Genetic differentiation of dengue vector *Aedes aegypti* in the small geographical scale of Banyumas District, Indonesia based on Cytochrome Oxidase I

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Abstract. *Mohammed MA, Nuryanto A, Kusmintarsih ES. 2021. Genetic differentiation of dengue vector Aedes aegypti in the small geographical scale of Banyumas District, Indonesia based on Cytochrome Oxidase I. Biodiversitas 22: 675-683.* Aedes aegypti (Linnaeus, 1762) is a major vector of arboviruses. Currently, *Ae. aegypti* is spreading throughout the Banyumas District, Central Java, Indonesia and there is little information regarding the genetic variation of this species, yet the information is essential to develop effective vector control measures. The aims of this study was to evaluate the genetic differentiation of *Ae. aegypti* populations in South Purwokerto and Cilongok subdistricts. Mosquito larvae were collected using oviposition traps inside houses in South Purwokerto and Cilongok (Jastaba village). Twenty larvae of *Ae. aegypti*, ten from each location were identified and used for genetic analysis. Analysis of mitochondrial cytochrome Oxidase I (mtCOI) produced around 600 bp fragments, detecting ten haplotypes. The haplotype diversity and nucleotide diversity indices were higher in South Purwokerto samples. Tajima and Fu's statistics indicated that the sampled populations were in genetic equilibrium. Indices of demographic history were fit for a spatial expansion model. Higher genetic variation was observed within the population compared to between the populations. Furthermore, strong genetic difference was detected between the two populations, with highly significant F_S (p < 0.0001). Weak haplotype sharing occurred between the two populations assuming that the gene flow was facilitated by human transportation. All haplotypes were a cluster of single clades and closely related to haplotypes generated from previous studies in Central Java.

Keywords: *Aedes aegypti*, Banyumas, cytochrome oxidase I, genetic diversity

INTRODUCTION

Aedes aegypti (Linnaeus,1762) is the primary vector responsible for transmitting the dengue virus and other arboviruses (Black et al. 2002; Brady et al. 2012). According to World Health of Organization (2019), around 3.9 billion people in 128 countries are at risk of dengue infection with approximately 390 million per year. Indonesia is one of countries that has been experiencing an incidence of arbovirus diseases (Yoshikawa and Kusriastuti 2013). Currently, dengue virus is being spread in almost all Indonesian provinces. For example, Götz et al. (2016) and Wijayanti et al. (2016) described dengue disease incidence in the Java region. A molecular epidemiological survey in Banyumas District has revealed multiple dengue serotypes (Kusmintarsih et al. 2018). Banyumas District is one of the endemic areas of dengue hemorrhagic fever (DHF) in Central Java. According to Banyumas District Health Office (2020), around 16 villages in 2018 and up 50 villages in 2019 were affected by dengue disease.

Elimination of breeding sites is most effective way for control of *Ae. aegypti* vector (Nathan 1993), in addition to the traditional methods of fogging and using house insecticide, to prevent dengue virus (Vontas et al. 2012). Although insecticide fogging may reduce the density of adult mosquito population, it has little effect on the larval breeding sites. Insecticide treatment could reduce mosquito population size, speed up genetic bottleneck and accelerate genetic drift (Twerdochlib et al. 2012). The long-term use of insecticide has caused resistance against a variety of insecticides (Moyes et al. 2017).

Several molecular markers, such as Cytochrome Oxidase I and Restriction Fragment Length Polymorphism (RFLP) have been used to study genetic diversity among *Ae. aegypti* population (Scarpassa et al. 2008; Sharma et al. 2013) revealing that different factors such as environmental, urbanization, and human activities impact the genetic structure of vector species (Huber et al. 2002). Studies of genetic variations in *Ae. aegypti* population needs DNA markers that display a fast-evolving gene, so that the evolution and relationship among the vector species can be detected (Beebe et al. 2005). Mitochondrial DNA is more powerful than nuclear DNA for population genetic studies, so far, it encodes a gene with a high rate of evolution and rarely undergoes recombination (Avise et al. 1987; Beebe et al. 2005). In particular, mitochondrial Cytochrome oxidase I (mtCOI), is a powerful tool for assessing *Ae. aegypti* genetic studies (Anoopkumar et al. 2019). Cytochrome oxidase I has been used in population genetic studies of *Ae. aegypti* in different geographic regions (da Silva et al. 2012; Paupy et al. 2012; Seixas et al. 2013; Gandhi et al. 2020). In Banyumas District,

information about the genetic variation of *Ae. aegypti* is not yet available, so studies on genetic diversity and gene flow patterns are essential for dengue vector control. For instance, gene flow information is useful for identifying the level of insecticide-resistant gene dispersal (Rasheed et al. 2013).

