GENETIC DIVERSITY OF *AEDES AEGYPTI* (DIPTERA: CULICIDAE) ISOLATED FROM FIVE CITIES IN NORTH COAST AREA OF CENTRAL JAVA, INDONESIA

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Abstract. The tropical mosquito *Aedes aegypti* is the major vector in the transmission of human arboviruses, including chikungunya, dengue and yellow fever viruses. All provinces in the Indonesian archipelago have reported incidences of dengue. In order to study the genetic diversity and the dispersal of *Ae. aegypti* in Central Java, genetic analyses based on mitochondria cytochrome oxidase 1 gene (mt*COI*) were performed on *Ae. aegypti* isolated in five cities along the national North Coast road of Central Java. Seventeen representative mt*COI* fragments from either larvae or adult mosquitoes were PCR-amplified, sequenced and analyzed for sequence polymorphism, haplotype and genetic differentiation. A phylogenetic tree was constructed using maximum likelihood method and general time reversible model. There were seven haplotypes and the presence of different haplotypes in the cities was indicative of the heterogeneity of *Ae. aegypti* in Central Java. Gene flow and genetic differentiation analyses revealed no differentiation among populations from the cities. The possible gene flow between populations may reflect an active dispersal of *Ae. aegypti* among the cities as a result of movement of traffic along the national North Coast road of Central Java.

Keywords: *Aedes aegypti*, haplotype, mitochondria *COI,* Central Java, Indonesia

INTRODUCTION

Aedes (Stegomyia) *aegypti* (L.) is the principal vector for urban yellow fever (YF) and dengue (DENV) viruses. Originating from Africa, this mosquito has expanded geographically throughout the tropical and subtropical regions of the world (Gubler, 2011). The species was introduced to Southeast Asia and the Pacific islands during World War II. The dramatic global geographic expansion of this species along with the increase in human population growth, urbanization and globalization have increased the risk for outbreaks of dengue epidemics.

Although there is available dengue vaccine, it is registered for widespread use only in a limited number of Southeast Asian countries (Wichmann *et al,* 2017) and thus, dengue prevention strategy

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still need to be combined with mosquito vector controls. Control measures should target dengue-competent mosquito populations to interrupt DENV transmission. Knowledge of the genetic structure and gene flow among *Ae. aegypti* populations provides useful clues to help track and even prevent movements of associated genetic traits such as vector competence (Urdaneta-Marquez and Failloux, 2011).

Mitochondrial (mt)DNA is commonly used for molecular evolution studies in insects and has proved to be particularly useful for detecting mosquito genetic divergence and dispersal history (Kambhampati and Rai, 1991; Kambhampati, 1995; Mousson *et al*, 2005). In particular, mitochondrial cytochrome oxidase 1 gene (mt*COI*) has been found useful for genetic studies of *Anopheles* and *Aedes* mosquitoes (Cook *et al*, 2005; Walton *et al*, 2000). Mt-*COI* has also been reported to be useful for DNA barcoding, which complements taxonomy-based identification of mosquito species (Chan *et al*, 2014).

Indonesia has been witnessing a dramatic spread of *Ae. aegypti* in the archipelago resulting in dengue disease hyperendemicity (Kraemer *et al*, 2015; Rašić *et al*, 2015). The four DENV serotypes were shown to be circulating in all provinces of the country (Setiati *et al*, 2006). The clinical, virology and demographical features of dengue in Semarang, Central Java, and its surrounding regions have previously been reported (Fahri *et al*, 2013). Here, we investigated the genetic diversity of *Ae. aegypti* isolated in five cities in coastal areas of Central Java. This should aid in tracking the spread of this dengue virus vector from this region into other areas of Java, which could be facilitated by vehicles using the national North Coast road leading to cities and provinces in Java Island.

