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

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Abstract. *Aedes aegypti* is a major vector of arboviruses, such as yellow fever, chikungunya and dengue virus. Banyumas Regency is one of the most affected areas with a dengue virus disease in Central Java. The species has spread throughout the Banyumas areas; however, there is no information about the genetic variability of this species, yet this is essential for strengthening the ongoing vector control measures. Therefore, population differentiation and gene flow analysis for 603 base-pair fragments of mitochondrial cytochrome Oxidase I were conducted among twenty *Ae. aegypti* specimens from Purwokerto and Cilongok in Banyumas regency. Ten haplotypes were detected as clustering into a single Clade. The Purwokerto samples exhibited higher haplotype and nucleotide diversity than Cilongok, and moderate gene flow has occurred between the two populations. Tajima and Fu's statistics indicated that the sampled populations are in genetic equilibrium. Indices of demographic history were fit for a spatial expansion model; Purwokerto population had gone far past the expansion from their lineage than the more recent expansion in Cilongok. Higher genetic variation was observed within the population compared to between the populations. Furthermore, strong genetic differentiation was detected between the two populations. Even so, the estimate of population pairwise F_{ST} (0.178) was highly significant ($p < 0.0001$). The significant genetic differences could be due to the physical environment, insecticide treatment and variability of the mosquitoes' breeding structure between both populations. this study assume the limited effectiveness of the vector control measures.

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Keywords: *Ae. aegypti*, genetic differentiation, Banyumas regency, Central Java

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INTRODUCTION

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I recommend to read: Gandhi et al. Molecular analysis of mitochondrial cytochrome oxidase I gene of *Aedes aegypti* L. mosquitoes. Journal of Asia-Pacific Entomology 23 (2020) 51–59

Aedes aegypti is the main vector responsible for transmission of the dengue virus and other arboviruses (Black et al. 2002). 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; in Java Island especially, dengue incidence has been reported frequently (Sayono et al. 2017). Banyumas Regency is one of the endemic areas of dengue hemorrhagic fever (DHF) in Central Java, and according to the Banyumas district health office, around 104 dengue cases were reported in Banyumas Regency in 2020.

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Currently, the Ministry of Health of Indonesia has launched a program called Mosquito Nest Eradication (PSN: Pemberantasan Sarang Nyamuk) which involves mosquito habitat management through destroying their breeding sites. This is done in addition to the traditional methods of fogging and using house insecticide to prevent dengue virus. Although insecticide fogging may decrease 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. 2017). This is as such in spite of the fact that long term use of insecticide has caused resistance against a variety of insecticides to develop in the mosquito population (Moyes et al. 2017). Studies on the patterns of genetic structure and gene flow in *Ae. aegypti* population are important for dengue vector control, and the information on gene flow is especially

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useful for identifying the level of insecticide dispersal (Rasheed et al. 2013) for better understanding of the circulating dengue virus transmission in Banyumas Regency.

Mitochondrial DNA is commonly used in population genetic studies of *Ae. aegypti* in various geographic scales and dengue endemic areas (Gonçalves et al. 2012). Cytochrome oxidase I (COI), the largest of the three mitochondrial-encoded cytochrome oxidase subunits, is a useful tool for *Ae. aegyti* genetic studies (Anoopkumar et al. 2019). In Java, few studies described the population structure of *Ae. Aegypti*. 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 it concluded that the species exhibited considerable genetic diversity. Additionally, the study of Rašić et al. (2015) in Yogyakarta region revealed that the *Ae. aegypti* populations were spatially structured and seasonally stable.

This preliminary survey was aimed to assess the genetic diversity of *Ae. aegypti* population in the small geographic scale of Purwokerto and Cilongok in Banyumas Regency by analyzing mitochondrial Cytochrome oxidase I to determine the level of genetic differentiation and gene flow.

MATERIALS AND METHODS

Description of the study area

Banyumas Regency (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 km^2, and the region is inhabited by 1.85 million people. Banyumas Regency consists of 27 sub-districts with a total of 331 villages. The climate in Banyumas is distinguished by a tropical monsoon climate. Purwokerto which is the capital city of Banyumas lays 55 m above sea level (asl) and covers an area of around 38.58 km^2. The average temperature in Purwokerto is approximately 26.9 °C, and about 2284 mm of precipitation falls annually. Cilongok is the largest sub-district in Banyumas Regency, which lays 209 m ASL and covers an area of 105,34 km^2. It has heavy rainfall during the year with an average annual rainfall of 3669 mm and an average annual temperature of 25.8 °C.

