

SEQUENCE ANALYSIS OF THE CYTOCHROME OXIDASE I GENE IN *Aedes albopictus* ISOLATED FROM TAMAN BUKIT KINRARA AND PJS7, SELANGOR

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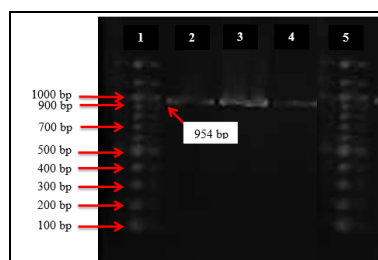
Nurul-Ain Ismail^a, Norazilla Omar^a, Mayamin Hamsidi^a, Nazri Che Dom^a, Rodziah Ismail^a, Abu Hassan Ahmad^b, Afiq Zakia^a, Siti Nazrina Camalxaman^{a*}

*Corresponding author
sitinazrina@salam.uitm.edu.my

^aFaculty of Health Sciences, Universiti Teknologi MARA, 42300 Puncak Alam, Selangor, Malaysia

^bSchool of Biological Sciences, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia

Graphical abstract



Abstract

Aedes albopictus is one of the most invasive mosquitoes in the world that harbors and can transmit many arboviruses, most notably dengue and chikungunya virus. In recent time, *Ae. aegypti* has gained more attention during dengue outbreaks compared to its counter parts, obscuring the role of *Ae. albopictus* as a vector. Moreover, existing data regarding *Ae. albopictus* is also currently limited in Malaysia. Hence, the present study was conducted to determine the genetic diversity of *Ae. albopictus* using molecular techniques from two dengue infested areas in Subang Jaya, Selangor, namely Taman Bukit Kinrara (TBK) and PJS7. Cytochrome oxidase I (COI) gene from field collected mosquitoes were analyzed and compared to the USM laboratory strain (F135) together with sequences from the GenBank. Results from this study revealed that the field collected mosquitoes from TBK and PJS7 are genetically similar with each other. However, the samples exhibited polymorphism with the laboratory strain by 47 variable nucleotide sites. Our local samples are related with the *Ae. albopictus* populations from India, probably due to its migration across these two regions via several human activities. This study shows that the COI gene is a valuable marker for the detection of *Ae. albopictus* and can be utilized to study its worldwide geographical distribution. Further analysis is strongly recommended using larger sample size and different localities to validate and substantiate our findings.

Keywords: *Aedes albopictus*, cytochrome oxidase I gene, genetic diversity, phylogenetic analysis

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1.0 INTRODUCTION

Dengue related cases have substantially increased these past couple of years, affecting millions of people worldwide. In Malaysia, cases has increased by four times since 2013 (1), mainly caused by *Ae. aegypti* and *Ae. albopictus* (2). *Ae. aegypti* has previously received more limelight as a vector (3).

This indoor mosquito reaches contact with human blood for development of their eggs (4). *Ae. albopictus* is becoming an increasing threat and a major public concern in many countries in recent times due to its rapid invasion and ability to transmit infections (5).

Ae. albopictus is otherwise known as the "Asian tiger mosquito" pertaining to its geographical origin from the edge forest of Southeast Asia. It is

preferentially found outdoors and has been considered as a rural vector (6). *Ae. albopictus* was first recorded in Netherland and Italy in 2007, (7) and repeated invasion of this mosquito species has been well documented in Germany (8). Similar scenarios have been implicated in Malaysia as postulated by Dieng *et al.* (4).

In the absence of any commercially available vaccine, vector control strategies have become an important tool to curb its expansion and to alleviate risks. The present study was conducted to determine the genetic diversity of *Ae. albopictus* from two dengue infested areas at Subang Jaya using the mitochondrial DNA encoding cytochrome oxidase 1 (CO1) gene as a genetic marker. The data obtained would probably predict the future expansion of this species within Subang Jaya areas.

2.0 MATERIALS AND METHODS

2.1 Mosquito Sampling and Rearing

Mosquito eggs were collected using ovitraps from Taman Bukit Kinrara (TBK) and PJS7, Selangor. The study locations were selected based on frequent report of dengue cases and its classification as hotspot areas defined by three temporal risk indices (9). Samples were then transported back to the Vector Laboratory in the Department of Environmental Health, UiTM Puncak Alam and were reared until adulthood. Adult female *Ae. albopictus* were morphologically identified as described by Gerberg (10) and fixed in 95% (v/v) ethanol for subsequent analysis. In this study, the USM laboratory strain, was employed as a control.

