Genetic variation of *Aedes aegypti* mosquitoes across Thailand based on nuclear DNA sequences

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**A R T I C L E   I N F O**

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**A B S T R A C T**

The *Aedes aegypti* L. mosquito is the primary vector of dengue viruses in Thailand, where dengue disease is a major public health problem in both urban and rural areas. Understanding the genetic variation of *Ae. aegypti* populations can help to understand the distribution, population structure and gene flow of this species. Single nucleotide polymorphism (SNP) markers were used to analyze the genetic variation of 21 *Ae. aegypti* populations collected across six geographic locations in Thailand. Nuclear DNA sequences of four putative neutral fragments located on different chromosomes were examined. An average of 14 SNPs per kb was detected per population. Tajima’s D statistical test showed no significant deviation from the neutral equilibrium model in the majority of populations, suggesting that the detected patterns of variation were under random mutation and genetic drift equilibrium. Relatively low genetic differentiation was detected between all mosquito populations.

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**Introduction**

Dengue is the most common and widespread mosquito-borne viral infection in the world (Gubler, 2002; Bhatt et al., 2013). Dengue viruses, the cause of dengue fever and more severe disease, are transmitted to humans by the infective bite of the female *Aedes aegypti* (L.) mosquito, the most important species among potential natural vectors in the subgenus *Stegomyia* and moreover, this mosquito species is also responsible for transmitting chikungunya and Zika viruses throughout Southeast Asia, Africa and the Americas (Higgs and Vanlandingham, 2015; Paixão et al., 2016). In Thailand, *Ae. aegypti* was first reported in 1907 and is believed to have spread from Southeast Asia into the Pacific Region during World War II (Gubler, 1998). This mosquito species is widespread in urban and rural areas of Thailand and is a major public health threat and contributor to disease burden in communities (Bureau of Epidemiology, Department of Disease Control, 2014).

The study of population genetics involves the comparison and estimation of temporal heredity changes for describing patterns of genetic diversity in natural populations and developing genotypic maps. Data from population genetic studies of *Ae. aegypti* are useful for a better understanding the epidemiology of dengue transmission and improving vector control. Genetic structure analysis allows identification of genetic differentiation between individuals in subpopulations across areas (Brown et al., 2011; Gloria-Soria et al., 2016).

Mitochondrial DNA (mtDNA) is a commonly used genetic marker for studying molecular diversity in animals. However, the appearance of nuclear mitochondrial pseudogenes (Numt) in the nuclear genome of *Ae. aegypti* may result in over amplification of mtDNA or even the targeting of actual mtDNA sequences (Hlaing et al., 2009), potentially causing serious complications when analyzing population genetic studies using mtDNA alone (Hazkani-Covo et al., 2010). The use of nuclear DNA is intended to overcome this potential problem with *Ae. aegypti*. As others have demonstrated, the use of nuclear DNA for studying *Ae. aegypti* population genetics appears an acceptable alternative for avoiding some inherent limitations with using mtDNA (Crawford et al., 2017; Pless et al., 2017).
In this study, single nucleotide polymorphism (SNP) markers were used to analyze patterns of genetic variation and population differentiation in four putative neutral fragments (Ts1, AelMUC1, ApoLp-2 and CPA) of *Ae. aegypti*. The Ts1 (Transferrin) gene is located on chromosome I and is involved in iron transport (Harizanova et al., 2005). The AelMUC1 (Mucin-like protein) gene is located on chromosome II and is involved in the degradation of proteins in the digestive tract (Isoe et al., 2009). The four analyzed genes were chosen based on previous study (Paduan and Ribolla, 2009) that represented a suitable marker for *Ae. aegypti* genetic study. Moreover, the reliability of polymerase chain reaction (PCR) amplification was also a concern for the markers selected in this study.

**Materials and methods**

**Mosquito samples and DNA extraction**

Immature and adult stage mosquitoes were collected across Thailand between 2012 and 2014 (Fig. 1; Table 1). Larvae were collected in artificial containers and adults were trapped using sweep nets in and around homes. In total, 21 mosquito populations were collected from six geographic locations of Thailand, including two insular populations (Phuket and Chang islands). Immature mosquitoes were reared to adults. All adult specimens were identified to the species level using standard morphological characters (Rueda, 2004), and stored at −20°C until genomic DNA extraction using GF-1 Tissue DNA Extraction kits (Vivantis; USA). Ten adult female mosquitoes from each location were used for DNA extraction and further analysis.

