/or NS1 and 18% positivity by NS1 only). These confirmed cases occurred almost evenly among all age groups with most cases affected adults aged between 21 and 30 years (Fig. 1A). Forty-seven out of 57 confirmed cases were positive for DENV by real-time RT-PCR. Among these 47 patients, 24 (51%) were male and 23 (49%) were female. We observed more secondary infections (57%) compared to primary infections (Table 1). Serotyping revealed the presence of all four DENV serotypes. The DENV-3 was the predominant serotype (26 or 55%), followed by DENV-1 (11 or 23%) and equal number of DENV-2 and -4 cases (5 or 11% each) (Fig. 1B).

From 47 dengue-confirmed patients with known infecting serotypes, 28 (60%) were DHF and 19 (40%) were DF. We did not find DSS and expanded dengue syndrome in this study. Most of the patients (32 or 68%) experienced thrombocytopenia (platelet count <100,000/μL), while severe thrombocytopenia (<50,000/μL) was observed in 15 patients (32%). The characteristics of the patients together with proportion of the infecting serotypes, diagnosis, infection status, severity, and hematological data are shown in Table 1.

DENV genotypes and phylogenetic relationships

Full-length E gene sequences of 16 isolates, comprising all four serotypes, were successfully obtained. The E gene sequences have been deposited into GenBank repository and granted accession number of KY709181–KY709196. Three out of 11 DENV-1 isolates were genotyped according to Goncalvez classification [19] and identified as Genotype I (Fig. 2). Two of them were grouped together in a lineage which include Indonesian strains imported to Taiwan and Okinawa–Japan in 2014 [23,24] (Fig. 2). The other DENV-1 Purwokerto isolate was closely related to strain isolated in Tokyo–Japan in 2014. These isolates were grouped together in a lineage consisting of strains from Indonesia city of Surabaya isolated in 2010 (Fig. 2).

Among five DENV-2 isolates detected in this study, four were genotyped. All of these isolates belonged to Cosmopolitan genotype according to Twiddy classification [20] (Fig. 3). Although grouped into single Cosmopolitan genotype and collected within the same study time, these 4 isolates were further differentiated into three distinct lineages. One isolate was closely related with strains from Bali isolated in 2011–2012 and grouped together with strains

Table 1
Distribution of demographic and clinical parameters for RT-PCR confirmed dengue patients in Purwokerto, Indonesia, 2015.

Parameters	N	DENV-1 (N = 11)	DENV-2 (N = 5)	DENV-3 (N = 26)	DENV-4 (N = 5)	p Value[a]
Gender						
Male	24	5	2	15	2	0.781[a]
Female	23	6	3	11	3	
Infection type						
Primary	20	4	3	12	1	0.578[a]
Secondary	27	7	2	14	4	
NS1 antigen detection[d]						
Positive	19	4	3	12	2	0.864[a]
Negative	26	7	2	14	3	
Severity						
DF	19	5	3	11	0	0.227[a]
DHF	28	6	2	15	5	
Hematology data[b]						
Thrombocyte (/μL)	NA	88,182 ± 68,111	123,200 ± 73,916	75,209 ± 48,474	77,400 ± 48,624	0.376[c]
Hemoglobin (g/dL)	NA	12.99 ± 1.76	14.24 ± 2.07	13.84 ± 1.67	12.44 ± 1.38	0.197[c]

[a] Pearson Chi-squared test.
[b] Mean ± STDEV.
[c] One way ANOVA test.
[d] Two samples have no information of NS1 antigen detection.

