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Genetic structure of the dengue vector *Aedes albopictus* (Skuse) from different developed settlements in Penang Island, Malaysia based on microsatellite markers

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Abstract. The medically important mosquito, *Aedes albopictus* is native to Asia and has become a major health concern in most Asian countries including Malaysia. Being recognized as a dengue vector, a clearer understanding of how mosquito populations are geographically connected, may therefore represent a profound yet significant understanding of control strategies. There are no documented reports on the genetic structure of *Ae. albopictus* populations from different developed settlements inferred from microsatellite DNA markers in Malaysia, particularly in Penang Island (Northern Peninsular Malaysia). Here, we assessed the molecular population genetics of *Ae. albopictus* in terms of their allelic variation, genetic diversity and population structure. A total of 42 mosquitoes were sampled from Jelutong, Batu Maung and Balik Pulau which represented urban, suburban and rural areas in Penang Island respectively and analysed for polymorphism at six microsatellite loci. All of the microsatellite markers were successfully amplified and were polymorphic, showing low genetic structure among geographic populations (F_{ST}= 0.0362). It is supported with admixture individuals observed in STRUCTURE and FCA and this suggests that high gene flow has been experienced between populations. These findings implicate passive dispersal through human-aided transportation; as a factor shaping the genetic structure of *Ae. albopictus* populations in Penang Island.

INTRODUCTION

Aedes albopictus is an important vector of dengue virus (Nelder *et al.*, 2010) in tropical areas and currently the most invasive mosquito species in the world (Zhong *et al.*, 2013). Despite its typical lifetime flight range of 200m (Hawley, 1988), it is able to colonize new areas rapidly, probably due to increasing intercontinental trade. This mosquito typically prefers suburban and rural areas, where the larval habitat occurs in natural containers containing waters such as tree holes, leaf axillae, or bamboo internodes, and artificial containers like tin cans, water tanks, or used tires (Usmani-Brown *et al.*, 2009). The ability to dwell in flexible environments permits its rapid colonization and widespread distribution. With its over 1.6 million inhabitants living in 1048 km^2, Penang Island has been undergoing intensive urbanization over time, resulting in the presence of numerous potential breeding sites which has also

lead to the expansion of the *Ae. albopictus* habitat (Li *et al.*, 2014). The numerous breeding sites (for females to lay eggs) and dense human populations (for females to feed on blood), tend to limit mosquito dispersal, thereby contributing to genetic differentiation (Huber *et al.*, 2002b).

Control of the disease vectored by mosquitoes has mainly been carried out by vector control, most commonly by killing the vectors with various biocides, but control programs based on this strategy have widely been inadequate (Lam, 1993). Extended use of insecticides for dengue control may favour insecticide resistance in mosquito populations (Pasteur and Raymond 1996); furthermore, rebuilding from selected resistant individuals gives rise to a population genetically different from that present originally (Huber *et al.*, 2002b). Therefore, knowledge on the geographical genetic variation in *Ae. albopictus* populations in relation to dengue transmission would be informative.

Population genetic studies of *Ae. albopictus* have been carried out worldwide as the species continues to spread and displace *Aedes aegypti* in some areas (Gratz, 2004). Different genetic markers have been used to study the population genetic structure of *Ae. albopictus*, such as cytochrome oxidase subunit 1 (COI) gene (Kamgang *et al.*, 2011; Zhong *et al.*, 2013; Beebe *et al.*, 2013; Zawani *et al.*, 2014; Ismail *et al.*, 2015), microsatellite loci (Kamgang *et al.*, 2011; Beebe *et al.*, 2013) and mitochondrial DNA regions, including nicotinamide adenine dinucleotide (NAD) dehydrogenase subunit 5 (Usmani-Brown *et al.*, 2009; Kamgang *et al.*, 2011).

Among the numerous molecular markers available in population genetics, microsatellites are one of the most powerful tools developed in recent years (Porretta *et al.*, 2006). Microsatellites are highly variable genetic markers that have been widely used at the intraspecific level in population genetic studies. Microsatellites are also used as popular markers in insect studies because of the high polymorphic, high abundance and highly variable nature of their loci in the genome (Behura, 2006). These features provide the foundation for their successful application in a wide range of fundamental and applicable fields (Chistiakov *et al.*, 2006).