Although no information about the population structure of *Ae. aegypti* is available in Banyumas District, Central Java; one exception is the study reported by Yohan et al. (2018), which analyzed small samples of *Ae. aegypti* from the north coast of Central Java and concluded that the species exhibited considerable genetic diversity. This preliminary survey was aimed to assess the level of genetic differentiation and gene flow of *Ae. aegypti* population in the small geographic scale of South Purwokerto and Cilongok in Banyumas District by analyzing mitochondrial Cytochrome oxidase I.

MATERIALS AND METHODS

Description of the study area

Banyumas District (7.4832 S, 109.1404 E) is located in the southwest of Central Java Province, Indonesia (Figure 1), with a total area of 132,760 km², and 1.85 million people inhabit the region. Banyumas District consists of 27 sub-districts with a total of 331 villages.

A tropical monsoon climate distinguishes the climate in Banyumas. Purwokerto, the capital city of Banyumas lays 55 m above sea level and covers around 38.58 km². Cilongok is the largest sub-district in Banyumas District, lays 209 m asl. and covers an area of 105,34 km² (Central Bureau of Statistics 2017). According to the Indonesian Meteorological, Climatological, and Geographical Agency (2020), the average temperature in South Purwokerto is approximately 26.9°C, and about 2284 mm of precipitation falls annually. While in Cilongok, an average annual rainfall of 3669 mm and an average annual temperature of 25.8°C.

Research samples

Mosquito larvae were collected using oviposition traps (ovitraps) from houses in South Purwokerto and Cilongok (Jastaba Village) in the period from April to August 2019. Ovitraps are small pots filled with tap water to allow the female *Aedes* species mosquitoes to lay their eggs (Fay and Perry 1965). Along with study, 25 ovitraps were placed inside three houses in Cilongok and four houses in South Purwokerto for two to three weeks. Ovitraps were transferred to laboratory, and larvae reared at room temperature to third instar. *Ae. aegypti* larvae were identified based on a described morphological key (Stojanovich 1965). Around 20 larvae of *Ae. aegypti* were clearly identified and used in molecular analysis Table 1.
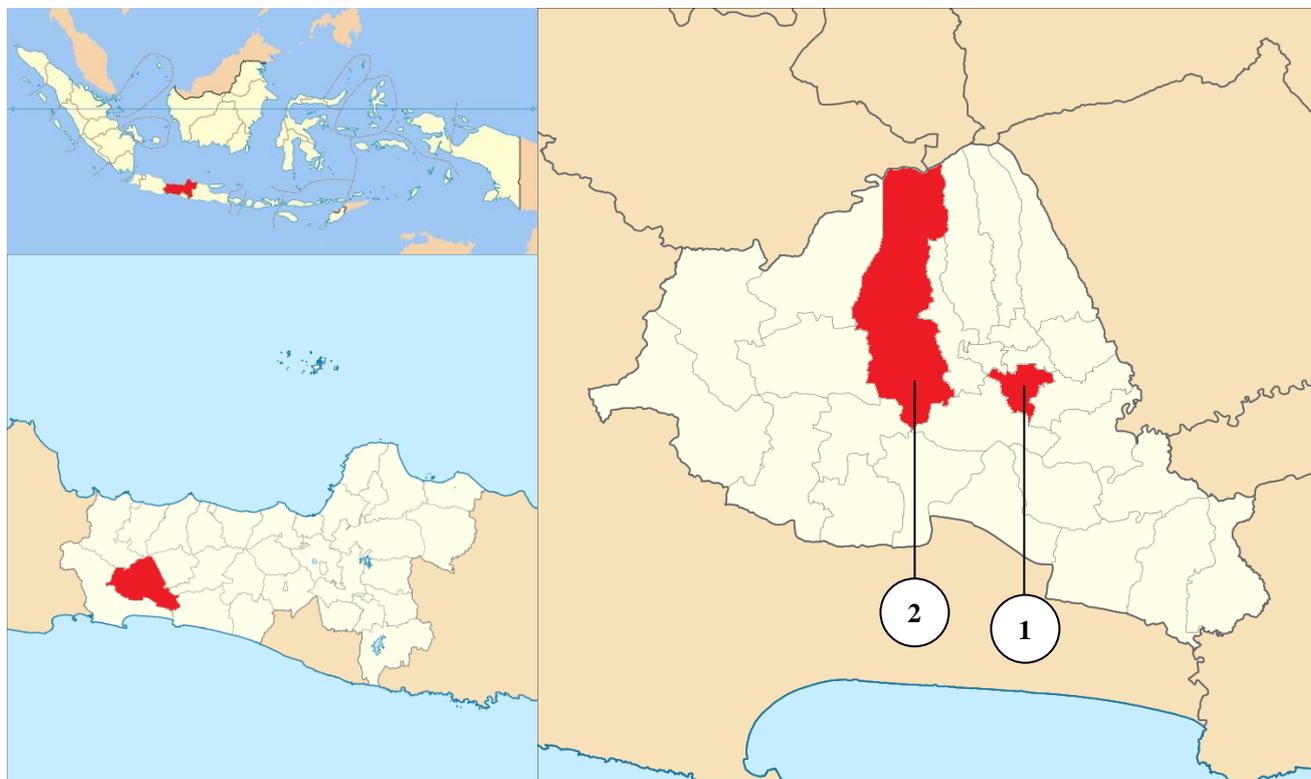