2.2 Mitochondrial DNA Analysis

After gradual rehydration, total DNA from the whole bodies of individual mosquito was extracted using DNeasy® Blood & Tissue kit (Qiagen, Germany). The CO1 gene in the samples was amplified using the designed primer set: CO1F (5'-TGGAACAGGGTGAACGGTTT-3') and CO1R (5'-TAACGTCGAGGTATCCGGC-3'). The thermal amplification was performed in 50 µL reaction that consists of 25 µL of HotStarTaq Master Mix (Qiagen, Germany) containing 2.5 units of HotStarTaq DNA polymerase, 1X of PCR buffer with 1.5 mM of MgCl₂ and 200 µM of each dNTP; 0.1 µM of each CO1 primer and 2 µL of DNA template. The reaction was conducted with the following thermo profiles: pre-denaturation at 95°C for 1 minute, 35 cycles of denaturation at 94°C for 1 minute, annealing at 68°C for 1 minute and extension at 72°C for 1 minute. The final extension was performed at 72°C for 4 minutes. The amplicons were directly detected on 1.5% (w/v) agarose gel electrophoresis. The amplified fragments were then purified using GeneJET Gel Extraction and

DNA Cleanup Micro kit (Thermo Scientific, Lithuania) and subjected to sequencing.

2.3 Phylogenetic Analysis

The obtained sequences were analyzed using Chromas Lite 2.0 software and aligned with the reference sequences of CO1 available in the GenBank database using the ClustalX 2.1 (11). Phylogenetic analysis was conducted using MEGA v.5.2.2 (12) and the phylogenetic tree was inferred using maximum-likelihood method with 1000 bootstrap replicates of Tamura 3-parameter model (13).

3.0 RESULTS

Amplification of the CO1 gene using the designed primer yielded a 954 bp for both samples; TBK and PJS7 (Figure 1). Further sequencing of both amplicons confirmed the detection of *Ae. albopictus* in all analyzed samples.

Polymorphism was analyzed through multiple sequence alignment of the CO1 gene between the samples alongside reference sequences retrieved from the GenBank. Our findings show the both sample sequences are indistinguishable between one another and displayed strong genetic similarities. Nevertheless, differences in the nucleotide sequences are evident when compared to the USM laboratory strain, LS, sequence. Both of the sample sequences revealed polymorphisms with 47 variable nucleotide sites (Figure 2).

Aligned sequences of the CO1 gene (486bp) was used to construct a dendrogram as depicted in Figure 3. Based on the variations, our results signifies that *Ae. albopictus* from different regions are clustered into four well-defined groups, namely Group 1, Group 2, Group 3 and Group 4. Among the groups, Group 1 is the largest group that comprises of several populations of *Ae. albopictus* from different countries with different environmental climates.

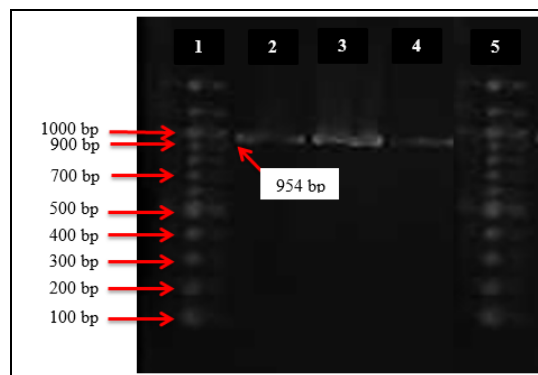


Figure 1 PCR products of the CO1 fragments were electrophoresed on a 1.5% (w/v) agarose gel. Lane 1 and 5: 100 bp DNA ladder (New England Biolabs, UK). Lane 2: USM laboratory strain (F135). Lane 3: Sample of TBK. Lane 4: Sample of PJS7

Meanwhile, the individuals from Indonesia are resolved in Group 2. Although both samples and the laboratory strain seem to share the same internal node, they eventually have diverged over the time. They are grouped into two groups, namely Group 3 and Group 4. Within Group 3, mosquito samples of TBK and PJS7 are more closely related to each other as compared to mosquitoes of Chetakkal, India. All these three forms a clade that is a sister to the clade composed of the laboratory strain, including the Indian populations from Mananthawadi, Mumbai and Saharanpur.

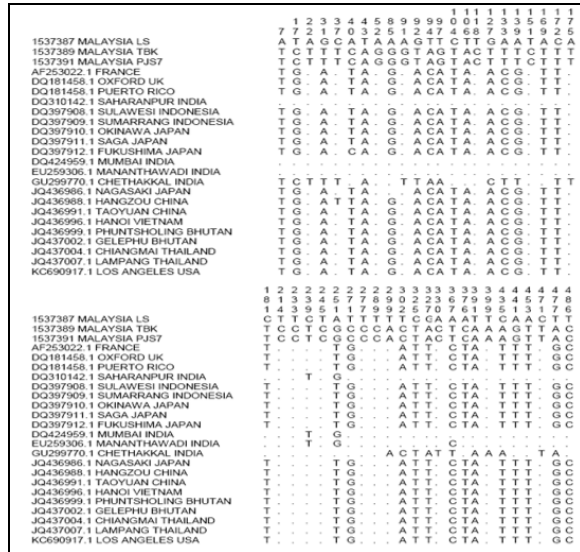


Figure 2 Sequence alignment showing variable nucleotide sites of the CO1 gene of *Ae. albopictus*. The number at the top of the alignment indicates the position of nucleotides in the sequence