A previous study examining the population genetic structure of *Ae. albopictus* in Penang Island by Zawani *et al.* (2014) using the COI gene marker revealed no variation in the genetic structure of *Ae. albopictus* mosquitoes in Penang area, suggesting further studies using different genetic markers are needed instead of the COI gene which was used in their study in order to confirm their hypothesis. Studies on the genetic variation in vectors is crucial as the data provides raw material for change within populations under pressure and may provide material useful for developing sound control strategies. Therefore, this study was aimed to examine the patterns of genetic structure of *Ae. albopictus* dwelling in different developed settlements (urban, suburban and rural) areas in Penang Island, inferred from microsatellite markers.

MATERIALS AND METHODS

Mosquito collections

The samples of *Ae. albopictus* were collected as larvae using ovitraps from three different areas that represented urban, suburban, and rural settings in Penang Island. The average distance between each area is approximately 21 km (Figure 1).

Urban area is an area that surrounds a city having very little vegetation, many buildings and houses close to each other, high densities of human inhabitants, potable water is often stored indoors for domestic usage due to insufficient water supply (Rahman, 2012). The urban location chosen for this study is Jelutong (5°23'15.9"N 100°18'29.2"E) which is located at the Northeast Penang Island District (Figure 1). It is an urban area containing little vegetation with the land use types being primarily residential and commercial buildings close to each other and having public services.

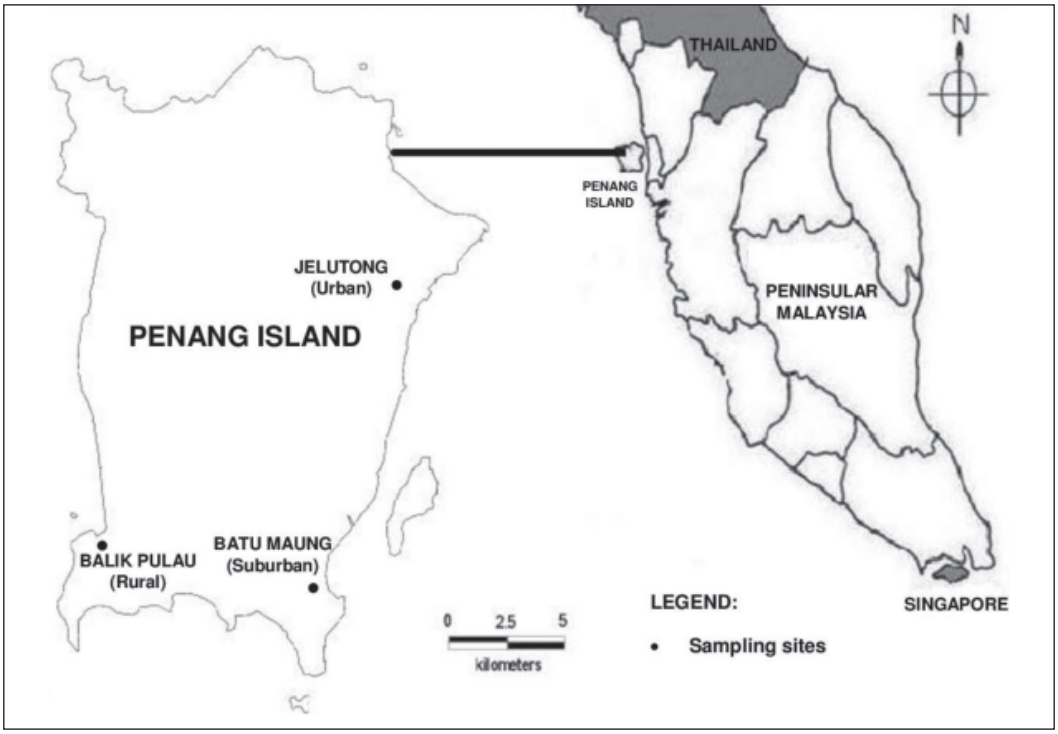

Figure 1. A map of Penang Island showing the location of sites from which *Aedes albopictus* was collected.

Whereas, suburban area refers to a residential area that is in close proximity the main city area and generally consists of some vegetation, less buildings and population density, but greater house yards in comparison to the urban area (Rahman, 2012). The selected suburban location is Batu Maung (5°17'14.5"N 100°16'59.5"E) (Figure 1), an area with land use that includes a mixture of residential and manufacturing buildings, and several small fishing villages. This study site is located at the Southwest Penang Island District.

Rural areas are large and open, isolated from the city, with high vegetation cover such as small forests. The number of houses is smaller compared to urban and suburban areas due to low human population density. The rural study site in this study is Kampung Pulau Betung in Balik Pulau (5°18'13.4"N 100°11'54.0"E) (Figure 1) that has an economy based on fishing and is located at the Southwest Penang Island District.