**DNA amplification and sequencing**

DNA samples were amplified using PCR with four primer pairs of the coding regions described (Ts1, AelMUC1, ApoLp-2 and CPA genes), and PCR product sizes of 1095, 429, 321 and 261 bp, respectively. Primer pairs of the Ts1, AelMUC1 and ApoLp-2 genes were obtained from previous work (Paduan and Ribolla, 2009); whereas, primers for the CPA gene (forward: 5’ TGGACGGCCCTC-GAATCAC 3’ and reverse: 5’CAGCTCCAACAGCCCTCAGAC 3’) were newly designed using the exon-primed, intron-crossing method (He and Haymer, 1997). A total volume of 25 μl comprised the PCR mixture, containing approximately 100 ng of DNA template, 10X buffer, 50 mM MgCl2, 10 mM dNTPs, 10 μM forward primer, 10 μM reverse primer and 1 Unit Taq polymerase (Vivantis; USA). The PCR products were purified and sent to Macrogen Inc. (Seoul, Korea) for sequencing. Sequences were aligned using the BioEdit 7.2.5 software (Hall, 1999) and edited manually afterwards.

**Population genetic analyses**

Genetic diversity parameters number of SNPs, haplotype ratio, nucleotide diversity, and statistical tests of neutrality (Tajima’s D test) (Tajima, 1989), and Fu and Li’s D test (Fu and Li, 1993) were estimated using the DnaSP 5.1 software (Librado and Rozas, 2009).

The total fixation index (FST) value based on the method of Hudson et al. (1992) and pairwise FST values based on Slatkin (1995) were used to measure population differentiation. The levels of genetic differentiation within and among the 21 populations, and within and among the six geographical regions were estimated using the analysis of molecular variance (AMOVA), with the Arlequin 3.5 program (Excoffier and Lischer, 2010).

**Results**

**Genetic diversity**

The genetic diversity of 21 *Ae. aegypti* mosquito populations across Thailand was estimated based on four putative neutral DNA sequences: Ts1, AelMUC1, ApoLp-2 and CPA fragments. In total, 106, 27, 10 and 23 SNPs were detected from the sequence lengths of 1,095, 429, 321 and 261 bp sequences, respectively. A mean of 14 SNPs per kilobase (Kb) was observed from the distribution of SNPs among the four nuclear DNA genes. Estimates of nucleotide diversity (π) ranged from 0.009 (Apolp-2) to 0.019 (AelMUC1), and (θw) ranged from 0.005 (Apolp-2) to 0.016 (Ts1) (Table 2).
Consistently low levels of genetic diversity ($\pi$ and $\theta_w$) were detected from the Bangkok population in all four genes (Fig. 2A–D). Moreover, the lowest levels of nucleotide diversity were also seen in the Bangkok population compared with the estimated diversity of populations from the other six locations in Thailand (Fig. 3).

Statistical neutrality tests (Tajima’s D and Fu and Li’s D (one-tailed) tests) were used to measure deviation from neutral equilibrium expectation. Fragment sequences of the Tsf, AelMUC1 and ApoLp-2 genes showed positive D values, while fragment sequences of the CPA gene produced a negative value (Table 2). A positive Tajima’s D value indicates an excess of intermediate frequency polymorphisms in the population, while a negative Tajima’s D value indicates an excess of low frequency polymorphisms. No significant Tajima’s D value was found in fragment sequences of the Tsf, ApoLp-2 and CPA genes ($p > 0.05$), suggesting that these fragments are under neutral equilibrium control. In contrast, a significant positive Tajima’s D value (1.968) was found in a fragment sequence of the AelMUC1 gene ($p < 0.05$), indicating it is not under neutral equilibrium control. Moreover, it was also found that most of the 21 populations showed no significant deviations from the neutral equilibrium model. Fu and Li’s D tests indicated none of the fragments had a significant deviation from the neutral equilibrium model. A positive Fu and Li’s value was observed for fragments of the AelMUC1, ApoLp-2 and CPA genes, indicating an excess of polymorphisms on internal branches (intermediate frequency polymorphisms). In contrast, a negative Fu and Li’s D value was shown in a fragment of the Tsf gene, indicating an excess of polymorphisms on external branches of the genealogy (singletons).