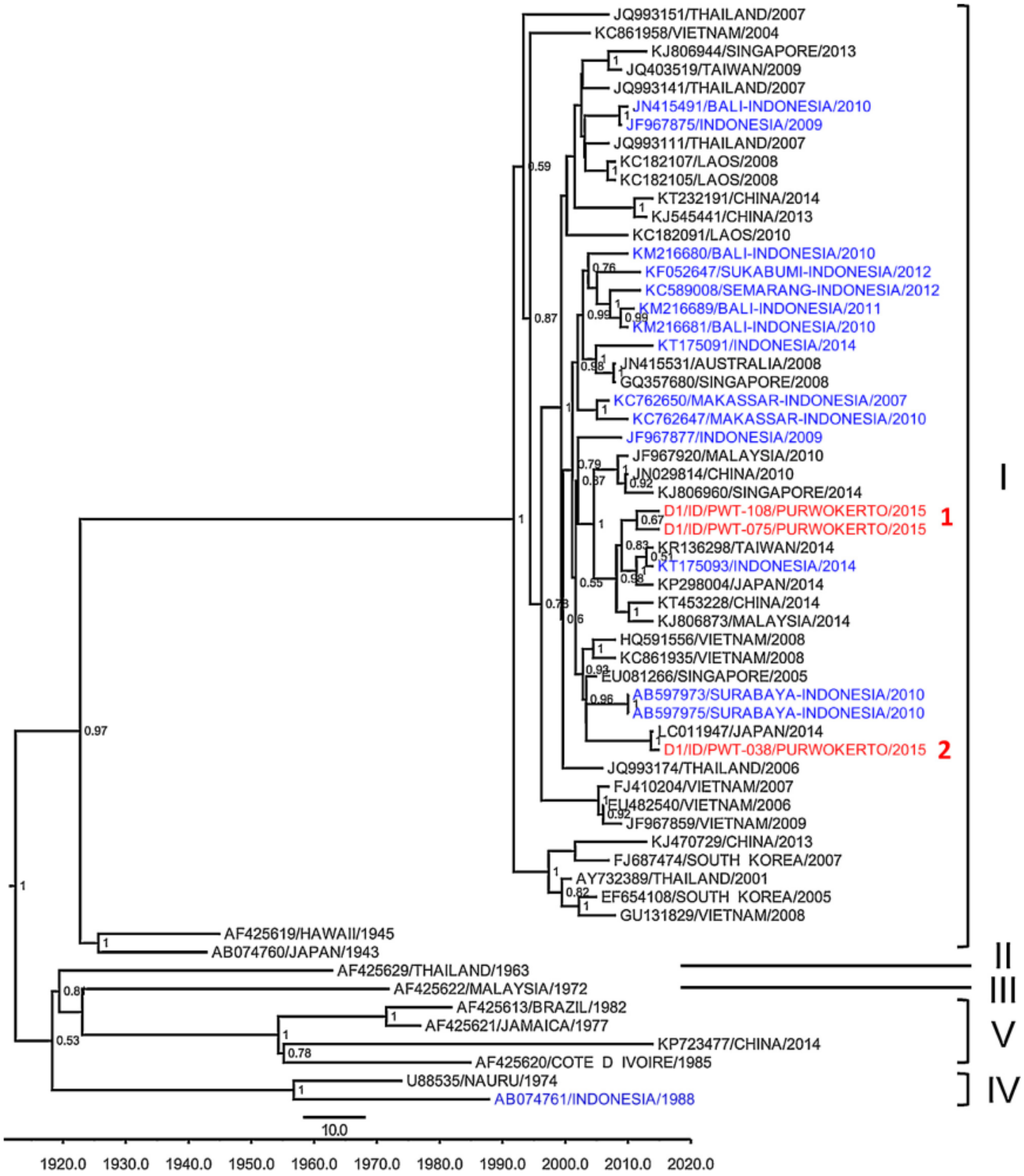

Fig. 2. Phylogeny of the most closely related DENV-1 genotypes generated by Bayesian inference method as implemented in BEAST using GTR + G + I evolution model calculated using E gene sequences. The red labels indicated the isolates from Purwokerto and the blue labels indicated other strains from Indonesia. Arabic numbers in red denote the lineages.

from Indonesian city of Makassar isolated in 2007–2008 [25] and Singapore isolated in 2004 (Fig. 3, lineage 1). Two isolates formed a monophyletic lineage consisting of mostly strains from Indonesian Sumatra in 2010 [26] and Jakarta in 2009 (unpublished), Singapore, Brunei and China (Fig. 3, lineage 2). The remaining isolate was grouped with other Indonesia strains from Sukabumi isolated in 2012 [27] and Jakarta in 2004 [28] and 2013 [29].