Each sample from all these localities consisted of pooled larvae from five to fifteen ovitraps, to avoid inbreeding. Fourteen adults from each locality were used in this study (total sample number, n = 42). After five days, the mosquito larvae were transported back to the insectary and were transferred to a tray containing water and food (El-garj *et al*., 2015) for the larvae to develop to adulthood under a temperature of 28.6 ± 1.8°C and a relative humidity of 65-80%. The *Ae. albopictus* wild strain females from each locality were identified morphologically following the method of Rattanarithikul and Panthusiri (1994) and were preserved in individual tubes containing 70% alcohol at -20°C freezer until used for molecular analysis.

DNA extraction

Genomic DNA was extracted from a single adult mosquitoes using the DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Each mosquito was homogenized with the help of a microtube pestle in a 1.5 ul microcentrifuge tube before extracting the

DNA. A volume of 180 ul Buffer ATL and 20 ul proteinase K were added and thoroughly mixed using the vortex mixer. This step was followed by incubation at 65ºC with occasional pulsing of the samples to ensure even dispersal. The sample was further vortexed for another 15 s until the mosquitoes were completely lysed.

A volume of 200 ul of Buffer AL was added to the sample and thoroughly mixed using the vortex. Then, 200 ul of ethyl alcohol (96-100%) was added and vortexed again. The mixture was pipetted into a DNeasy mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min. The flow-through and the collection tube were discarded. The DNeasy mini spin column was then placed into a new 2 ml collection tube.

A volume of 500 ul of Buffer AW1 was added and centrifuged at 8000 rpm for 1 min. The flow-through and collection tube was again discarded and the DNeasy mini spin column was placed into a new 2 ml collection tube. A volume of 500 ul of Buffer AW2 was added and centrifuged at 12000 rpm for 5 min to dry the DNeasy membrane. The flow-through and collection tube were discarded. Then, the DNeasy mini spin column was placed into a new 1.5 ml microcentrifuge tube. A volume of 200 ul of Buffer AE was added and centrifuged at 8000 rpm for 1 min to elute the DNA. The extracted gDNA was stored in -20ºC until ready for the amplification process.

DNA amplification

Genetic polymorphism was assessed at six microsatellite markers which are AealbA9**,** AealbB51, AealbB52, AealbB6, AealbD2, and AealbF3 following Porretta *et al.* (2006) (Table 1). Singleplex Polymerase Chain Reactions (PCRs) were carried out using a PCR thermal cycler PTC-100 Programmable Thermal Controller (MJ Research, Inc.) in 50 µl reaction mixtures according to the manufacturer's guidelines (Promega Corporation, USA), containing 10 µl of 5X Green or Colorless GoTaq® Flexi Buffer, 3 µl of $MgCl_2$ solution (25 mM), 1 µl PCR nucleotide mix (10 mM), 1 µl of each primer, 0.25 µl of GoTaq® DNA Polymerase

Table 1. Six microsatellite DNA loci developed for the *Ae. albopictus* by Porretta *et al.* (2006) were used in this study

Locus	GenBank Accession No.	SSR motif	Primer sequences (5'-3')	$T_{a(°C)}$
AealbA9	DQ366022	$(AC)_4GCAT(AC)_2TC(AC)_8CCAA$ $(AC)_2CG(AC)GT(AC)C(AC)AT(AC)$	F: TGGGACAAGAGCTGAAGGAT R: 5'6-FAM_CTCGTTCTCTAC TCTCTCCGTT	52
AealbB51	DQ366023	$(AC)_3T(AC)_2AA(AC)AAA(AC)_3$ $AA(AC)AT(AC)_2T(AC)_2$	F: 5'6-FAM_TCCACGTGGTAT AACTCTGA R: GTAGTTGTCCAATTAACATCG	50
AealbB52	DQ366024	$(AC)A(AC)A(AC)_2...(AC)_6...(T)_3$ $G(T)_5G(T)_5G(T)_4GGG(AC)_3$	F: GGGTCTAGAAGTAATAGCGATG R: 5'6-FAM_GCATTCTTTGCTT CTGTTTGC	50
AealbB6	DQ366026	$(AC)_1AT(AC)_7GC(AC)_2GCAT$ $(AC)_6AG(AC)$	F: ATGAGGTGACCCTTTTGTGC R: 5'6-FAM_AAATTTTATAGGG CCCTCGG	50
AealbD2	DQ366021	$(A)_{16}(AC)_9GC(AC)_{22}$	F: 5'6-FAM_GAATCCCACACAGCG TCTTT R: GGTCGCTTGACACCTTGAAT	55
AealbF3	DQ366027	$(AC)_6AT(AC)_3AAAA(GC)_2$	F: 5'6-FAM_CTCGTGAGTACGTTC CGTGA R: AGGGAAACAAGGACTTCATCA	53

(5u / µl), 3 µl of template DNA, and 30.75 µl of double distilled water.