MATERIALS AND METHODS

Study sites and sample collection

Mosquito specimens were collected as larvae or reared-adult mosquitoes from five regions/cities along the North Coast part of Central Java, namely, Brebes, Demak, Jepara, Kudus, and Semarang (Fig 1) during August-September 2013. Larvae were collected from artificial water-containers in households surveyed. Identification of *Ae. aegypti* isolates was performed by assessment of morphological features (Chan *et al*, 2014). Collected samples were frozen and stored at -20°C until used.

Mt*COI* amplification and sequencing

Individual larvae or adult mosquitoes representing each breeding container in each city were macerated using MagNA Lyser Green Beads (Roche, Mannheim, Germany) and 350 µl aliquot of lysis buffer in a MagNA Lyser homogenizer (Roche) at 6,000 rpm for 15 minutes. Following centrifugation (4,000*g* for 5 minutes), 200 µl aliquot of the homogenate was extracted using MagNA Pure LC DNA Isolation Kit I (Roche) in an automated MagNA Pure LC 2.0 extraction system. DNA in 50 µl of elution buffer was stored at -20°C until used. The 5′ segment of mt*COI* was PCR amplified as described by Beebe *et al* (2005). The forward primer (5′-TAGTTCCTTTAATATTAGGAGC -3′) was designed to lie 245 bp into the *COI* 5′ region and the reverse primer (5′-TAATATAGCATAAATTATTCC-3′) 813 bp into *COI*, generating a 590-bp *COI* fragment. PCR mixture (50 µl) contained 10X Expand Hi-fi PCR buffer (Roche), 25 mM $MgCl_2$ (Promega, Madison, WI), 10 mM dNTP Mix (Promega), Expand High Fidelity DNA polymerase (Roche), and 50-100 ng of DNA template. Thermocycling was conducted in a GeneAmp PCR

System 9700 instrument (Applied Biosystems, Foster City, CA) as follows: initial denaturation step at 95°C for 2 minutes; 40 cycles of 95°C for 30 seconds, 50°C for 1 minute; and 72°C for 1.5 minutes; with final step at 72°C for 10 minutes and storage at 4°C. Amplicons were separated by 2% agarose gel-electrophoresis, visualized by staining with ethidium bromide (EtBr), purified from gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced in both directions using BigDye Dideoxy Terminator sequencing kits v3.1 (Applied Biosystems) in a 3130xl Genetic Analyzer (Applied Biosystems) at the Eijkman Institute, Jakarta. Sequences were assembled using SeqScape v.2.5 software (Applied Biosystems) with additional manual adjustments when necessary and deposited to GenBank (Table 1).

MtCOI haplotyping and population genetic structure and gene flow analysis

The mt*COI* sequences were identified as *Ae. aegypti* using BOLD Systems origin querying database (Ratnasingham and Hebert, 2007) and then analyzed for polymorphisms and haplotypes. Sequences were grouped according to their collection locations (except for the single sample from Demak) and analyzed for their population genetic structure differentiation and gene flow. Sequence polymorphism analysis, haplotyping, gene flow, and genetic differentiation among populations were performed using DnaSP software v.5.10 (Rozas and Rozas, 1995; Librado and Rozas, 2009). The Fixation index measured by F-statistics (F_{ST}) values were calculated to estimate genetic differentiation between populations. Population differentiation based on differences in allele frequencies were estimated using Nei's G_{ST} values (Nei, 1973). Significant F_{ST} values were analyzed based on the null hypothesis of no differentiation between populations. The relationship among haplotypes was computed as a distance map based on pairwise difference to generate a minimum spanning tree (MST) and network (MSN) among haplotypes. Distance map data were prepared using Arlequin v.3.5 software (Excoffier and Lischer, 2010). MST and MSN were generated using HapStar v.0.7 (Teacher and Griffiths, 2011).