For DENV-3, seven out of 26 isolates were successfully sequenced and identified as Genotype I (Fig. 4) according to Lanciotti classification [21]. Similar with DENV-2, these isolates were also clustered into three different lineages. Two isolates were grouped together and closely related to Indonesia strain from Surabaya in 2013 [30]. Three isolates were grouped together in a lineage consisting of strains from Jakarta, Bali and other city in Indonesia isolated from travelers returning to Western Australia [31]. The remaining two isolates were grouped together and closely related to strain from Semarang, Central Java [14] and strains form

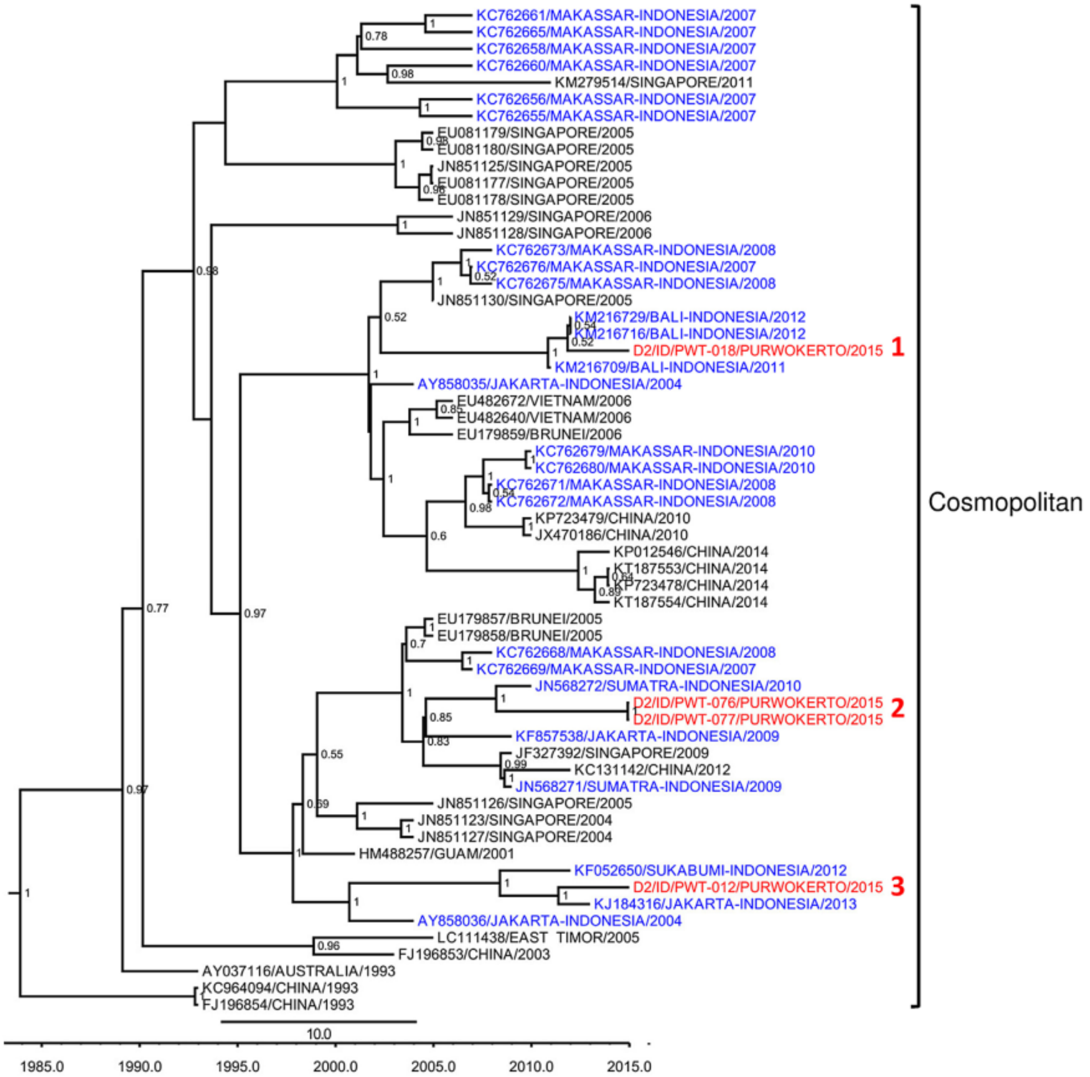

Fig. 3. Phylogeny of the most closely related DENV-2 Cosmopolitan genotype generated by Bayesian inference method as implemented in BEAST using GTR+G+I evolution model calculated using E gene sequences. The red labels indicated the isolates from Purwokerto and the blue labels indicated other strains from Indonesia. Arabic numbers in red denote the lineages.