**Genetic structure**

Genetic differentiation among the 21 mosquito populations was estimated using the total $F_{ST}$ values of Hudson et al. (1992). A relatively low level of genetic differentiation was detected from all

**Table 2**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sample size</th>
<th>Length (base pair)</th>
<th>Number of SNPs</th>
<th>Haplotype ratio</th>
<th>Nucleotide diversity</th>
<th>Test of neutrality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\pi^a$</td>
<td>$\theta_w^b$</td>
</tr>
<tr>
<td>Tsf</td>
<td>204</td>
<td>1095</td>
<td>106</td>
<td>0.42</td>
<td>0.017</td>
<td>0.016</td>
</tr>
<tr>
<td>AelMUC1</td>
<td>208</td>
<td>429</td>
<td>27</td>
<td>0.29</td>
<td>0.019</td>
<td>0.011</td>
</tr>
<tr>
<td>ApoLp-2</td>
<td>208</td>
<td>320</td>
<td>10</td>
<td>0.06</td>
<td>0.009</td>
<td>0.005</td>
</tr>
<tr>
<td>CPA</td>
<td>208</td>
<td>261</td>
<td>23</td>
<td>0.19</td>
<td>0.011</td>
<td>0.015</td>
</tr>
</tbody>
</table>

SNP = single nucleotide polymorphism.

* $p < 0.05$.

a Nei (1987).

b Watterson (1975).

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Fig. 1. Collection sites (by province) of sampled *Ae. aegypti* populations in Thailand.
fragments, suggesting low evolutionary separation between the 21 population samples across Thailand (Table 3).

In AMOVA analysis, low variation among all populations (0.37%) demonstrated a low degree of genetic differentiation (Table 4). Additionally, the genetic separation between the mainland and the two island populations was estimated using pairwise FST values for all four gene fragments. The Chang Island population showed no genetic differentiation from mainland populations (FST = 0.000), while the Phuket Island population showed a non-significant differentiation (FST = 0.0059).

Population clustering of the 21 mosquito populations using the combined data of the four fragment genes was tested using BAPS analysis (Fig. 4). The best cluster pattern for the 21 populations was five (K = 5) with the best log of marginal likelihood. Across the majority of populations, the highest proportion cluster was 36%. This cluster indicated the appearance of a common shared haplotype in 85.7% of sampled populations across Thailand, including the northeastern, northern, southern, eastern, central (Bangkok) and western (Tak province) regions. The least common cluster (5%) was found only in southern and eastern populations (also with a relatively low frequency), indicating a unique haplotype in these populations.

Discussion

Using four nuclear genes, the mean number of SNP sites among 21 populations across Thailand was 14 per kb, corresponding well
with the average number of SNP sites (12 per kb) in a previous study examining 25 nuclear genes in *Ae. aegypti* (Morlais and Severson, 2003). The use of specific SNP sites allows the analysis of variation in a single nucleotide that occurs at a specific position in the genome, and where each variation is present to some appreciable degree within a population. Single nucleotide alterations are usually considered to be point mutations that have been evolutionarily successful enough to recur in a significant proportion of the population of a species.

In terms of nucleotide diversity, both the $\pi$ and $\Theta_W$ values for the Bangkok population showed a consistent low level of diversity in all genes, and the lowest level of diversity among the six geographical locations. The relatively low level of genetic diversity in Bangkok may have been the result of more frequent mosquito control activities in large urban areas. Such control, typically involving the application of insecticides to control immature and adult mosquitoes, may have periodically suppressed mosquito numbers sufficiently to decrease genetic diversity (intraspecific breeding); a finding coincident with the lower number of reported dengue cases (lower transmission) in the central regions of Thailand (Bureau of Epidemiology Department of Disease Control Ministry of Public Health, 2014). In Bangkok, *Ae. aegypti* samples were collected from only one site (in Khlong Toei district). This district is a densely populated, economically depressed community, where most
houses are of modest-to-poor construction and in close proximity to each other (Tonn et al., 1969). These circumstances allow mosquitoes to move more easily from one house to another. Thus, the samples collected from water containers in this locality could have contained siblings (from the same parent), potentially leading to the lower level of genetic diversity seen. However, it was also possible that the immature stage samples collected from the water containers might have represented multiple parental sources.

