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DNA barcoding and phylogenetic relationship for *Aedes aegypti* on Java Island, Indonesia

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ABSTRACT

Objective: This study aimed to identify *Aedes* (*Ae*.) *aegypti* mosquitoes in four areas on Java Island, Indonesia, and to characterize their phylogenetic relationship based on the cytochrome oxidase subunit I (COI) gene.

Methods: Mosquito specimens were collected from four geographical locations on Java Island using oviposition traps based on dengue outbreaks. *Ae. aegypti* larvae were morphologically identified and analyzed for their COI sequence. Sequences polymorphism was measured using DnaSP. Kimura two-parameter was used in MEGA X to estimate pairwise genetic distances. The median-joining haplotype network was generated using PopART, and the maximum likelihood tree was constructed using iTOL.

Results: A total of 40 *Ae. aegypti* specimens were identified, with 13 haplotypes and 10 polymorphic sites detected. The number of haplotypes was higher in Jakarta (H=6) and lower in Bandung (H=2). The mean intraspecific genetic distance ranged from 0.1% to 1.0%, while the mean inter-specific distance varied between 0.3% to 0.9%. The phylogenetic tree revealed two clades and significant genetic differentiation occurred between *Ae. aegypti* populations, with a fixation index ranging from 0.00 to 0.50. The greatest genetic distance was found between *Ae. aegypti* populations in Bandung and Semarang.

Conclusions: A significant genetic difference was observed among *Ae. aegypti* populations, suggesting that human activities and ecological conditions may affect the genetic differentiation of this species.

KEYWORDS: *Aedes aegypti*; Cytochrome oxidase subunit I; Genetic differentiation; Java Island

1. Introduction

Aedes (*Ae*.) *aegypti* is a mosquito that acts as the principal vector for the transmission dengue and other arboviruses[1]. This species is widely distributed in Indonesia, particularly on Java Island, which is the most populated island in Indonesia[2]. The prevalence of this species has often been associated with outbreaks of dengue virus in this region[3,4,5]. Although, variability in breeding sites and ecological conditions can affect the biology and diversity of the mosquito species[6], water storage practices are significantly associated with the distribution of *Ae. aegypti* and the risk of exposure to arboviral disease[7]. A precise identification of mosquito species is essential to validate taxonomy and for vector control. Identification of *Ae. aegypti* mosquito often relies on morphological features, which could be challenging to distinguish cryptic species due to shared characteristics[8,9,10]. DNA barcoding is therefore, a useful tool for accurate identification[11,12]. The mitochondrial cytochrome oxidase I subunit (COI) is an effective tool in DNA barcoding[13,14], and widely used in phylogenetic studies of *Ae. aegypti*[10,15,16].

Significance

Aedes (*Ae*.) *aegypti* mosquito is an important vector of arboviral diseases, and species identification is a crucial step in the control of the vectors. Information of *Ae. aegypti* populations and their phylogenetic relationship was inadequate on Java Island, Indonesia. The present study identified two cytochrome oxidase subunit I clades and high genetic differentiation among *Ae. aegypti* populations.

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Table 1. Climate data of sampling areas in 2020.

Sampling site	Coordinates	Elevation (m)	Humidity (%)	Rainfall (mm)
Bandung (Jatinangor)	6.9175° S, 107.6191° E	650 m	90	198
Semarang	7.0051° S, 110.4381° E	373 m	79	149
Jakarta (South Jakarta)	6.2088° S, 106.8456° E	355 m	76	206
Bogor (Tanah Sereal)	6.5515° S, 106.7883° E	330 m	85	223



Figure 1. Location of the four sampling areas on Java Island.

The two subspecies, *Ae. aegypti aegypti* (predominant in tropical/subtropical regions) and *Ae. aegypti formosus* reported only in Africa[17] have shown difference in vector competence. When using COI, significant genetic differences were found between the subspecies[10]. In a previous study, Mohammed *et al.*[18] reported low genetic difference among *Ae. aegypti* in Purwokerto and Cilongok in Banyumas, Indonesia.