another cities in Indonesia [26], as well as those from Malaysia [32] and China in 2010.

We genotyped two out of five DENV-4 isolates as Genotype II according to Lanciotti classification [22]. These isolates were grouped together with strains from Indonesia, Southeast Asia, and Micronesia (Fig. 5). One of them was closely related to the strain from Bali in 2010 [31], other city in Indonesia in 2009, and imported cases in Taiwan in 2009 [32]. The other isolate was grouped together with strain from Indonesia in 2010 [26].

Discussion

Similar with many other urban areas in Indonesia, Purwokerto is endemic for dengue and ravaged by the disease annually. While dengue is endemic, to the best of our knowledge, no dengue virological data in Purwokerto have been reported. Therefore, our study provides the first information on the serological profile, serotype distribution, virus genetic diversity and the origins of the DENV circulating in the area.

In our study population, most (76%) of patients were positive for dengue IgM/IgG antibodies while 54.2% of them were positive for NS1 antigen and/or RT-PCR detection. Moreover, about 60% of patients were secondary infection. In our previous study, we observed the seropositivity of samples from different cities/region in Indonesia ranged from 38.5% to 93.1%, with the overall percentage for Indonesia was 73.7% [33]. The seropositivity of 76% in Purwokerto city is still within the observed range and comparable to the seropositivity of Indonesia. Altogether, these reflect the considerable burden of dengue in the community. As urbanized area, Purwokerto has a greater risk of dengue infections compared to its surrounding area in Banyumas regency [13]. Urbanization becomes