Figure 1. Map showing sampling sites in Banyumas District, Central Java, Indonesia. 1. South Purwokerto and 2. Cilongok

DNA extraction, COI amplification and sequencing

Individual larvae were placed in a 1.5 ml tube, and DNA was extracted using genomic DNA extraction mini kit-tissue (Geneaid) as prescribed by the manufacturer instructions. The COI gene was amplified using the forward primer 5-TAGTTCCTTTAATATTAGGAGC-3 and the reverse primer 5-TAATATAGCATAAATTAT TCC-3) generating around 600 bp fragment (Beebe et al. 2005). PCR amplification was performed by a thermal cycler (Primus 25® advanced-PEQLAB) using Go-Taq Green Master Mix (Promega) mixture contained with 50 μl of (Go-Taq Polymerase, dNTPs, MgCl$_2$), 0.4 uM of each primer and 50-100ng of DNA template. The cycling consisted of 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 a final step at 72°C for 10 minutes and storage at 4°C. The fragments were separated by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide (B*orst 2005).* A 40 uL of PCR unpurified product from each sample was sent to 1st base DNA sequencing service.

The COI haplotypes and genetic differentiation analysis

Twenty nucleotide sequences were assembled using Bio-Edit, and multiple sequence alignment was done with ClustalW using MEGA X (Kumar et al. 2018). The sequences' similarity for fragments generated in this study was compared in the GenBank database using a Blastn search. Accession numbers were obtained after all sequences generated in this study were submitted to GenBank (Table 1).

The nucleotide sequence for both populations was analyzed by DNAsp.v6 software (Rozas et al. 2017). The basic sequences' statistics measured were haplotype diversity Hd, nucleotide diversity π, number of polymorphic sites *S,* and the average number of nucleotide differences *k*. The Tajima neutrality test *(D)* (Tajima 1989) and the Fu test (Fu 1997), which give information on selective neutrality in populations, were computed in DNAsp.v6. The examination of deviation from neutrality by both statistics was based on 1000 coalescent simulations.

The hypothesis of the expansion model was measured based on pairwise nucleotide differences between haplotypes were compared to the expected frequency distribution to test the effect of population growth or mismatch distribution (Roger and Harpending 1992; Harpending 1994). Estimations of raggedness index R and sum square deviation (SSD) were computed in Arlequin ver.3.5 (Excoffier and Lischer 2010) with 1000 replicates of parametric bootstrapping to estimate deviation from the expansion model. Other parameters measured were the time of expansion (τ), which was estimated with 95% confidence intervals (CI), the mutation parameters, (theta initial θ_0 and theta final θ_1), and the number of migrants *M* (Hamilton et al. 2003; Excoffier 2004).