Mt*COI* phylogenetic analysis

Multiple sequence alignment was performed using MUSCLE (Edgar, 2004) to generate sequence alignments of the mt*COI* fragments with reference sequences downloaded from GenBank. Further editing, trimming and alignment generated sequence alignment lengths of 503 nt. Phylogenetic relationships among specimens were determined using the maximum-likelihood (ML) method employing MEGA5 (Tamura *et al,* 2011). A test run was performed to identify the best-fit model using jModelTest v.2.1.4 software (Posada, 2009). Trees were inferred using general time reversible (GTR) model with Gamma distributed (4 discrete categories) rates (GTR+G). Branch supports were estimated by bootstrapping with 1,000 replicates.

RESULTS

Seventeen mt*COI* gene sequences obtained from larvae/mosquitoes collected from five cities in the north coastal region of Central Java Province could be divided into seven different haplotypes (Table 1) with 18 sites of nucleotide polymorphisms (Table 2). Two sequences of *Ae. albopictus* samples from Jakarta were used as outgroups. Haplotype H1 was the most predominant haplotype consisting of eight samples from Brebes, Demak, Jepara, Kudus, and Semarang, followed

Table 1
Sample characteristics and mitochondria cytochrome oxidase I gene haplotypes of *Aedes aegypti* isolated from five cities in Central Java, Indonesia.

No.	Sample ID	City	Morphology	GenBank accession No.	Haplotype
1	ID/BRB/L001	Brebes	Larvae	KP334259	H1
2	ID/BRB/L002	Brebes	Larvae	KP334260	H2
3	ID/BRB/L003	Brebes	Larvae	KP334261	H3
4	ID/BRB/L004	Brebes	Larvae	KP334262	H1
5	ID/DMK/L001	Demak	Larvae	KP334263	H1
6	ID/JPR/L004	Jepara	Larvae	KP334265	H3
7	ID/JPR/A411C4	Jepara	Adult	KP869124	H1
8	ID/JPR/A421C7	Jepara	Adult	KP869125	H1
9	ID/JPR/A431C4	Jepara	Adult	KP869126	H1
10	ID/KDS/L001	Kudus	Larvae	KP334264	H4
11	ID/KDS/A311C1	Kudus	Adult	KP869121	H5
12	ID/KDS/A321C1	Kudus	Adult	KP869122	H6
13	ID/KDS/A331C1	Kudus	Adult	KP869123	H1
14	ID/SMG/A001	Semarang	Adult	KP334266	H1
15	ID/SMG/A003	Semarang	Adult	KP334267	H7
16	ID/SMG/A004	Semarang	Adult	KP334268	H3
17	ID/SMG/A009	Semarang	Adult	KP334269	H3
18	ID/JKT/A001*	Jakarta	Adult	KP334274	*Ae. albopictus*
19	ID/JKT/A002*	Jakarta	Adult	KP334275	*Ae. albopictus*

*Used as outgroups.

by haplotype H3, with four isolates from Brebes, Jepara, and Semarang (Fig 1).

Overall haplotype diversity (Hd) for the 17 samples was 0.750 with nucleotide diversity (per site) (π) of 0.00526 and average number of nucleotide differences (κ) of 2.647. Analysis of Hd and π in each city showed the presence of two or more haplotypes, with Kudus having the highest Hd (1.0), κ (6.167), number of segregating sites (S = 12), and π (0.01226) (Table 3). The π value of each city population was relatively low. Tajima's D and Fu and Li's D and F neutrality tests showed low values that are not statistically significant, as is the case with F-statistics (F_S) values (Table 3). Analysis based on F_{ST} values shows no significant differentiation among mosquito populations. Similar profile was observed for G_{ST} values (Table 4).

Haplotype network analysis based on pairwise difference generated an MST and MSN where each mutational step is represented, where the distance matrix among haplotypes showed one or more mutational step(s) for a single common lineage of haplotype H1 (Fig 2). The highest number of mutational steps ($n = 8$) was observed between haplotype H1 and H5.

Maximum likelihood phylogenetic tree of *Ae. aegypti* mtCOI 503 nt fragments from the five cities along the north coast of Central Java Province revealed clustering of the samples into a single clade with low

Table 2
Mitochondria cytochrome oxidase I gene polymorphisms of *Aedes aegypti* haplotypes isolated from five cities in Central Java, Indonesia.