Samples were initially denatured at 94°C for 3 min, and then processed through 35 cycles, consisting of a denaturation step at 94°C for 45 s, an annealing step for 45 s according to T_a (°C) as given in Table 1 and an extension step at 72°C for 45s. The final elongation step was at 72°C for 5 min. An additional extension was performed at 72°C for infinite time.

Amplification products were then resolved in 2% agarose gel electrophoresis and visualized under ultraviolet light using GelDoc-It® TS 310 Imaging System (Ultraviolet Products Ltd.) to verify if the band was present before being subjected to fragment analysis. The amplification products were sent out to Mytacq Bioscience Enterprise (Selangor, Malaysia) for fragment analysis service using Applied Biosystems 3730XL DNA Analyzer. The scoring was done by using GeneMapper ID v3.2 (Applied Biosystems) with the assistance of Peak Scanner Software v1.0 (Applied Biosystems).

Data analysis

For each population, genetic variability parameters (allelic frequencies, observed and expected heterozygosities under Hardy-Weinberg equilibrium) and genotypic differentiation among populations and estimation of F_{IS} (inbreeding coefficient) values were assessed using FSTAT version 2.9.3.2 (Goudet, 1995) and GENEPOP software version 4.2 (Raymond and Rousset, 1995) respectively. Differentiation between populations was examined by F-statistics, computed according to Weir and Cockerham (1984), using GENEPOP software version 4.2 (Raymond and Rousset, 1995).

A hierarchical analysis of molecular variance (AMOVA) was performed on all populations to infer the relative attribution of variance among populations, among individuals within populations and within individuals using Arlequin version 3.5.2.2 (Excoffier *et al.*, 2005). To assess isolation by distance between three populations used in this study, the Mantel test (Sokal, 1979) was performed. This analysis was conducted to determine whether the matrix of genetic distance by population pairwise difference (F_{ST}) correlates with the matrix of geographic distance (km) by using Arlequin version 3.5.2.2 (Excoffier *et al.*, 2005).

The Factorial Correspondence Analysis (FCA) was done as a complementary approach to a univariate test like F_{ST} since multilocus population genetic data are multivariate in nature (Guinand, 1996). It was employed to assess population subdivision on pairwise genetic distance among 42 individuals from three *Ae. albopictus* populations. GENETIX version 4.05 (Belkhir *et al.*, 2004) was used to perform FCA based on genotypic data obtained for individuals from the populations.

To determine and assign individuals to their respective source populations based on multilocus genotypic data, STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000) was utilized where the number of groups, k was probabilistically determined using the program. An assumption of correlated allele frequency among populations (Falush *et al.*, 2003) and admix model was used with the burn-in period each at 10,000 of 10 iterations. An examination of $K = 1$ to 3 was performed and the true number groups, K was determined. The highest peak of $\Delta K = K$ was chosen, where k is the most probable number of groups for the entire dataset.

RESULTS

Genetic variability

A total of 42 individuals from three populations of *Ae. albopictus* in Penang Island, Malaysia were successfully genotyped and scored for all six microsatellites loci. No evidence for scoring error due to null allele, large dropout or stuttering was detected after assessing with Micro-checker software (Van Oosterhout *et al.*, 2004).

All six loci investigated were found to be polymorphic, showing a number of distinct alleles ranging from 2 (AealbB51 and AealbB52) to 14 (AealbA9 and AealbB6)

(Table 2) which were similar to a study by Kamgang *et al.* (2011) who found allele number per locus ranging from 2 at locus AealbB51 to 17 at loci AealbA9 and AealbB6. Locus AealbB51 and AealbB52 were the least polymorphic, being monomorphic in most populations, which might be due to the presence of null alleles.

Across all loci, the total number of alleles (N_A) detected and the mean allelic richness (A_R) ranged from 26 (Jelutong) to 31 (Batu Maung and Balik Pulau) and from 4.333 (Jelutong) to 5.167 (Batu Maung and Balik Pulau) respectively. The mean observed heterozygosity (H_O) of alleles per locus ranged from 0.077 to 0.082 while the mean of expected heterozygosity (H_E) ranged from 0.079 to 0.099 for *Ae. albopictus* as shown in Table 2.