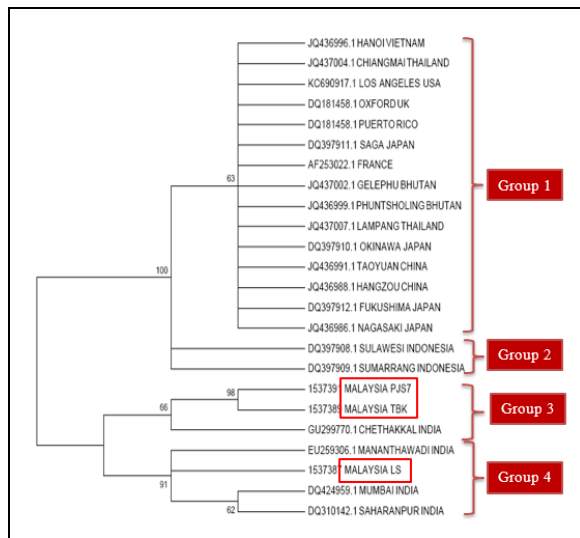


Figure 3 Evolutionary relationship of *Ae. albopictus* based on the CO1 gene analysis. The evolutionary relationship was inferred using maximum-likelihood method with 1000 bootstrap analysis using MEGA v.5.2.2 software. The analysis involved 24 nucleotide sequences with the total of 456 positions in the final data sets

4.0 DISCUSSION

Determination of genetic variations among *Ae. albopictus* is important in the designation and advancement of vector surveillance. With this regard, the pilot study was conducted to detect *Ae. albopictus* for the purpose to describe its genetic diversity and geographical distribution using the CO1 gene. This mitochondrial gene is a well-known marker that has been extensively used in the genetic study of *Ae. albopictus* worldwide (14)(15), due to its highly conserved nature across the taxa (16). To date, Futami *et al.*, (17) has used the CO1 gene to identify mosquito species and to determine their geographical distributions in Costa Rica and Panama.

Based on Figure 1, PCR products of the described primer consisted of 954 bp. DNA extracted were intact and high purity. As stated by Gibson *et al.* (18), the conditions of the DNA samples of the specific target are crucial factors for successful amplification to occur. The designed primer proved to be successful and reproducible, hence can be used in subsequent barcoding studies.

Sequence variation was detected in both samples, TBK and PJS7, which presumptively indicates they inherit similar genetic traits. This observation could be manifested independently by several factors. First, passive transports of the mosquito via human activities could expand its population size, thus hindering its genetic variability within the CO1 gene (19). Secondly, it may be attributed by *Wolbachia* infection, which could alter the polymorphisms of the CO1 gene in *Ae. albopictus* (20). This view narrates the ones by Shaikevich and Zakharov (21), whose study of CO1 polymorphisms focused on *Culex* species. Thirdly, reduction of mosquito population size following exposure to insecticides in tandem with vigorous vector control efforts could also be a contributing factor (22).

The observed variation in the CO1 gene between the samples and the USM laboratory strain may occur due to base substitution mutations namely transition and transversion during the process of reproduction. Nevertheless, based on Figure 2, the transition of (C <--> T) is more pronounced compared to the transition of (A <--> G). This finding is different to that of Shaikevich and Zakharov (21), whereby polymorphism of CO1 gene in *Culex* species was mainly due to the nucleotide transition of (A <--> G).

Phylogenetic analysis was performed in order to evaluate the evolutionary relationships of *Ae. albopictus* based on the CO1 sequence, yielding four well-defined groups (Figure 3). TBK and PJS7 exhibit close genetic similarities and are therefore clustered into the same group (Group 3), which is closely related with the *Ae. albopictus* populations from India. This can be correlated with the migration of mosquitoes across these two regions via several human activities such as human travelling or via international trades of goods (23). Furthermore, India

also imports natural rubber from Malaysia for the production of car tires. With respect of Subang airport as a trade hub, such activities may further intensify the mosquito to migrate and well facilitate its colonization in new habitats for breeding, thus disseminating its genetic traits and epidemic risks. This highlights the importance of targeted vector control to curb its future expansion of *Ae. albopictus* worldwide.

5.0 CONCLUSIONS

Overall, the designed primer proved successful in the detection of *Ae. albopictus* and could be used for subsequent mosquito analysis. Besides, the analysis of the *CO1* gene provided new insights into the genetic diversity and geographical distribution of *Ae. albopictus* which could catalyze further research on a larger scale. Since the present study only incorporates two dengue infested areas, further investigation with the greater sampling sites is strongly recommended as it could clearly portray the biodiversity of this mosquito in our country which in turn may help improve current vector control strategies.

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