Haplotype	*N*	Nucleotide position																		
		34	45	129	132	133	170	178	185	219	228	306	345	357	363	387	393	399	438
Haplotype 1 (H1)	8	G	G	T	T	C	A	A	T	T	T	G	T	A	T	C	C	A	A
Haplotype 2 (H2)	1	.	.	C	C	A	T	C	.	.	.	.	.	.	.	.	.	.	.
Haplotype 3 (H3)	4	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Haplotype 4 (H4)	1	T	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	.	.
Haplotype 5 (H5)	1	.	.	.	.	.	.	.	.	.	C	A	C	G	C	T	.	G	G
Haplotype 6 (H6)	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.
Haplotype 7 (H7)	1	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.

N, number of haplotype member.

Table 3
Haplotype and nucleotide diversity values and number of segregating sites of *Aedes aegypti* isolated from four cities in Central Java, Indonesia.

City	*N*	H	Hd	κ	π	S	D	D*	F*
Brebes	4	3	0.833	3.000	0.00596	6	-0.80861	-0.80861	0.731
Jepara	4	2	0.500	0.500	0.00099	1	-0.61237	-0.61237	0.172
Kudus	4	4	1.000	6.167	0.01226	12	-0.58365	-0.58365	-0.253
Semarang	4	3	0.833	1.667	0.00331	3	0.16766	0.16766	-0.133

N, sample size; H, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; κ, average number of differences; S, number of segregating sites; D, Tajima's statistics; D*, Fu and Li's D* statistics; F*, Fu and Li's F* statistics.

Table 4
Genetic differentiation between populations of *Aedes aegypti* isolated from four cities in Central Java, Indonesia estimated using pairwise genetic distance values.

	Brebes	Jepara	Kudus	Semarang
Brebes	-	-0.08475	-0.02326	-0.05263
Jepara	-0.07692	-	0.04000	0.01538
Kudus	0.00901	0.01235	-	0.01124
Semarang	-0.03704	-0.08333	-0.01075	-

Numbers indicate fixation index values measured as F_{ST} (below diagonal) and G_{ST} (above diagonal).