Mosquito samples were collected using ovitraps from inside houses in Purwokerto and Cilongok in the period from April to July 2019.

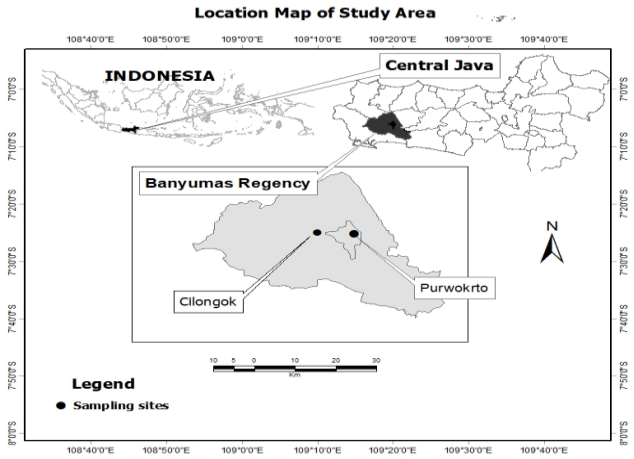

Figure 1. Map showing sampling sites (Purwokerto and Cilongok) in Banyumas regency, Central Java, Indonesia

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 as described by Beebe et al. (2005) using the forward primer 5-TAGTTCCTTTAATATTAGGAGC-3 and the reverse primer 5-TAATATAGCATAAATTATTCC-3) generating around 600-bp fragment. 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%

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Add detail information on research samples: Cilongok and Purwokerto: village, district? The samples collected from a location or several locations within Cilongok and Purwokerto, respectively? How many samples did you collect? then, how many samples were analyzed?

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Coordinate is represent Banyumas Regency, but the authors are also placed Indonesia map dan Central Java map in similar box

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agarose gel-electrophoresis and visualized by staining with ethidium bromide (EtBr). How to purify gel contained COI gene? 40 ul of PCR product from each sample was sent for DNA sequencing.

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The COI haplotypes and genetic differentiation analysis

The nucleotide sequences obtained in this study 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. All sequences obtained from your research should be submitted to GenBank database for publication, so you should submit all sequences to GenBank and present accession number in a Table. Example Table…. COI sequences of Ae. aegypti from ……. retrieved from NCBI.

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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)* and the Fu test, 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.

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To test the hypothesis of the expansion model observed, 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 100 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*).

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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) using Arlequin.

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Median-Joining haplotype network was constructed using a network software (2004–2019 Fluxus Technology Ltd) to evaluate the relationships between haplotypes. Phylogenetic relationships among samples were estimated by neighbor-joining (NJ) methods using Mega X. Trees were inferred with the Tamura3-parameters model by 1000-replicate bootstrapping. To estimate level of phylogenetic relationship with *Ae.aegypti* from other regions, four cytochrome oxidase I sequences originating from Semarang were retrieved from GenBank (KP334267.1, KP334266.1, KP334268.1 and KP334269.1) and used to construct a phylogenetic tree. *Ae. albopictus* (GenBank: KP334275.1) was used as outgroup.

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RESULTS AND DISCUSSION

Haplotype distribution

A total of 20 samples of *Ae. aegypti* were sequenced, ten from each site (Purwokerto and Cilongok). The length of the sequences after alignment were 603 base pairs. A total of 10 haplotypes and 9 polymorphic sites were identified (Table 1). 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 Purwokerto samples and H 8, and H10 were exclusive to the population of Cilongok (Table 2).

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The samples were obtained from a location or several locations on each site

Table 1. Polymorphic sites in ten haplotypes obtained from 603 bp fragment of *Ae. aegypti* specimens

Haplotypes	Polymorphic sites									
	9	**45**	**147**	**159**	**303**	**309**	**525**	**593**	**594**	**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 2. Haplotype distribution for *Ae. aegypti* samples in Purwokerto and Cilongok

Haplotypes	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

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. Purwokerto samples had higher values, which are as follows: h =7, Hd =0.933, S=8, K = 2.933 and π = 0.005 (Table 3).

Neutrality Test

Tajima D statistics were (0.084) and (0.093) $p > 0.05$, while Fu's test resulted in values of (0.506) and (1.340) $p > 0.02$ for Purwokerto and Cilongok respectively (Table 3). Positive non-significant values indicating the observed polymorphism were consistent with the neutral mutation model.

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Mismatch Distribution

According to the significant values of Raggedness index R and SSD for Purwokerto and Cilongok samples (Table 4), the hypothesis of sudden 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 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 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).