Hardy-Weinberg equilibrium (HWE) and inbreeding coefficient (F_{IS})

Each locus was tested separately for significant departure ($P < 0.05$) from HWE. The observed heterozygosity varied from 0.0200 to 0.1200 while the expected heterozygosity ranged from 0.0192 to 0.1200 (Table 3). Deviations from the HWE were found in 10 out of 18 tests in the three populations. These were observed in locus AealbA9 (Batu Maung and Balik Pulau), locus AealbB51 (Jelutong), locus AealbB52 (Batu Maung and Jelutong), locus AealbB6 (Batu Maung and Balik Pulau), locus AealbD2 (Batu Maung), locus AealbF3 in Balik Pulau and Jelutong. All these departures were associated with positive F_{IS} values, reflecting heterozygosity deficits. Inbreeding coefficient (F_{IS}) over all loci showed that most of the populations had high deficiency of heterozygosity. F_{IS} with 0 value indicates no inbreeding. While, F_{IS} value of 1 means the presence of complete inbreeding.

Genetic comparison of populations of *Aedes albopictus* in Penang Island

We examined the population structure of *Ae. albopictus* mosquitoes collected from urban, suburban and rural locality by analysing 6 loci exhibiting departures of HWE. Significant differentiation among populations, (F_{ST}) was observed between all *Ae. albopictus* pairwise comparisons for all loci (Table 4). According to Wright's values when $F_{ST} = 0$ there is no genetic divergence, $F_{ST} > 0$-0.05 indicates low genetic differentiation, $F_{ST} > 0.05$-0.15 indicates moderate differentiation and $F_{ST} > 0.15$-0.25 indicates high genetic differentiation.

As shown in Table 4, low genetic differentiation was observed between *Ae. albopictus* populations from Batu Maung and Jelutong as well as Batu Maung and Balik Pulau with $F_{ST} = 0.0352$ and $F_{ST} = 0.0219$ respectively. Conversely, a moderate value was observed between *Ae. albopictus*

Table 2. Allelic richness (A_R), no. of alleles (N_A) and the mean allele no. of observed (H_O) and expected heterozygosity (H_E) at each locus

Locus	Population	Jelutong (Urban)	Batu Maung (Suburban)	Balik Pulau (Rural)	Total
	N	14	14	14	42
AealbA9	A_RN_A	6.0006	10.00010	7.0007	9.22014
AealbB51	A_RN_A	2.0002	1.0001	1.0001	1.9202
AealbB52	A_RN_A	2.0002	2.0002	1.0001	1.9952
AealbB6	A_RN_A	7.0007	9.0009	9.0009	9.25014
AealbD2	A_RN_A	4.0004	5.0005	7.0007	6.91511
AealbF3	A_RN_A	5.0005	4.0004	6.0006	5.3108
Mean	A_R	4.333	5.167	5.167	5.768
	N_A	4.333	5.167	5.167	8.500
	H_O	0.082	0.080	0.077	0.080
	H_E	0.079	0.082	0.099	0.087

Table 3. Population genetic diversity as measured by observed (H_O) and expected (H_E) heterozygosities and F_{IS}

Locus	Population	Jelutong (Urban)	Batu Maung (Suburban)	Balik Pulau (Rural)
	N	14	14	14
AealbA9	H_O	0.1200	0.0800	0.1000
	H_E	0.1081	0.1081	0.1170
	F_{IS}	-0.1143	0.2676	0.1503
	P	0.2069	0.0010*	0.0177*
AealbB51	H_O	0.0000	0.0000	0.0000
	H_E	0.0488	0.0000	0.0000
	F_{IS}	1.0000	–	–
	P	0.0010*	–	–
AealbB52	H_O	0.0000	0.0000	0.0000
	H_E	0.0666	0.0192	0.0000
	F_{IS}	1.0000	1.0000	–
	P	0.0002*	0.0370*	–
AealbB6	H_O	0.1200	0.1100	0.0900
	H_E	0.1081	0.1200	0.1092
	F_{IS}	-0.1143	0.0863	0.1818
	P	0.3507	0.0000*	0.0390*
AealbD2	H_O	0.0700	0.0600	0.0800
	H_E	0.0722	0.0885	0.0862
	F_{IS}	0.0319	0.3305	0.0756
	P	0.1219	0.0183*	0.1571
AealbF3	H_O	0.0200	0.0700	0.0400
	H_E	0.0755	0.0788	0.0837
	F_{IS}	0.7426	0.1165	0.5315
	P	0.0000*	0.0518	0.0000*

H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient (high positive values indicate deficiency of heterozygotes, while small or negative values indicate excess of heterozygotes); P, significance of deviation from HWE ($P < 0.05$); *, significant deviation from HWE.