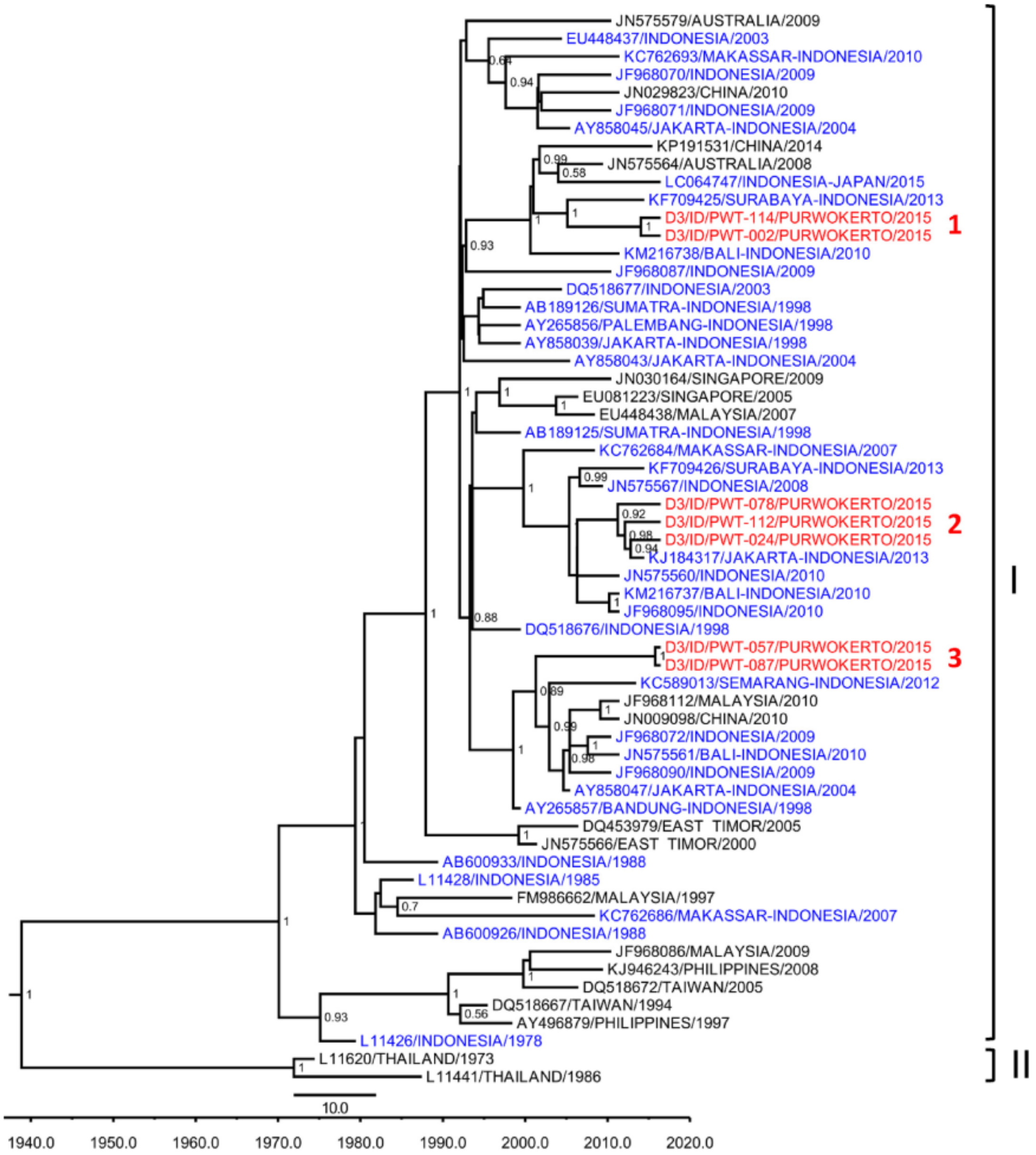

Fig. 4. Phylogeny of the most closely related DENV-3 genotypes generated by Bayesian inference method as implemented in BEAST using GTR + G + I evolution model calculated using E gene sequences. The red labels indicated the isolates from Purwokerto and the blue labels indicated other strains from Indonesia. Arabic numbers in red denote the lineages.

a prevalent factor for dengue infections as the city has become economically more developed since the establishment of universities in the city which increase the number of urban population, especially students, in the city.

In term of disease manifestation, we revealed the occurrence of both DF and DHF. We observed majority of dengue cases (60%) were DHF. This was similar to study in Semarang in 2012 in which 74% of the cases were DHF [14]. The relationship of infecting serotype with clinical manifestation has been reported [34,35]. We did not find possible relationship of infecting serotype with demography, hematology, and clinical manifestation (Table 1). We also did not find correlation of infecting serotype with thrombocytopenia as previously reported in Singapore [36]. Association between DENV-1 and DENV-3 with primary infection which has been reported in Thailand [35], however, this was not observed in Purwokerto. The possible relationship of clinical manifestation with demography and hematology were also analyzed (data not shown). Again, no significant correlation was found among them. We are aware that the limited sample size obtained in this study may affect the reliability of the data analysis, therefore, no confident conclusion can