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Table 3. Genetic variability indices for the *Ae. aegypti* samples from Purwokerto and Cilongok

Population	N	H	Hd	k	π	S	Tajima D	*p* value	Fs	*p* value
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; H = 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 4. Estimates of the demographic and spatial expansion model, sum of squared deviation (SSD) and Harpending's raggedness index (*R*) indices

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Demographic expansion	Tau CI 95%	Theta0 CI95%	Theta1CI95 %	SSD	SDD *p* value	R index	R *p* value
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**
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.

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AMOVA analysis

AMOVA results showed greater variation (82.15%) within the population and lower variation (17.85%) among population ($p < 0.02$) (Table 5). The population pairwise fixation index (F_{ST}) was 0.178 ($p < 0.0001$) indicating that a strong genetic structuring occurred in the sampled population of *Ae. aegypti*.

Table 5. Analysis of Molecular Variance (AMOVA) for Cytochrome Oxidase I (COI) of *Ae. aegypti* samples from Purwokerto and Cilongok

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 F_{ST} value was 0.178; *p* value = 0.00000+-0.0000

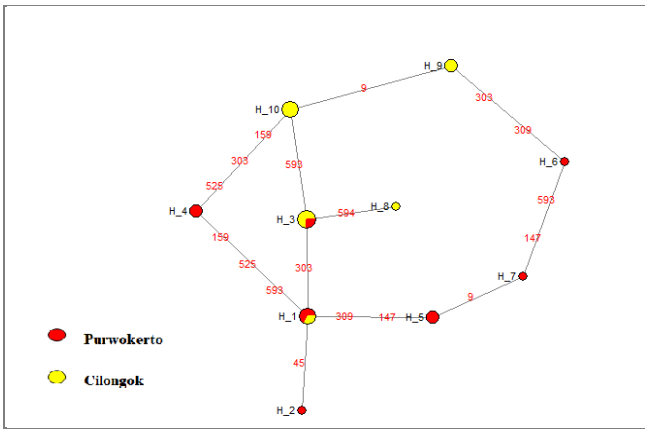

Figure 2. Haplotype network of cytochrome oxidase I of the *Ae. aegypti*. The nodes represent the proportion of individuals in each haplotype, each line in the network represents a single mutation, and numbers are position of mutation between two haplotypes

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 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. Purwokerto haplotypes were separated by a higher number of mutations, while Cilongok haplotypes were closely related and separated by a fewer number of mutations.

Place Figure 2 here

Phylogenetic analysis

Neighbor-Joining (NJ) phylogenetic tree indicated that the haplotypes were clustered into a single clade supported by weak bootstrapping values (Figure3). H3, H8 to H10 were similar to *Ae. aegypti* isolate from Semarang (KP334268.1 and KP334269.1), while H1 and H2 were closely related to KP334266.1. H4 to H7 (Purwokerto) had no match with four haplotypes from Semarang. These results may suggest H1 to H3 and H8 to

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H10 were of similar origin compared to *Ae. aegypti* of Semarang. In contrast, H4 to H7 were probably a different *Ae. aegypti* introduction or a newly evolved mutation.

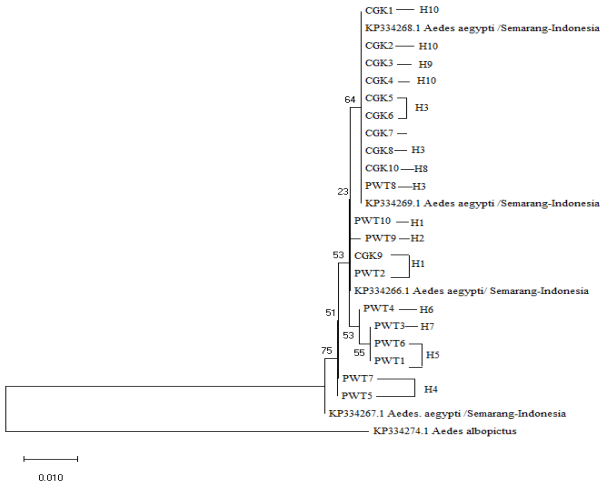

Figure 3. Neighbor-joining (NJ) tree of *Ae. aegypti* cytochrome oxidase I gene from Purwokerto (PWT), Cilongok (CGK) and Semarang (retrieved from GenBank) using the Tamura 3-parameter genetic distance model with bootstrap support which was estimated from 1000 repetitions