Table 4. Matrix of pairwise estimates of microsatellite-based F_{ST} across *Ae. albopictus* populations in Penang Island

Locations	Jelutong (Urban)	Batu Maung (Suburban)	Balik Pulau (Rural)
Jelutong	0		
Batu Maung	0.0352	0	
Balik Pulau	0.0502	0.0219	0

populations from Balik Pulau and Jelutong with $F_{ST} = 0.0502$, however, the value is still considered low. Overall differentiation across the three populations used in this study was indicated by a global F_{ST} of 0.0362. All pairwise comparisons F_{ST} revealed insignificant differentiation in all three populations. It is congruent with the molecular variance (AMOVA) performed on all three populations which revealed that 3.63% of the total genetic variance was discovered among the populations while 25.39% was within populations and 70.98% was within individuals indicates minimal

differentiation and genetical homogeneity (Table 5).

The Mantel's test for Isolation by Distance (IBD) provided further support for the presence of a geographical pattern of differentiation in the microsatellite data, clearly identifying a positive and significant relationship between the two factors (r = 0.8075, P = 0.3383) (Figure 2). Furthermore, although differentiation was present amongst sample sites, the magnitude of differentiation was low, with all pairwise comparisons among the Penang Island sites producing F_{ST} of 0.0362.

Genetic Structure

The Factorial Correspondence Analysis (FCA) plots illustrated the small difference and large overlap among individuals from urban, suburban and rural areas of Penang Island. Individuals' scores were plotted into three principal axes and it showed that the three populations can be clustered into one group as the separations were not absolute (Figure 3). Estimates of differentiation, AMOVA and FCA analysis all incorporate information on sampling location to infer differences and similarity among groups of individuals.

As a different approach, Bayesian clustering of individuals was used to infer population structure based on individual genotypes without geographical information. In Bayesian clustering analysis, we identified two clusters (K = 2), separating *Ae. albopictus* populations (Figure 4). No population structuring was detected in urban, suburban and rural *Ae. albopictus* populations, further suggesting lack of major genetic differentiations among these populations. Assignment of the populations into respective groups based on multi-locus

Table 5. Analysis of molecular variance (AMOVA) comparison within populations, among individuals within populations and within individuals of *Ae. albopictus* using six microsatellite markers

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among populations	2	7.190	0.05838	3.63
Among individuals within populations	39	76.464	0.40888	25.39
Within individuals	42	48.000	1.14286	70.98

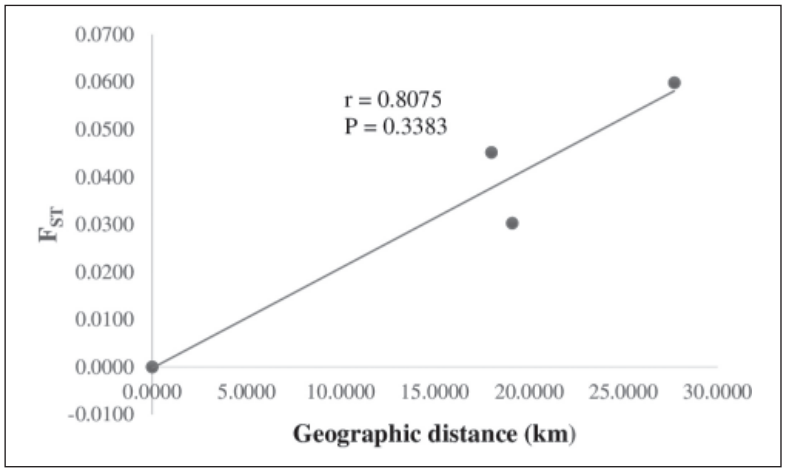

Figure 2. Relationship between pairwise estimates of genetic distance (F_{ST}) and geographical distance (km) for *Ae. albopictus* microsatellite data. Trendline shows the general pattern of increasing genetic distance with greater geographic distance (IBD).