Most of the 21 *Ae. aegypti* populations sampled showed no significant deviation from the neutral equilibrium model, suggesting that the pattern of genetic variation was most likely explained by the influence of mutation and random genetic drift interaction, rather than influenced by forces of natural selection. Comparing the mainland populations and the two island populations, mosquitoes from Chang Island showed no genetic difference from mainland samples, although the geographic distance between Chang Island and the mainland is greater than between Phuket Island and the mainland. Phuket Island showed slight, but no significant genetic differentiation from mainland populations either. This was not unexpected, since both islands have large and frequent interaction with the mainland and so the opportunity for regular re-introduction of outside populations of *Ae. aegypti* is substantial.

The present data suggested that the 21 *Ae. aegypti* populations sampled across Thailand presented no significant genetic differences between populations, the capacity to transmit dengue and other viruses, as well as strategies to control this important mosquito species could likely be uniform throughout the country.

The BAPS analysis of the fragment sequence data showed the appearance of a common haplotype among a majority (85.7%) of populations using the clustering model, indicating that samples shared similar genetic patterns. Five clusters appeared best for population clustering among the 21 locations across Thailand. A common haplotype appeared in most of the populations and was present in the northeastern, northern, southern, and eastern locations, as well as in the central (Bangkok) and western (Tak

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**Fig. 3.** Nucleotide diversity, $\theta_{w}$ (Watterson, 1975) and $\pi$ (Nei, 1987), of *Ae. aegypti* populations in six geographic regions of Thailand for gene fragments: *Tsf* (A), *AelMUC1* (B), *Apolp-2* (C) and *CPA* (D).

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**Table 3**

Total fixation index ($F_{ST}$) values of sampled *Ae. aegypti* populations across Thailand.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total $F_{ST}$ value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tsf</em></td>
<td>0.06</td>
</tr>
<tr>
<td><em>AelMUC1</em></td>
<td>0.02</td>
</tr>
<tr>
<td><em>Apolp-2</em></td>
<td>0.24</td>
</tr>
<tr>
<td><em>CPA</em></td>
<td>0.08</td>
</tr>
</tbody>
</table>

$^a$ Hudson et al. (1992).

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**Table 4**

Analysis of molecular variance with combined four genes and sampled *Ae. aegypti* populations.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Degrees of freedom</th>
<th>Variance component</th>
<th>Variation (%)</th>
<th>Fixation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among 6 geographic locations</td>
<td>5</td>
<td>0.066</td>
<td>0.37</td>
<td>$F_{CT} = 0.004$</td>
</tr>
<tr>
<td>Among populations within 6 geographic locations</td>
<td>15</td>
<td>1.950</td>
<td>10.77</td>
<td>$F_{SC} = 0.108^*$</td>
</tr>
<tr>
<td>Within populations</td>
<td>187</td>
<td>16.092</td>
<td>88.87</td>
<td>$F_{ST} = 0.111^{*}$</td>
</tr>
<tr>
<td>Total</td>
<td>207</td>
<td>18.108</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^p < 0.05$.  
$^a$ Fixation index among groups.  
$^b$ Fixation index among populations within groups.  
$^c$ Fixation index within populations.
province) populations, indicating clear genetic similarity among populations. A unique haplotype only appeared in some southern and eastern populations.

This study assists in understanding the contemporary genetic structure of *Ae. aegypti* populations in Thailand. However, the small sample size was a possible limitation to the conclusions and that future studies increasing the number of fragments and samples analyzed should provide. The strong similarity of mosquito populations between geographically separated regions in the country has possible epidemiological as well as vector control and pest management implications (such as genetic manipulation and insecticide resistance) for combating virus transmission by *Ae. aegypti*.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand.