The present study aimed to identify *Ae. aegypti* in four different geographical areas on Java Island, Indonesia, and to examine their phylogenetic relationship based on the COI gene sequence.

2. Subjects and methods

2.1. Mosquito collection

A cross-sectional survey was conducted in four areas: 1) Jatinangor (Bandung), 2) South Jakarta (Jakarta), 3) Tanah Sareal (Bogor) and 4) Semarang on Java Island, Indonesia (Figure 1). The selection of the areas was based on outbreaks of dengue virus[3,4,5], as well as geographical and ecological conditions (Table 1). The mosquito specimens were collected from January to June 2020. In each area, approximately 20 oviposition traps (ovitraps) were placed inside houses in dark and around humid zones. The ovitraps (plastic bowel) were filled with 300-400 mL of tape water and left for one week to allow female mosquitoes to lay their eggs. After one week, the water (containing mosquito stages) was recovered from ovitraps into labeled and sealed jars (1L), transported to the laboratory of Entomology, Jenderal Soedirman University, where the immature stages were reared at room temperature until they reached the third and fourth instar larvae for identification.

2.2. Identification of *Ae. aegypti* species

The identification of *Ae. aegypti* larvae were performed using the key of Leopolado and Rueda[19] based on the presence of strong black hoods on the sides of the thorax and pitchfork shape comb in one raw (Figure 2). *Ae. aegypti* specimens were stored in 1 mL sterile tube containing 70% isopropanol and kept at -20 °C.

2.3. DNA extraction and COI gene amplification

Genomic DNA (gDNA) was extracted from an individual larva using DNA extraction mini-kit (Tissue) according to the manufacturer's procedures. The quality of each gDNA was measured using a Nanodrop UV spectrophotometer.

The amplification of the COI gene was performed using primers (forward: 5-TAGTTCCTTTAATATTAGGAGC-3 and reverse: 5-TAATATAGCATAAATTATTCC-3), which amplify around 600 bp fragments of the partial COI gene fragment as described by Beebe *et al.*[20]. PCR was conducted using a thermal cycler (Primus 25® Advanced, produced by PEQLAB) with 50 µL of GoTaq®Green Master Mix (Promega) contained Go-Taq Polymerase, dNTPs, $MgCl_2$, 0.4 µM of each primer and 50-100 ng of DNA template. The cycling conditions were as follows: the initial denaturation phase at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 1 min, 72 °C for 1.5 min, and the final phase at 72 °C for 10 min with storage at 4 °C. Agarose gel-electrophoresis 1% was performed to separate the fragments by staining using Syber Green for visualization. Sanger DNA sequencing was performed by Apical Scientific, Malaysia.

2.4. COI sequence analysis

2.4.1. Sequence alignment and homology

The sequences were assembled using Bio-Edit and aligned with ClustalW in Molecular Evolutionary Genetics Analysis (MEGA X) v12[21]. A Basic Local Alignment Search Tool for nucleotides search was performed in GenBank and Barcode of Life Database (BOLD). The sequences were submitted to NCBI GenBank under accession numbers PP124964-PP125003.

Table 2. COI sequences of *Ae. aegypti* used in phylogenetic analysis.

GenBank ID	No. of sequence	Location/ Country	Source/Reference
PP124964 to PP124973	10	Jakarta	Present study
PP124974 to PP124983	10	Bandung	Present study
PP124984 to PP124993	10	Semarang	Present study
PP124994 to PP125003	10	Bogor	Present study
MT881571 to MT881590	20	Banyumas	[18]
KU207145.1	1	US-Honolulu	[24]
OL963828.1	1	Kenya	[25]
MN019006.1	1	Cape Verde	[26]
KT630412	1	Malaysia	[27]
MK265723	1	India	[28]