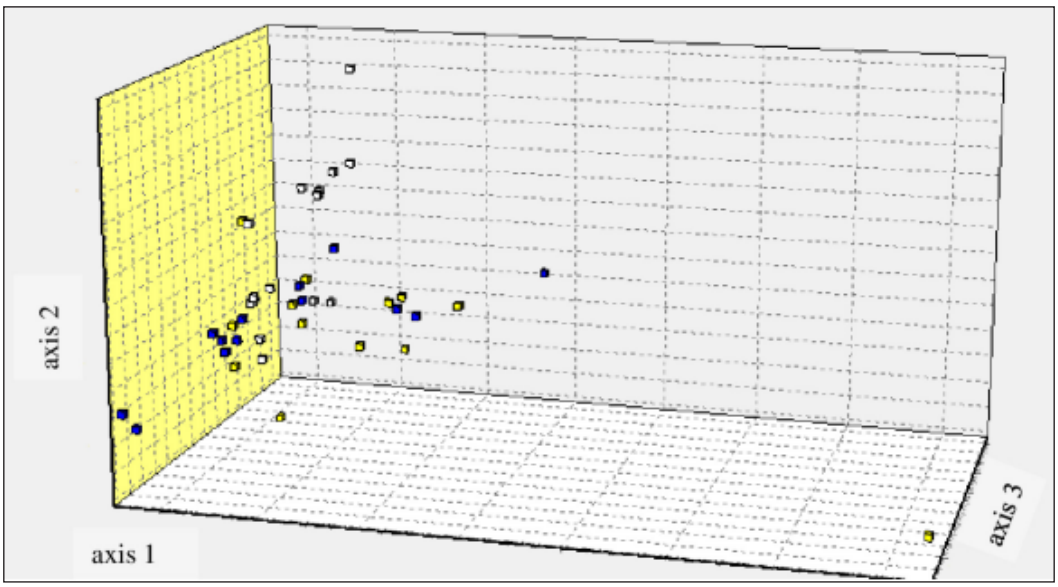

Figure 3. Results of Factorial Correspondence Analysis FCA: all individuals coloured by sample site.
Note: White-Jelutong (Urban), Yellow-Batu Maung (Suburban), and Blue-Balik Pulau (Rural).

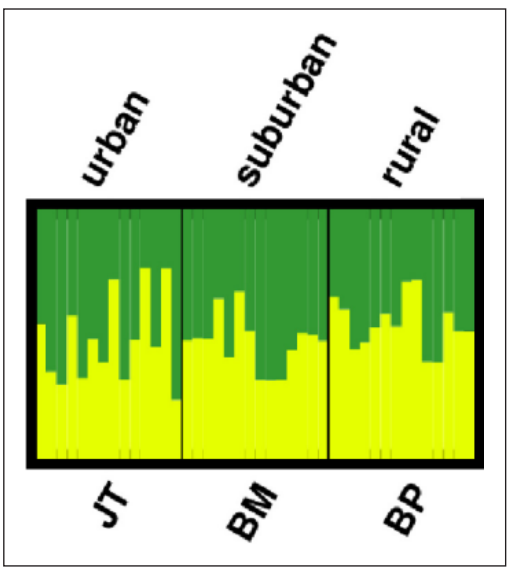

Figure 4. Graphs of cluster membership coefficients estimated by Bayesian inference of population structure (K = 2). Each color indicates a different cluster membership for each *Ae. albopictus* individual genotyped in this study.
Note: JT-Jelutong, BM-Batu Maung, and BP-Balik Pulau.

genotypic data revealed that the segregation of individual populations was correlated with geographical distribution. Figure 4 indicates the coloured bar each respectively to represent the existing genetic clusters and the grouping of each population and individual studied. Multiple colours in the bar plot indicated that they were admixed populations and this case was detected in all of the populations within Penang Island.

DISCUSSION

This is the first study of the genetic structure and differentiation of *Ae. albopictus* populations in Penang Island inferred from microsatellite markers. Mosquito samples from three different settings that represented three different populations in Penang Island were analysed using a set of six microsatellite DNA markers. These set of microsatellite loci have already been tested on an *Ae. albopictus* population from Indonesia (Porretta *et al.*, 2006). The number of alleles detected in this study was significantly higher at all loci with the exception of AealbB51 concordant to a study by Kamgang *et al.* (2011).

Overall, the observed level of polymorphism and the small number of deviations from HWE across the markers

as well as the samples suggested that this set of microsatellite markers is suitable and may be useful for population structure studies of *Ae. albopictus* in Malaysia. In fact, microsatellite markers are currently the most widely used markers to test ecological and evolutionary hypothesis in wild populations and have become an ideal tool for population genetics studies (Ismail, 2013). The high level of variability, co-dominance of alleles allowing assignments of allele frequencies for population level analysis and locus specificity of microsatellites have been the advantages of microsatellites in population genetics studies (Jarne and Lagoda, 1996; Sunnucks, 2000).

Null alleles and linkage disequilibrium

No evidence for scoring error due to null alleles, large allele dropout or stuttering was detected after assessing with Micro-checker software (Van Oosterhout *et al.*, 2004) and no evidence of linkage disequilibrium between loci was found. Therefore, all six loci were retained and used for subsequent analyses.