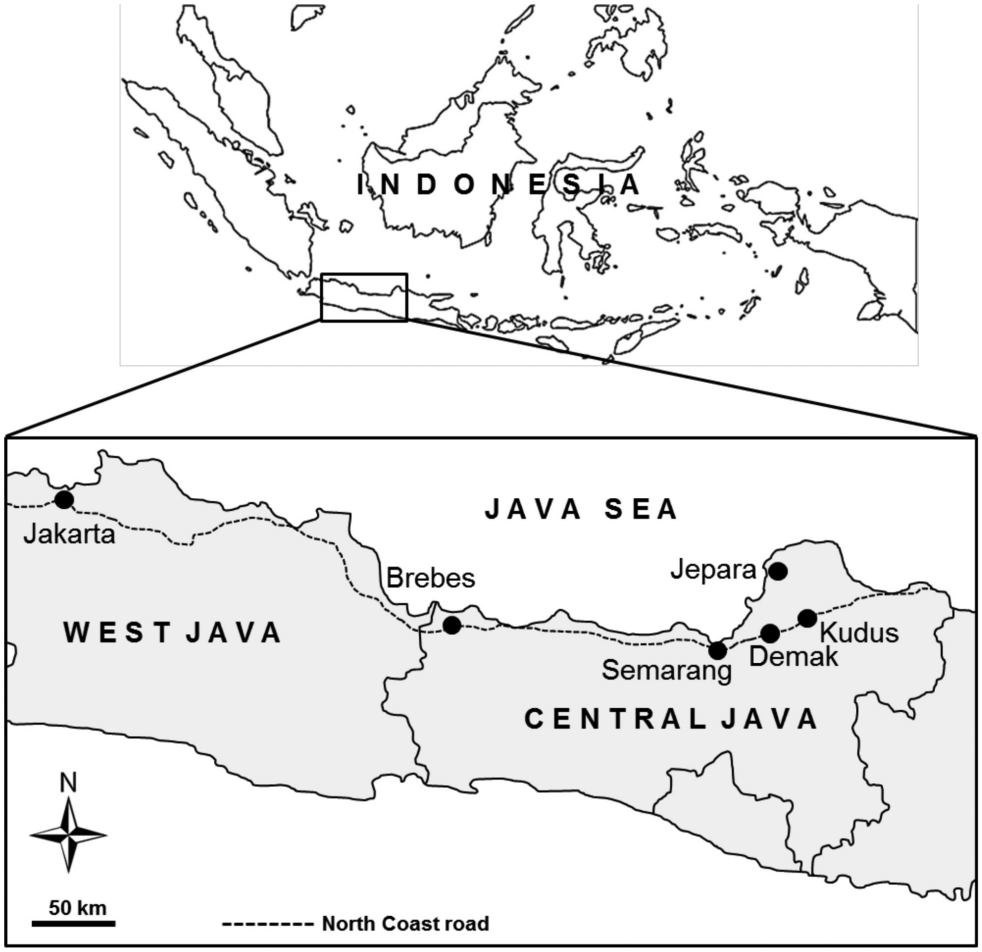

Fig 1–Sample collection sites in five cities along the North Coast of Central Java, Indonesia.

bootstrap support (Fig 3). Grouping of the samples into haplotypes could also be observed, with all haplotypes clustered with samples from other countries in Southeast Asia, Africa, Europe (Portugal), and Latin America.

DISCUSSION

Indonesia is one of the tropical countries that is experiencing an increasing incidence of vector-borne infectious diseases, such as dengue and Chikungunya, transmitted by *Aedes* mosquitoes (Yoshikawa and Kusriastuti, 2013). All provinces in the Indonesian archipelago have reported incidences of dengue, including the Central Java Province. Central Java is served by the North Coast Road, which is main route of land transportation and trade across Java Island. This national road is 1,430 km in length, connecting Jakarta, West Java, Central Java, and East Java provinces. Many cities in Central Java Province located along the North Coast Road and the high volume of movements

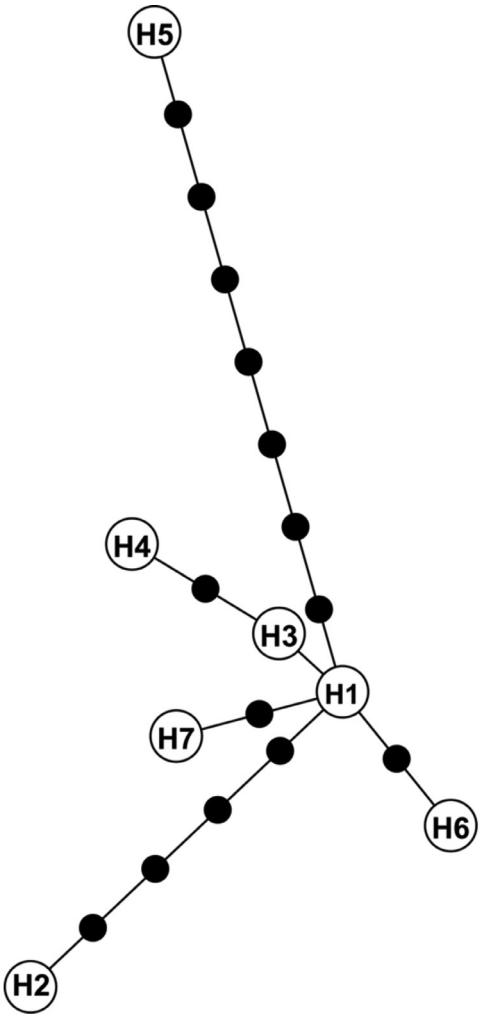

Fig 2–Minimum spanning tree (MST) and network (MSN) of seven *Aedes aegypti* mitochondria cytochrome oxidase I gene haplotypes from five cities in North Coast of Central Java, Indonesia. MST and MSN were generated using HapStar v.0.7(Teacher and Griffiths, 2011). Black dots along the lines indicate the numbers of mutations between two haplotypes.

of humans and goods along the road may aid in the dispersal of *Ae. aegypti* among cities in the region.