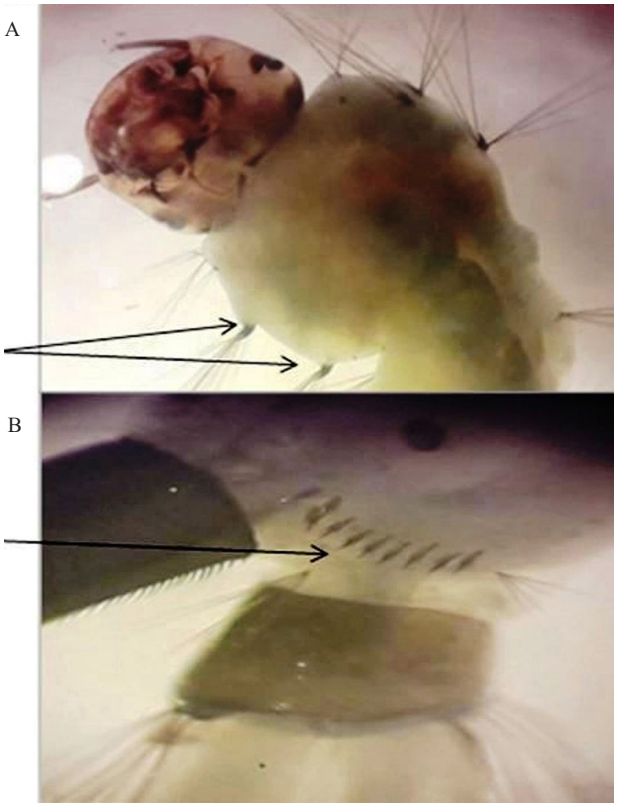


Figure 2. The morphological characters of *Ae. aegypti* larvae. Arrows in A indicate strong black hooks on the sides of the thorax; Arrow in B indicates pitchfork shape comb in one raw.

Table 3. Haplotype numbers, polymorphic sites, sites and frequency of COI sequence of *Ae. aegypti* populations.

Haplotype	Sequence	Location	Frequency
H1	A T C T T G T A T C	Bandung, Banyumas	11
H2	. . T	Banyumas, Bogor, Jakarta, Semarang	15
H3	. C T . . . . . . T	Banyumas	3
H4	. C T	Bandung, Banyumas, Bogor	15
H5	. . T . . . . . C .	Banyumas, Bogor	3
H6	G . T C . A . . C .	Bogor	1
H7	. . T C	Semarang	3
H8	. . T C . A C G C .	Semarang	3
H9	. . T C . A . G C .	Jakarta	2
H10	. . T . . . . G . .	Jakarta	1
H11	. . T C . . . G . .	Jakarta	1
H12	. . T C . . . G C .	Jakarta	1
H13	. . . . C	Jakarta	1

Note: "." represents identity with haplotype H1.

2.4.2. Sequence polymorphism and haplotype network

DNAsp v6 software[22] was used to determine the number of haplotypes, the number of polymorphic sites, and haplotype frequencies. Population Analysis with Reticulate Trees (PopART) v1.7[23] was used to construct a median-joining haplotype network based on the sequences obtained here which were then compared with COI sequences identified by Mohammed *et al*.[18] in Central Java.

2.4.3. The COI sequences divergence and phylogenetic relationship analysis

The Kimura two-parameter (K2P) model was used in MEGA X to measure intraspecifc and interspecific pairwise genetic distance. The 40 sequences generated in this study, 20 sequences from a previous study[18], and five sequences retrieved from NCBI GenBank; Accession numbers: KU207145.1[24], OL963828.1[25], MN019006.1[26], KT630412.1[27], and MK265723.1[28] were aligned and used for the genetic analysis (Table 2). For phylogenetic analysis, sequences were alignment by ClustalW tool at https://genome.jp/tools-bin/clustalw. The aligned sequences were saved as nwk format and used to construct the a maximum likelihood phylogenetic tree using the Interacive Tree of Life (iTOL) v7 at http://itol.emb.de/[29].

2.4.4. Gene flow and genetic difference

The pairwise genetic distance was calculated using Arlequin v3.5[30] to estimate the gene flow between *Ae. aegypti* isolates generated here and those from regions. Fixation index (Fst) along with their statistically significant *P* values ($P< 0.05$) were recorded.

2.4.5. Ethical statement

This study did not involve human participants or vertebrate animals; therefore, ethical approval was not required.