Discussion

Analysis of 603 bp fragments of Cytochrome oxidase I revealed a significant level of polymorphism among 20 *Ae. aegypti* samples; ten haplotypes were detected, and H1 and H3 were found in both locations. The indices of genetic diversity, haplotype diversity Hd and nucleotide diversity π were relatively higher in Purwokerto (Hd = 0.933; π = 0.005) than Cilongok (Hd = 0.844; π = 0.002). Mosquitoes of the inside city were genetically differentiated; the physical environment and the variability of mosquito breeding sites may influence the genetic diversity of the species. Insecticides used for vector control are quite often conducted in urban settings which may reduce population size and induces bottleneck and genetic drift effects (Urdaneta-Marquez and Anna-Bella 2011). Low genetic diversity could mostly result from a decline in population by insecticide treatment (Marcombe et al. 2013). However, some studies have revealed high genetic diversity of *Ae. aegypti* population in areas that frequently use insecticides (Paupy et al. 2000). Thus, sampled population of *Ae. Aegypti*, specifically in Purwokerto, did not undergo genetic bottleneck. 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 (e.g. Yogyakarta: π~ 0.0004 and 0.004 Rašić et al. (2015); North Coast of Central Java: π~ 0.00099 and 0.0122 Yohan et al. (2018)). Moreover, it is comparable to those reported worldwide such as 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).

The haplotype network revealed that Purwokerto samples had a higher number of haplotypes and mutations, despite the fact that Purwokerto is more urbanized and crowded, which may affect the structure of *Ae. aegypti* population (Huber et al. 2002). Higher numbers of mutations could be correlated with the use of insecticides for vector control which is potentially a 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 in a small and hilly elevated site, which suggests that inbreeding effects may result in weak genetic diversity as observed in Cilongok. The H1 and H3 were found in the population of Purwokerto and Cilongok assuming

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Please make deeper discussion to explain your results
Example: phylogenetic analysis should be discussed in the last discussion

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Did the references use COI gene? Please add previous studies conducted in Java or Indonesia

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common ancestral haplotypes in this region and evidence of gene flow between the two populations. Haplotype sharing commonly resulted from extensive gene flow and migration between the distant populations (Naim et al. 2020). The phylogenetic tree indicated clustering of the haplotypes into a single clade; in addition, the unrooted haplotype network supports the assumption of one lineage. The majority of haplotypes generated in this study were closely related to *Ae. aegypti* from Semarang (Yohan et al. 2018) which is a major harbor city connecting different regencies inside and out of Central Java. Presumably through human transportation, *Ae. aegypti* dispersed in areas of Central Java (Huber et al. 2004).

In this study, Tajima *D* statistics and Fu's value were positive non-significant (P> 0.05) in both populations (Table3); the positive Tajima *D* may indicate that the populations are in genetic equilibrium and balancing selection. Fu's test is more sensitive for population expansion and more powerful to measure population growth than Tajima. Positive non-significant Fu's may indicate alleles deficiency as a result of over-dominant selection or recent population bottleneck.

Mismatch distributions for demographic and spatial expansions showed multimodal distribution, suggesting that the populations were under demographic equilibrium. The hypothesis that the observed data fits the sudden expansion model was tested using the sum square deviations (Excoffier 2004) and the raggedness *R* index (Harpending 1994); the indices of SSD and raggedness index *R* were significant ($p<0.05$). This implies that 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 4). Purwokerto samples have had far distant spatial expansion from their older lineage when compared to the recent expansion showed in Cilongok samples. From these findings, the demographic history of *Ae. aegypti* is consistent with the hypothesis of past spatial expansion (Liao et al. 2010).

The AMOVA analysis was used to test genetic differentiation in which populations were grouped into 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 different environmental factors such as rain and rivers, close distance between the two sites (15km), connection by pathways, and people movement might have contributed to dispersal of the mosquitoes. This is assuming the fact that these factors in addition to physical environment and other human activities may influence the genetic structure of *Ae. aegypti*.

The results of this study indicate that the COI marker is a powerful tool for assessing the genetic structure and level of polymorphism in *Ae. Aegypti* population. The current study has implications for vector control efforts. Significant genetic diversity of *Ae. aegypti* showed in this study may suggest limited effectiveness of vector control measures especially in regard to insecticide treatment. The weak genetic connectivity occurring between the two populations may allow insecticide resistance to spread throughout by gene flow from the treated population to the untreated population.

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ACKNOWLEDGEMENTS

This study was supported by Universitas Jenderal Soedirman (UNSOED) research grant year?. The authors acknowledge the people who have supported this work especially in Cilongok.

REFERENCES

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