Genetic differentiation analysis was estimated based on the Fixation Index (F_{ST}) and hierarchical analysis to estimate genetic differentiation within and among the population. This is based on Analysis of Molecular Variance AMOVA (Excoffier et al. 1992) using Arlequin (Excoffier et al. 2010). Median-Joining haplotype network was constructed using a network software (2004-2019 Fluxus Technology Ltd) to evaluate the relationships between haplotypes. Partial sequences (forward) obtained in this study were used to build phylogenetic tree using neighbor-joining (NJ) method in Mega X software. Trees were inferred with the Tamura 3-parameters model by 1000 replicates bootstrapping. To estimate level of phylogenetic relationship with *Ae.aegypti* from other regions, partial cytochrome oxidase I sequence from Indonesia (KP334267.1, KP334266.1, KP334268.1 and KP334269.1), Pacific region (KT313650), Panama (KX171394), and *Ae. albopictus* (GenBank: KP334275.1) as outgroup were retrieved from GenBank and used to construct a phylogenetic tree.

Table 1. Sampling information and accession number of each *Ae. aegypti* sample

Sample code	Sampling site	Coordinates	No. of Larvae	GenBank accession No.
PWT10	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881571
PWT8	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881572
PWT7	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881573
PWT6	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881574
PWT5	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881575
PWT4	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881576
PWT3	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881577
PWT2	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881578
PWT1	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881579
PWT1	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881580
CGK10	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881581
CGK9	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881582
CGK8	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881583
CGK7	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881584
CGK6	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881585
CGK5	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881586
CGK4	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881587
CGK3	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881588
CGK2	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881589
CGK1	South Purwokerto, Banyumas District	7.4243 S 109.2396 E	1	MT881590

RESULTS AND DISCUSSION

Haplotype distribution

A total of 20 sequences were generated, 10 from each population (South Purwokerto and Cilongok). The length of the sequences after alignment was around 600 bp. A total of 10 haplotypes and 9 polymorphic sites were identified (Table 2). The haplotypes were distinguished by 7 transitions A⇔G or G⇔A at positions (9, 147, 159, 303, 309, 593 and 594), single C⇔T transition at position 45, and 1 single T⇔A transversion at position 525. The Haplotype H1 and H3 were found in both populations, while the H2, H4, H5, H6, and H 7 were unique to South Purwokerto samples, and H 8, and H10 were exclusive to the population of Cilongok (Table 3).

Polymorphism indices

The overall number of haplotypes (h) was 10, haplotype diversity (Hd) was 0.921, the average number of nucleotide differences (k) was 2.552 and nucleotide diversity (π) was 0.004. South Purwokerto samples had higher values, which are as follows: h =7, Hd = 0.933, S = 8, K = 2.933 and π = 0.005 (Table 4).

Neutrality test

Tajima D values for South Purwokerto and Cilongok were (0.084) and (0.093), respectively with $p > 0.05$, while Fu values were (0.506) and (1.340), respectively with $p > 0.02$ (Table 4). Positive non-significant values indicating the observed polymorphism were consistent with the neutral mutation model.

Mismatch distribution

According to the significant values of Raggedness index R and SSD for South Purwokerto and Cilongok samples (Table 5), demographic expansion would be rejected. In contrast, the values of R Index and SSD were non-significant and good fit for the spatial expansion model (SSD = 0.046, p = 0.310 and R= 0.162, p = 0.170) for South Purwokerto and (SDD = 0.063, p =0.0330 and R = 0.211, p = 0.340) for Cilongok. Longer expansion time and migration rate were observed in the South Purwokerto samples τ (95% C.I) = 11.813 and M (95%.C.I) = 152.881) implying long distant spatial expansion for older lineage. The Cilongok samples had shorter expansion time and smaller migration rate τ (95% C.I) = 7.527 and M (95% C.I) = 49.910).

AMOVA analysis

AMOVA results showed greater variation (82.15%) within the population and lower variation (17.85%) among population ($p < 0.02$) (Table 6). The population pairwise F_{ST} value was 0.178 ($p < 0.0001$), indicating that a strong genetic structuring occurred in the sampled population of *Ae. aegypti*.