3. Results

3.1. Morphological identification and sequence homology

Forty larvae were identified as *Ae. aegypti* based on their morphological characteristics. The partial COI gene sequences produced a fragment of approximately 560 bp. After editing, the sequence length was reduced to 492 bp. The GenBank accession numbers for the 40 sequences are provided in Table 2. The homology search revealed a 98%-100% similarity with COI sequences of *Ae. aegypti* in GenBank and BOLD database.

3.2. Haplotypes network and polymorphism

A total of 13 haplotypes and ten polymorphic sites were detected in the COI sequences. The most frequent haplotypes were H2 and H4, followed by H1 (Table 3). The number of haplotypes and their mutations are shown in Figure 3. Haplotype H2 was shared between Banyumas, Bogor, Jakarta, and Semarang; H4 was shared between Bandung, Banyumas, and Bogor; while H1 was shared between Banyumas and Bandung.

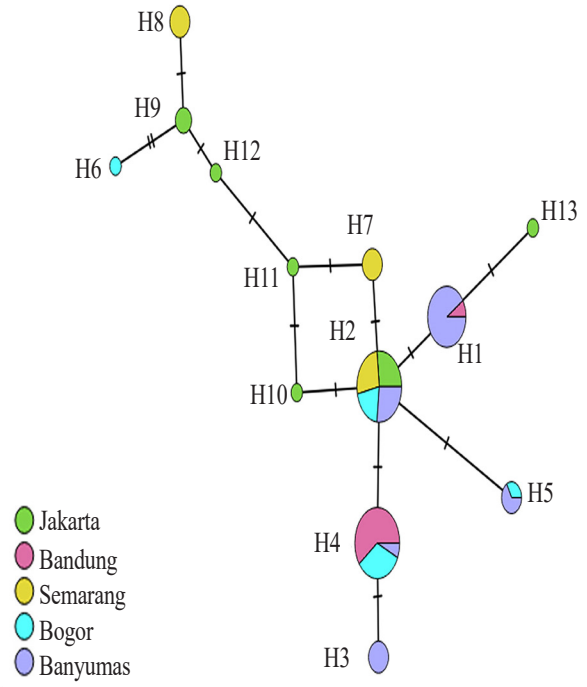


Figure 3. Median-joining haplotype network showing nodes representing the proportion of individuals in each haplotype and lines indicating mutational positions between two haplotypes.

3.3. The COI sequences divergence and phylogenetic relationships

The mean intra-specific distance obtained in this study was less than 2.0% (Table 4), with the minimum distance being 0.1% and the maximum distance 1.0%. The mean inter-specific distance ranged from 0.3% to 0.9%. When compared with *Ae. aegypti* from other regions, the lowest inter-specific distance was found between Bogor and Cape Verde (0.2%), Bandung and Cape Verde (0.2%), Banyumas and India (0.3%), while the highest distance (2.1%) was found between Bandung and Kenya.

The phylogenetic tree analysis identified two clades (Figure 4). The most of samples from the present study as well as specimens from India, Malaysia, Cape Verde, and the US were clustered in clade I. Within this clade, *Ae. aegypti* from Bandung, Bogor, and Banyumas were located in separate cluster. The clade II comprised specimens from Jakarta (three), Semarang (three) Bogor (one), Kenya (OL963828.1). No *Ae. aegypti* specimen from Bandung were found in clade II.

3.4. Gene flow and genetic difference

Fst between *Ae. aegypti* species in the present study are shown in Table 5. All of the Fst values were statistically significant ($P<0.05$). The Fst values varied as follows: Fst=0.00 ($P<0.05$) between Jakarta and

Table 4. Mean inter-specific (below the diagonal) and intra-specific (along the diagonal) genetic distance of COI sequences from this and other studies.