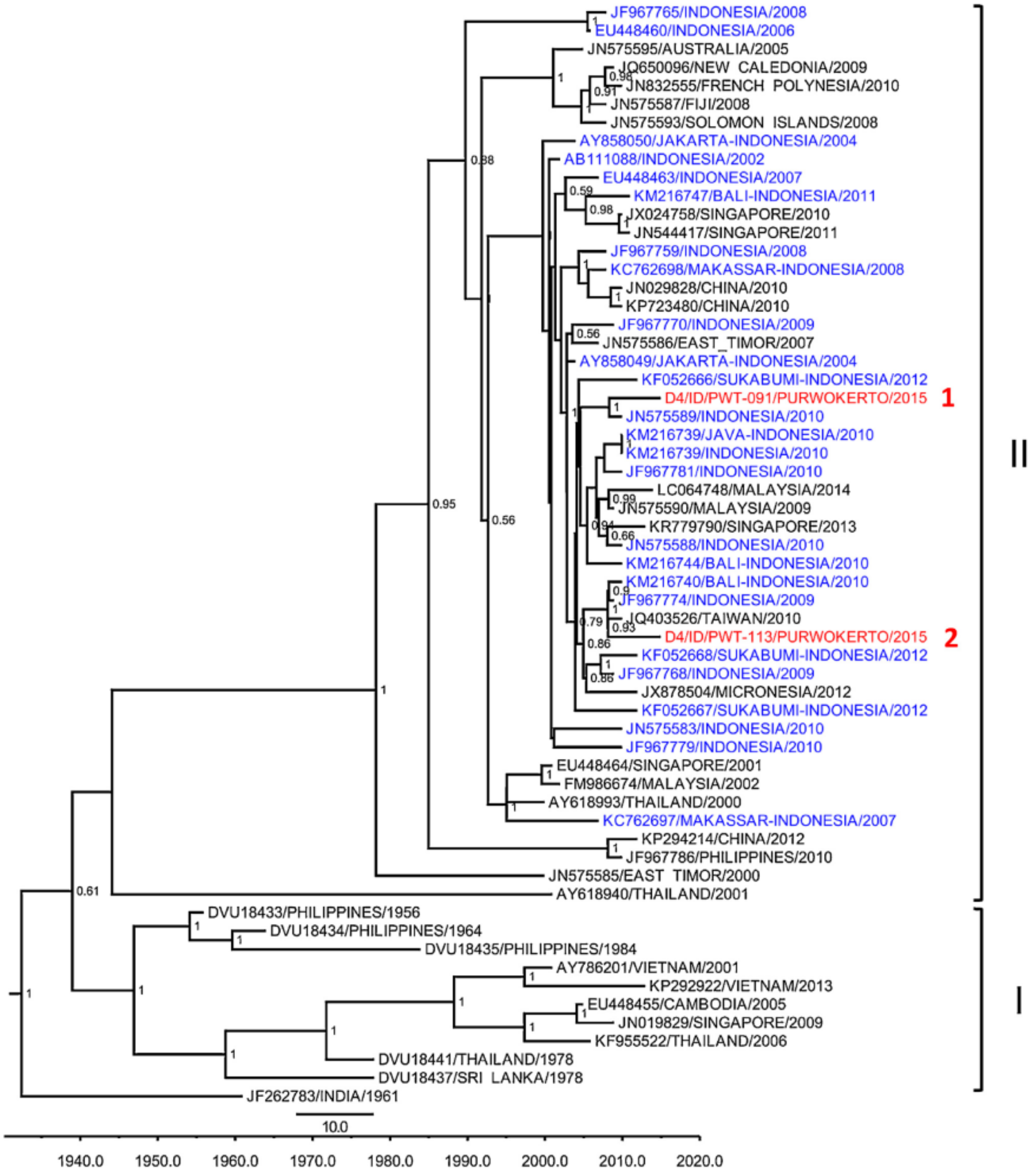

Fig. 5. Phylogeny of the most closely related DENV-4 genotypes generated by Bayesian inference method as implemented in BEAST using GTR + G + I evolution model calculated using E gene sequences. The red labels indicated the isolates from Purwokerto and the blue labels indicated other strains from Indonesia. Arabic numbers in red denote the lineages.

be drawn for the association of DENV serotypes with demography, clinical, and hematological data.

Our study was more focused on the virological aspects of DENV. To assess the dynamic of DENV in Purwokerto, we compared our data with data from nearby city of Semarang (located about 188 km from Purwokerto), because no historical virological data of dengue in Purwokerto are available. We observed distinct serotype predominance between the two cities. The majority of the DENV isolated in Semarang was DENV-1 [14] while the most predominant serotype in Purwokerto was DENV-3. The history of DENV-3 predominance was recorded in several cities in Indonesia including Jakarta [28], Palembang [37], and Bali in 2015 [38]. In another nearby city of Bandung (about 244 km from Purwokerto), DENV-4 was the most frequent serotype detected, followed by DENV-3 as the second most detected [39]. In other cities in Indonesia, different serotype predominance was also observed such as in Jakarta in 2010 [40], Surabaya in 2012 [41], and Jambi in 2015 [17]. Altogether, these findings reveal the differing DENV serotype predominance in different cities and thus demonstrate the spatial and temporal dynamics of DENV distribution in Indonesia.