Haplotype network

The Median-Joining haplotype network showed that the haplotypes were interconnected with more than one nucleotide substitution. Haplotypes represented in red color were generated from South Purwokerto, and the ones in yellow color are for Cilongok haplotypes (Figure 2). H1 and H3 were shared between both populations. The highest mutation number was eight, and it was found between H1 and H10, and there were 4 steps between H4 and H10. South Purwokerto haplotypes were separated by a higher number of mutations, while Cilongok haplotypes were closely related and separated by fewer number of mutations.

Phylogenetic analysis

Neighbor-Joining (NJ) phylogenetic tree indicated that the haplotypes were clustered into a single clade supported by bootstrapping values 65% (H3, H8, H9, and H10), 58% (H1, H2, H5, H6, H7, and H9), and 56% (H4) (Figure 3). H1 and H2 were similar to *Ae. aegypti* isolate from Semarang (KT313650 and KX1713940); H3 and H5 to H10 were close to haplotypes from Semarang (KP334268.1 and KP334269.1) and Panama and Pacific region. H4 was located in root of phylogenetic tree suggesting oldest haplotype in the area. The majority of haplotypes generated in this study were of similar origin compared to *Ae. aegypti* isolate *of* Semarang.

Table 2. Polymorphic sites in 10 haplotypes obtained from 603 bp fragment of *Ae. aegypti* specimens

Haplotypes	Polymorphic sites									
	9	4	1	1	3	3	5	5	5	
		5	4	5	0	0	2	9	9	
			7	9	3	9	5	3	4	Nᵃ
H1	A	C	G	A	A	A	T	A	G	3
H2	.	T	.	.	.	.	.	.	.	1
H3	.	.	.	.	G	.	.	.	.	4
H4	.	.	.	G	.	.	A	G	.	2
H5	.	.	A	.	.	G	.	.	.	2
H6	G	.	.	.	.	G	.	G	.	1
H7	G	.	A	.	.	G	.	.	.	1
H8	.	.	.	.	G	.	.	.	A	1
H9	G	.	.	.	G	.	.	G	.	2
H10	.	.	.	.	G	.	.	G	.	3

Note: Nᵃ = the number of individuals in each haplotype

Table 3. Haplotype distribution for *Ae. aegypti* samples in South Purwokerto and Cilongok, Banyumas District, Central Java, Indonesia

Haplotypes	South Purwokerto	Cilongok	Total
			Nᵃ
H_1	2	1	3
H_2	1	0	1
H_3	1	3	4
H_4	2	0	2
H_5	2	0	2
H_6	1	0	1
H_7	1	0	1
H_8	0	1	1
H_9	0	2	2
H_10	0	3	3
Total	10	10	20

Note: Nᵃ = the number of individual in each haplotype

Table 4. Genetic variability indices for the Ae. aegypti samples from South Purwokerto and Cilongok, Banyumas District, Central Java, Indonesia

Population	N	H	Hd	k	π	S	Tajima D	*p*-value	Fs	*p*-value
South Purwokerto	10	7	0.933	2.933	0.005	8	0.084	0.555	0.506	0.580
Cilongok	10	5	0.844	1.311	0.002	4	0.093	0.570	1.340	0.764
Total estimate	20	10	0.921	2.552	0.004	9				

Note: N: number of sequences; H: number of haplotypes; Hd: haplotype diversity; S: number of segregating sites; K: average number of differences, π: nucleotide diversity, D: Tajima statistics and Fs: Fu's neutrality test

Table 5. Estimates of the demographic and spatial expansion model, sum of squared deviation (SSD) and Harpending's raggedness index (*R*)

Demographic expansion	Tau CI 95%	Theta0 CI95%	Theta1 CI95%	SSD	SDD *p-value*	R index	R *p-value*
South Purwokerto	13.908	5.218	165.099	0.049	0.040	0.162	0.040
Cilongok	7.431	2.046	325.156	0.081	0.020	0.211	0.040
Spatial expansion	**Tau CI 95%**	**Theta CI95%**	**M CI95%**	**SSD**	**SSD *p-value***	**R index**	**R *p-value***
South Purwokerto	11.813	4.366	152.881	0.046	0.310	0.162	0.170
Cilongok	7.527	1.735	49.910	0.063	0.330	0.211	0.340

Note: Tau: expansion time, (Theta 0 and 1; initial and final mutation, M: rate of migration, and CI: confidence Interval

Table 6. Analysis of Molecular Variance (AMOVA) for Cytochrome Oxidase I (COI) of *Ae. aegypti* samples from South Purwokerto and Cilongok Banyumas District, Central Java, Indonesia