	Banyumas (*n*=20)	Bogor (*n*=10)	Semarang (*n*=10)	Bandung (*n*=10)	Jakarta (*n*=10)	India (*n*=1)	Malaysia (*n*=1)	Cap Verde (*n*=1)	Kenya (*n*=1)	US (*n*=1)	*Ae. albopictus* (*n*=1)
Banyumas	**0.030**										
Bogor	0.004	**0.004**									
Semarang	0.008	0.007	**0.008**								
Bandung	0.003	0.003	0.008	**0.001**							
Jakarta	0.008	0.008	0.009	0.009	**0.010**						
India[28]	0.003	0.005	0.008	0.004	0.008						
Malaysia[27]	0.005	0.005	0.007	0.004	0.007	0.004					
CapVerde[26]	0.003	0.002	0.006	0.002	0.006	0.002	0.002				
Kenya[25]	0.021	0.019	0.013	0.021	0.015	0.020	0.018	0.022			
US[24]	0.003	0.002	0.006	0.002	0.006	0.002	0.002	0.000	0.022		
Ae. albopictus[37]	0.137	0.136	0.135	0.138	0.132	0.138	0.141	0.136	0.131	0.136	

Table 5. The pairwise genetic distance values (Fst) among COI sequences in *Ae. aegypti* isolated from Java Island and other regions.

	Banyumas	Bogor	Semarang	Bandung	Jakarta	India	Malaysia	Cape Verde	Kenya	US
Banyumas	0.00									
Bogor	0.18*	0.00								
Semarang	0.34**	0.21*	0.00							
Bandung	0.37**	0.13*	0.50*	0.00						
Jakarta	0.27**	0.19**	0.00*	0.47**	0.00					
India	0.00**	0.24**	0.14**	0.77**	0.37**	0.00				
Malaysia	0.32**	0.24**	0.00**	0.80**	0.00 **	1.00**	0.00			
Cape Verde	0.00**	0.00**	0.00**	0.60**	0.00**	1.00**	1.00**	0.00		
Kenya	0.77**	0.70**	0.25**	0.93**	0.39**	1.00**	1.00**	1.00**	0.00	
US	0.00**	0.00**	0.00**	0.60**	0.00**	1.00**	1.00**	0.00**	1.00**	0.00

Note: *$P<0.05$; **$P<0.01$.

Semarang, Fst=0.19 (P<0.01) between Jakarta and Bogor, Fst=0.27 (P<0.01) between Jakarta and Banyumas, Fst=0.34 (P<0.01) between Semarang and Banyumas, Fst=0.37 (P<0.01) between Banyumas and Bandung, Fst=0.47 (P<0.01) between Jakarta and Bandung, and Fst=0.50 (P<0.01) between Bandung and Semarang. The highest distance was found between Kenya and Bandung (Fst=0.93; P<0.01). The lower Fst=0.00 (P<0.05) indicate the lack of genetic differentiation between *Ae. aegypti* populations, while the highest Fst=0.93 (P<0.01) implying great differentiation between the two populations.

4. Discussion

Ae. aegypti is an invasive species responsible for the transmission of dengue virus outbreaks in Java Island[3,4,5]. Accurate identification of this species is a prerequisite for controlling the vector populations. Previous studies on the identification and genetic differentiation of *Ae. aegypti* in Java Island have been inadequate, with the exception of Mohammed *et al*.[18] in Banyumas and Yohan *et al*.[31] in Central Java.

The present study identified *Ae. aegypti* populations in four different geographical areas on Java Island and examined their phylogenetic relationship based on COI gene sequences.

A total of 40 *Ae. aegypti* specimens were identified morphologically, producing 40 COI sequences. Overall, thirteen haplotypes were obtained. The number of haplotypes found here is slightly higher than in previous studies in Indonesia[18,31], which may be due to variations in the length of sequences, the gene sequence its self, or the number of sequences used in these studies. Haplotype sharing was observed between *Ae. aegypti* specimens from Jakarta and Semarang, Bogor and Banyumas, which could be due to interpopulation migration[15]. The haplotype sharing was absent between *Ae. aegypti* populations in Jakarta and Bandung.