Phylogenetic analysis was performed to determine the genotypes of DENV and the origin and relationships with other DENV strains from other regions in Indonesia and neighboring countries. Although Purwokerto DENV-1 viruses were grouped into single genotype, i.e. Genotype I, apparently, they were separated into two lineages, suggesting the genetic diversity of these strains. We did not find Genotype IV of DENV-1 which was previously found in other cities in Indonesia, such as in Sukabumi [27], Makassar [25], and Surabaya [41]. This finding enhances the notion of possible lineage replacement in DENV-1 in Indonesia from Genotype IV to Genotype I as this genotype was recorded to replace the older Genotype IV in recent studies in Jambi in 2015 [17], Sukabumi in 2013 [27], Semarang in 2012 [14], Makassar (2007–2010) [25], and Surabaya in 2009 [42].

The DENV-2 isolates from Purwokerto were identified as Cosmopolitan genotype. These isolates were grouped together with other Indonesia strains and those from Southeast Asia and China. Cosmopolitan genotype has been spread worldwide as it has been found in Southeast Asia, India, Middle East, Africa and Australia [20]. To date, this genotype is the common genotype circulating in Indonesia and has been found in Palembang [37], Jakarta [28], Surabaya [41,42], Semarang [14], Makassar [25], Sukabumi [27], Jambi [17], and Bali [38]. Based on the presence of various DENV-2 lineages in Purwokerto, it is plausible that the strains had undergone a local evolution, resulting in more genetically divergent strains as depicted by the presence of three separate lineages. One important finding about DENV-2 isolates in Purwokerto is the presence of isolate that is closely related with DENV-2 strains originated from Bali (Fig. 3, lineage 1) that were associated with the large outbreak in 2011–2012 [31]. The fact that this lineage is also present in Purwokerto may need to be continuously monitored for its potential to cause outbreak in the region as has been reported in Bali.

The DENV-3 isolates were classified as Genotype I. This genotype was the common genotype in Southeast Asia region [21]. Similar to DENV-2, the DENV-3 isolates also show a considerable genetic diversity as depicted by the presence of three distinct lineages. The Purwokerto DENV-3 isolates were closely related to other Indonesia strains especially those from Semarang [14], Jakarta [28], and Surabaya [42]. We did not observed the close-relatedness with DENV strains from outside Indonesia (except the imported cases in China), which suggest that these predominant viruses were of local origin and not imported from other countries.

For DENV-4 isolates, phylogenetic analysis revealed that the two isolates were grouped into Genotype II and formed two separate lineages. This genotype was frequently found in Southeast Asia and America [22]. The Purwokerto's DENV-4 isolates were closely related to virus sampled in Indonesia in 2009–2010, and the imported virus strain collected in Taiwan presumed to be originated from Indonesia in 2010 [32]. These phylogenetic data suggest the endemic nature of DENV-4 in Purwokerto.

In conclusion, we provide the first molecular and virological characteristics of DENV in Purwokerto, Banyumas regency, Central Java, Indonesia. Although the serotype predominance of DENV in Purwokerto was different from the nearby cities, the genotypes of the isolates were apparently similar to those commonly found in other cities in Indonesia. The phylogenetic and genotypic analyses suggested that an endemic cycle of transmission has been established in Purwokerto with all four DENV serotypes circulating. DENV isolates imported from other countries was not detected. High number of travel and urbanization might be the contributing factors to the distribution of serotype and genotypes. Purwokerto is one of the transit route connecting cities in Java Island, especially for land transport. Continuous DENV molecular and virological surveillance efforts will be useful to further understand the dynamic of dengue disease in this region and contributes to the disease prevention and management program.

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Competing interests

None declared.

Ethical approval

Ethical approval for the involvement of human participants was granted by the Research Ethics Commission of the Faculty of Medicine, Universitas Jenderal Soedirman, approval No. 065/KEPK/IV/2015.

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