Source of variation	d.f.	Variance components	Variation (%)	p-value
Among populations	1	0.712 Va	17.85	0.00196
Within populations	18	3.277 Vb	82.15	
Total	19	69.400	3.990	

Note: d.f: degree of freedom. The obtained FST value was 0.178; p-value : 0.00000+-0.0000

Figure 2. Haplotype network of cytochrome oxidase I of the Ae. aegypti. The nodes represent the proportion of individuals in each haplotype, each line in the network represents a single mutation, and numbers are position of mutation between two haplotypes

Discussion

Analysis of 600 bp fragments of Cytochrome oxidase-I revealed a significant level of polymorphism among 20 *Ae. aegypti* samples generating 10 haplotypes, H1, and H3 were found in both locations. The majority of haplotypes fell into South Purwokerto samples (H1 to H7), while in Cilongok (H3 and H8 to H10). The indices of genetic diversity, haplotype diversity Hd and nucleotide diversity π were relatively higher in South Purwokerto (Hd = 0.933; π = 0.005) than Cilongok (Hd = 0.844; π = 0.002). These differences could be attributed to difference in physical environment and the variability of mosquito breeding sites which influence the species genetic diversity (Nei et al. 1975). Insecticides used for vector control are quite often conducted in urban settings, which can reduce population size and induces bottleneck and genetic drift effects (Urdaneta-Marquez and Anna-Bella 2011). In general, low