The sampling sites in Jatinangor (Bandung) is located at high altitudes (650 m above sea level) with a climate characterized by high humidity, ranged from 80% to 90%[2]. In contrast, Semarang and Jakarta are located on the coast of Java, have flat topography (below 370 m), and average humidity ranging from 75% to 85%[2]. The lack of haplotype sharing and the low number of haplotypes found in Bandung could be due to bottleneck or genetic isolation[32]. On the other hand, the relatively higher number of haplotypes in Jakarta and Bogor might be due to population expansion or excess of recently evolved haplotypes[15, 33]. In general, *Ae. aegypti* populations outside Africa are younger and have undergone founder effects[34].

The identification of *Ae. aegypti* mosquito can be confused due to the shared morphological features[9]; therefore, the estimates of pairwise genetic distance using K2P is important for precise identification[35]. The mean intraspecific and interspecific distance found in this study was less than 2%. The mean intraspecific distance was ranged from 0.1% to 1.0%, while inter-specific was 0.3% to 0.9%. These values are below the 3.0% threshold commonly used to distinguish between culicidae species based on COI sequence[36], indicating that they belong to *Ae. aegypti* species. Furthermore, mean inter-specific distance for *Ae. aegypti* species obtained here and *Ae. albopictus*[37] was ranged from 13.1% to 13.8%.

Figure 4 shows that the maximum phylogenetic revealed a monophyletic tree clustered into two clades. Clade I, the most prevalent, comprised of majority of samples, while clade II was less prevalent and included samples from Jakarta, Semarang, and Kenya, with genetic distance ranging from 0.013 to 0.015. This clade II corresponds to H6, H8 and H9 in the haplotype network.

Within the clade I, *Ae. aegypti* specimens from Bandung, Bogor, and Banyumas were closely related to Malaysia, Cape Verde, and the US (mean distance ranging from 0.002 to 0.005). These results agrees with Yohan *et al*.[31], who found similarities between *Ae. aegypti* populations in Central Java and in South-East Asia.

The two clades of *Ae. aegypti* found in this study are consistent with those found in the previous studies in Africa, South America, and Asia[33,34,38,39]. Similarly, Brown *et al*.[40] and Moore *et al*.[41] found that the *Ae. aegypti* populations consist of two clades and share a common ancestor from Africa.

In this study, pairwise genetic distance was calculated to estimate genetic difference among *Ae. aegypti* populations. Generally, pairwise Fst values range from 0 (no genetic differentiation) to 1 (strong genetic differentiation)[42]. The lowest genetic distance (Fst=0.00; P<0.05) was found between *Ae. aegypti* specimens from Jakarta and Semarang (Table 5), and 0 indicates no genetic differentiation between *Ae. aegypti* populations. The highest Fst=0.50 (P<0.01) was found between Semarang and Bandung, suggesting a strong genetic differentiation and lack gene flow between these populations. When comparing with *Ae. aegypti* from East Africa, a moderate genetic differentiation was found between Semarang and Kenya (Fst=0.25; P<0.01), and complete differentiation was observed between Bandung and Kenya (Fst=0.93; P<0.01). These findings are consistent with the monophyletic phylogenetic tree, which divided into two clades. Moreover, genetic connectivity was found between *Ae. aegypti* specimens from Semarang, Bogor, and Jakarta with those from Malaysia, the US and Cape Verde. Given that Jakarta and Semarang are connected by the North coast road and international sea/airports. Thus, human movement could have assisted the expansion of *Ae. aegypti* vectors into this regions.

5. Conclusions

The great genetic differentiation found among *Ae. aegypti* populations in Java Island could indicate a potential variations in vector capacity and the transmission of re-emerging arboviral disease.

Although, the mitochondrial DNA is commonly used in phylogenetic studies, the existence of divergent lineage due to incomplete lineage sorting, as described across different species[43] could pose a challenge. Therefore, combining mitochondrial DNA analysis with nuclear marker would provide a more robust inference for the *Ae. aegypti* species evolutionary history.