Different genetic strains of *Aedes* can have different colonizing ability and vector competency in transmitting dengue virus (Bennett *et al,* 2002; Ocampo and Wesson, 2004). As mt*COI* has been proven to be a practical tool to study the genetic diversity and spread of *Ae. aegypti* (Beebe *et al,* 2005), examination of a fragment of this gene was employed in the study of genetic diversity of *Ae. aegypti* mosquitoes collected from five cities located in Central Java along the North Coast Road. The most frequent haplotype, H1, was well dispersed geographically among the five cities and served as the single common lineage of *Ae. aegypti* along the north coast of Central Java. However, significant nucleotide differences in certain haplotypes, *eg*, haplotypes H2 and H5 with 5 and 8 mutations, respectively, might reflect recent introduction of these haplotypes into the region. Cities in the north coast of Central Java are mainly cities with ports, *eg*, Semarang city that has a large international port. One unique haplotype, H5, showed a close relationship with a sample from Vietnam. The diverse genotypes of *Ae. aegypti* was shown also by the presence of multiple haplotypes in each city. Of particular interest is the genetic diversity of *Ae. aegypti* in Kudus city that manifested the highest haplotype diversity (Hd) value compared to other four cites, with the presence of four different haplotypes, *ie*, common haplotype H1 and three other unique haplotypes (H4, H5, and H6).

The estimated nucleotide diversity within a city population was low and there was an absence of genetic differentiation between city populations. The

Fig 3–Maximum likelihood tree and haplotype grouping based on mitochondria cytochrome oxidase 1 gene of *Aedes aegypti* isolated from five cities in Central Java, Indonesia. Seventeen samples (red) from the study were analyzed together with reference sequences downloaded from GenBank. Two *Ae. albopictus* isolates (blue) collected from Jakarta were used as outgroups. Bootstrap values of more than 50% are shown. Bar indicates rate of nucleotide substitutions per site. H, haplotype.

relatively low F_{ST} values in all five cities might indicate extensive gene flow and migration between mosquito populations among these cities although they are located many kilometers apart. The fact that nucleotide diversity was low in contrast to haplotype diversity may be explained by rapid population growth from ancestral populations (Dantur Juri *et al*, 2014). Taken together, these data showed that *Ae. aegypti* dispersal may be aided by movements between cities in Central Java by means of the North Coast road.

The weakness of this study lies in the low number of samples, which might contribute to possible misinterpretation of data leading to ambiguous conclusions. However, the *Ae. aegypti* mt*COI* sequences will be of benefit to the genetic data of *Ae. aegypti* from Indonesia, as to the best of our knowledge, there is only a few mt*COI* sequences available in the public database. Information on the genetic makeup of *Ae. aegypti* will be useful in tracing the introduction of disease-spreading mosquitoes in a particular area, as in the case of *Ae. aegypti* and *Ae. albopictus* incursions into Australia (Beebe *et al*, 2005; Beebe *et al*, 2013).

In summary, the partial sequences of *Ae. aegypti* mt*COI* isolated from five cities in Central Java has enabled identification of the considerable genetic diversity of this mosquito species in this region of Indonesia. The haplotypes distribution within the studied populations and genetic differentiation between populations depict a dispersal of *Ae. aegypti* mosquitoes between cities, possibly sided by the coast road linking these cities. Further extensive sampling and analysis will be required to verify and add further information on *Ae. aegypti* genetic diversity in Central Java and other provinces in Indonesia.

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