genetic diversity could mostly result from a decline in population size (Marcombe et al. 2013). However, some studies have revealed high genetic diversity of *Ae. aegypti* population in areas with frequently use insecticides (Paupy et al. 2000). In this study, the sampled population did not undergo genetic bottleneck assuming the control measures had little impact on reducing mosquito populations. High haplotype diversity and low nucleotide diversity found in this study may indicate expansion after a period of low population size followed by population growth which enhances the evolution of new mutations (Avise et al. 1984). The nucleotide diversity recorded in this study is slightly similar to those recorded in previous studies, for example in North Coast of Central Java: π~ 0.00099 and 0.0122 (Yohan et al. 2018). In addition to those reported worldwide i.e., Africa: π = 0.013 (Bennett et al. 2016), Brazil: π = 0.00748 (Fraga et al. 2013), Bolivia: π = 0.00135 (Paupy et al. 2012), Colombia: π = 0.001 to 0.008 (Jaimes-Dueñez et al. 2015) and Malaysia: π = 0.002 to 0.03 (Naim et al. 2020).
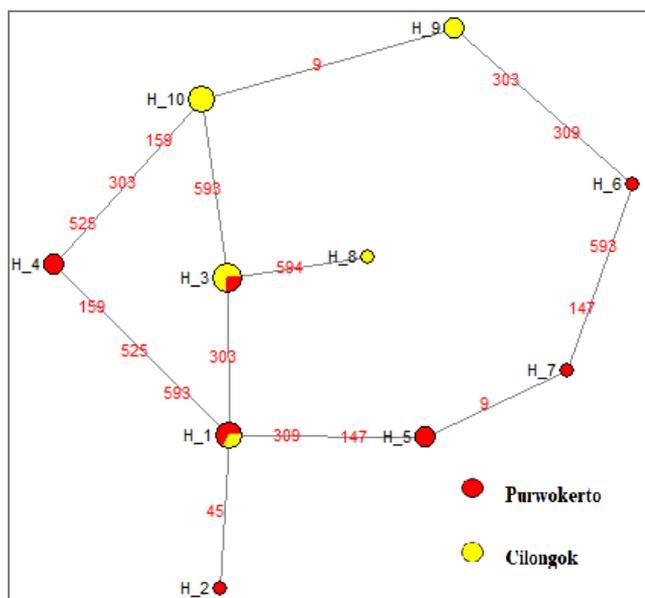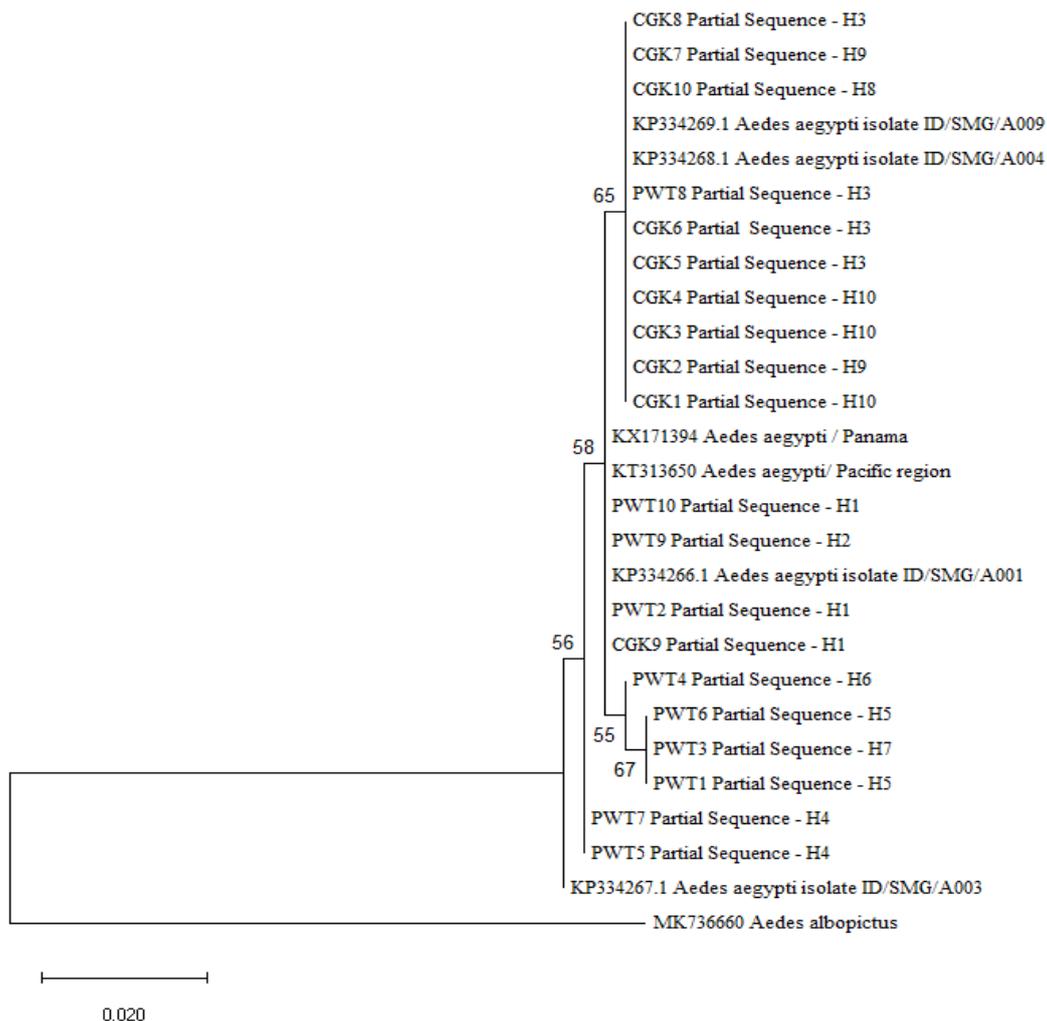
Figure 3. Neighbor-joining (NJ) tree for forward sequences of Ae .aegypti CO-I from South Purwokerto (PWT), Cilongok (CGK) of Banyumas District, Central Java, Indonesia, and sequence retrieved from GenBank. using 1000 bootstrap replication