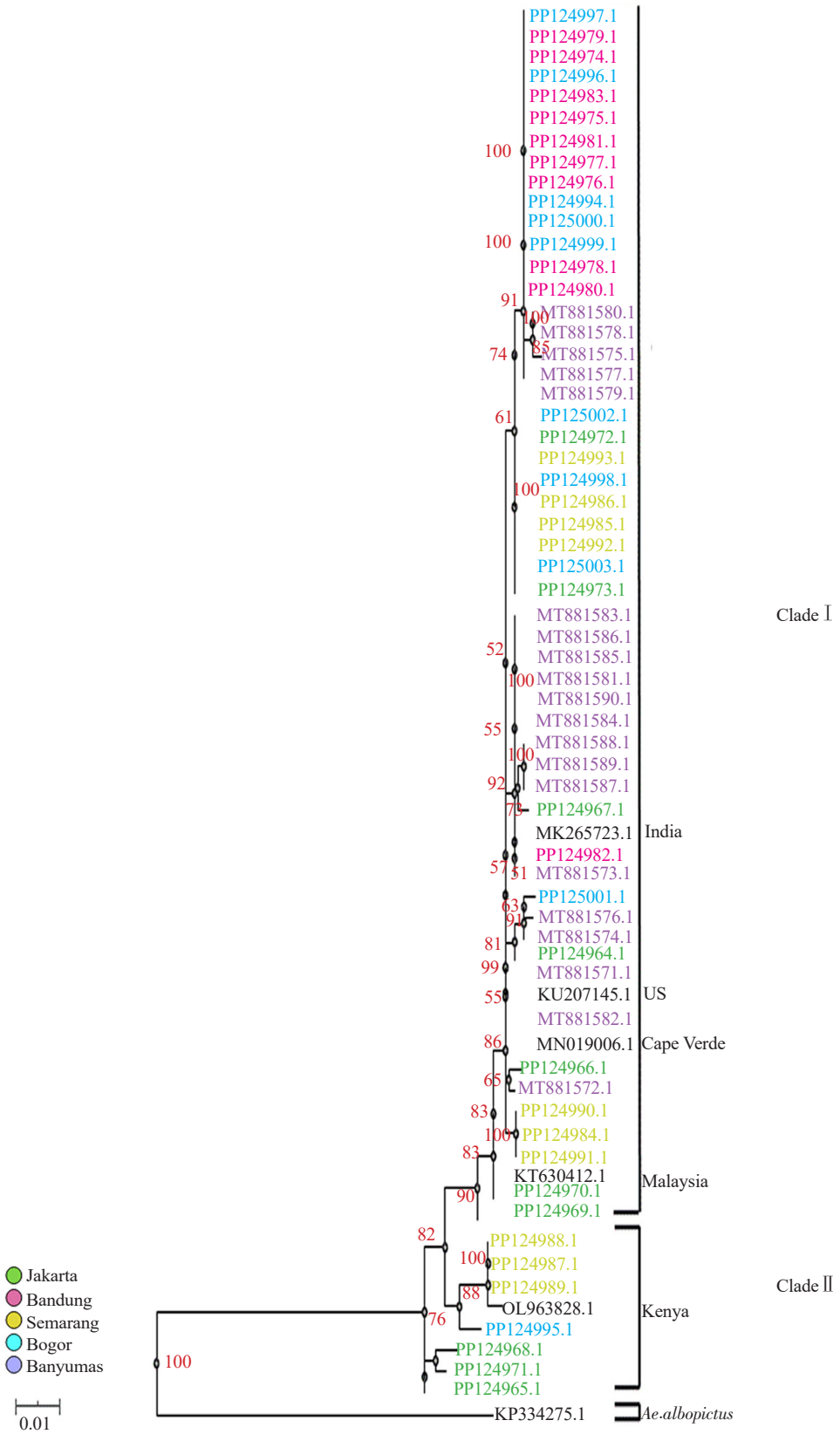


Figure 4. The maximum likelihood phylogenetic tree of COI sequences of *Ae. aegypti* species. Bootstrap values<50% were omitted.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

Mohammed MA conducted conception, design, experimental analysis, data analysis and interpretation, manuscript writing. Kusmintarsih ES and Nuryanto A were involved in conception, design, data interpretation, manuscript editing, revision and approval.

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