Tajima D statistics and Fu's value were positive non-significant (P > 0.05) in both populations (Table 4); the positive Tajima D may indicate that the populations are in genetic equilibrium and balancing selection (Brito et al. 2002). Fu's test (Fu 1997) is more sensitive for population expansion and more powerful to measure population growth than Tajima test (Tajima 1989). Positive non-significant Fu's may indicate alleles deficiency as a result of over-dominant selection or recent population bottleneck (Fu 1997; Liao et al. 2010).

Mismatch distributions for demographic and spatial expansions showed multimodal distribution, suggesting that the populations were under demographic equilibrium. The indices of SSD and raggedness index R were significant ($p < 0.05$). This implies there is a deviation from the demographic expansion model assuming that the populations were under demographic equilibrium. In contrast, the analysis failed to reject the spatial expansion model for both populations due to non-significant values of SSD and R ($p > 0.05$) assuming that the sampled populations underwent spatial expansion. Moreover,

different expansion times (τ) and rates of migration (M) were observed (Table 5). South Purwokerto samples have had far distant spatial expansion from their older lineage when compared to the recent expansion showed in Cilongok samples (Harpending 1994; Excoffier 2004). From these findings, the demographic history of *Ae. aegypti* is consistent with the hypothesis of past spatial expansion (Liao et al. 2010). Additionally, the phylogenetic tree revealed South Purwokerto haplotypes were older than recently derived haplotypes in Cilongok. A previous report (Rašić et al. 2015) indicated that human transportation; environmental factors could facilitate the expansion of *Ae. aegypti*, however, this suggestion is not fully understood within the context of the current study; the optimum temperature, humidity, and abundance of breeding sites are factors that may sustain adaptation and expansion of *Ae. aegypti* to this region (Seixas et al. 2019).

The AMOVA analysis was used to test genetic differentiation in which populations were grouped into South Purwokerto and Cilongok. The analysis revealed that the genetic variation within the population was (82.15%)

and among the population was (17.85%), and this is significant *(p <0.02)*. Likewise, the value of population pairwise F_{ST} (0.178%) was highly significant (*p<0.0001*) indicating that great genetic differentiation occurred between the sampled populations. In this study, moderate gene flow has occurred between the two populations (H1 and H3). A combination of factors such as rainwater and rivers, close distance between the two sites (15 km), connection by pathways, and people movement might have contributed to dispersal of the mosquitoes (Lima Jr & Scarpassa 2009; Nazri et al. 2013). This is assuming that these factors andand physical environment and other human activities may influence the genetic structure of *Ae. aegypti* (Huber et al. 2002; Guagliardo et al. 2014).

The haplotype network revealed that South Purwokerto samples had a higher number of haplotypes and mutations, although, South Purwokerto is capital city of Banyumas; population density around 234,400 peoples and urbanized compared to suburb in Cilongok (Central Bureau of Statistics 2011), the physical environment could offer diverse range of mosquito breeding sites thus eventually affect the structure of *Ae. aegypti* population (Huber et al. 2002; Nazri et al. 2013). High haplotype events could be correlated with insecticides for vector control, which is a potential factor associated with the evolution of new mutations (Li et al. 2012). Limited haplotypes and mutations were found in Cilongok, in which the mosquito collection was performed at a hilly and elevated site of 143 m above sea level, which suggests that inbreeding effects may result in weak genetic diversity, as observed in Cilongok (Chakraborty et al. 1992). The H1 and H3 were found in the population of South Purwokerto and Cilongok assuming common ancestral haplotypes in this region and due to evidence of gene flow between the two populations. Haplotype sharing commonly resulted from extensive gene flow and migration between distant populations (Naim et al. 2020).

The phylogenetic tree indicated clustering of the haplotypes into a single clade (Figure 3), Additionally, the unrooted haplotype network supports one lineage's assumption, as well as previous studies, have reported a single clade in Central Java (Yohan et al. 2018). The H3, H8, H9, and H10 were closely related to previously isolated *Ae. aegypti* from Semarang city in Indonesia (KP334268.1, and KP334269.1). Semarang is a major harbor connecting different Regencies inside and out of Central Java. Presumably, through human transport, mosquitoes' stages are dispersed in Central Java areas (Huber et al. 2004; da Silva et al. 2012). The H4, located at the root of the phylogenetic tree, forming a separate sub-group. The H1 and H2 were more similar to haplotypes from Panama (KX171394) and the pacific region (KT313650) and Semarang (KP334266.1), indicating that same isolate of *Ae. aegypti*. Previously, different studies revealed that *Ae. aegypti* samples from Southeast Asia (Vietnam and Indonesia), the Pacific region (French Guiana), and South America (Bolivia and Mexico) were shared a similar ancestor (Failloux et al. 2002; Gorrochotegui-Escalante et al. 2002; Paupy et al. 2012; Yohan et al. 2018).

In conclusion, this study indicates that the COI marker is a powerful tool for assessing the genetic differentiation and gene flow in *Ae. aegypti* population in South Purwokerto and Cilongok of Banyumas District, Indonesia. A moderate level of gene flow found between the two populations may allow insecticide-resistant genes to spread throughout the gene flow.

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