Population genetic diversity

The numbers of observed alleles for many of the markers were generally higher than those reported by Porretta *et al.* (2006) for one *Ae. albopictus* population studied (Toili, Indonesia). Porretta *et al.* (2006) reported an average of 4.6 alleles per microsatellite locus (range 3-9), whereas we found an average of 8.5 alleles per locus (range 2-14) in three populations. In contrast, Kamgang *et al.* (2011) reported an average of 9.3 alleles per locus (range 1-12) in 12 populations slightly higher than our findings. This might be due to the large number of populations included in their study. The observed heterozygosity was not consistent in all the populations and was often much lower than the expected heterozygosity, indicating an excess of homozygote genotypes at most loci (Table 3).

Hardy-Weinberg Equilibrium (HWE)

Most of the loci showed deviations from HWE due to heterozygote deficiencies, but the occurrence was random among the populations. Heterozygosity deficits are usually caused by inbreeding, selection, Wahlund effect (when there is either a lumping together of separate gene pools), and null alleles (Huang *et al.*, 2008). In this present study, there was no consistent pattern according to locus or population and unequal numbers of heterozygote deficiencies and heterozygote excess were observed. This could be due to population subdivision rather than the existence of null alleles based on the Micro-checker results (Mohamed, 2013).

Genetic differentiation and population structure

All of our samples collected in the urban, suburban and rural area in Penang Island were less differentiated (F_{ST} = 0.0362). However, the samples were assessed according to the urbanization level; mosquitoes from Jelutong (urban) and from Balik Pulau (rural) (F_{ST} = 0.0502) were moderately and significantly differentiated, whereas the mosquitoes from Jelutong (urban) and Batu Maung (suburban) (F_{ST} = 0.0352) were less differentiated correspondingly with the mosquitoes from Batu Maung (suburban) and Balik Pulau (rural) (F_{ST} = 0.0219). Several explanations could be offered for the difference. It has been shown that the type, the density and the location of the breeding sites, as well as the human density as well as human activities in one particular area, shape the mosquito population structure (Huber *et al.*, 2002a; Paupy *et al.*, 2005; Goubert *et al.*, 2016). For instance, in rural areas like Balik Pulau which is known as the back of the island, *Ae. albopictus* dispersal is limited by hills and cultivated forests separating habitations. This is supported by the result of IBD (r = 0.8075, p = 0.3383 Mantel test, 1000 permutations) which shows the positive correlation between geographic distance and genetic differentiation among three populations of *Ae. albopictus*.

Conversely, in urban as well as suburban areas such as Jelutong and Batu Maung respectively, the residential areas are packed and closer to each other.

Especially in the urban areas the number of breeding sites is much higher due to rapid development, and evenly distributed thereby allowing the mosquitoes to be more easily dispersed (Huber *et al.*, 2003). It is also evident that vector control using insecticides by the vector control unit, leads to a disturbance in mosquito populations, by means of modifying their genetic structure (Huber *et al.*, 2003).

The lower F_{ST} estimates in the populations in this study were probably caused by shorter distance between urban to suburban area as well as suburban to rural area (18.0 and 19.1 km respectively) and a lack of hilly topography as gene flow barriers (Chen *et al.*, 2004). The genetic differentiation between populations was small but statistically significant (F_{ST} = 0.0362). Thus, there is a small degree of genetic isolation between populations. The low genetic differentiation between populations in this study is in agreement with the positive relation found between mosquito differentiation (pairwise F_{ST} estimate) and geographic distances (Figure 2). Geographic distances could explain the level of differentiation obtained but it is not a general rule (Failloux *et al.*, 2002; Ravel *et al.*, 2002). The normal flight range of *Ae. albopictus* can be up to 800 m during its entire life-time. However, the distance of mosquito populations in this study ranges from 18 to 27.7 km, farther than the normal flight range of the mosquitoes. According to Nor Atikah (2017), *Ae. albopictus* females from the urban area of Penang Island could fly up to 250 m in the duration of three days. Thus, only passive migration through human transportation has been considered responsible for mosquito dispersal and exchange (Pasteur *et al.*, 1995; Lehmann *et al.*, 1996; Failloux *et al.*, 1997; Huber *et al.*, 2003; Fonseca *et al.*, 2010).

These results indicate highly effective migration and gene flow of *Ae. albopictus* between populations in Penang Island. Passive dispersal and gene flow also has serious implications and impact on many other existing and potential control strategies such as through the spread of insecticide-resistant alleles (Lenormand and Raymond, 1998; Gubler, 2002), sterile males (Lewis and van den Driessche, 1993), disease-refractory genes (James *et al.*, 2006) or *Wolbachia* (Hoffman *et al.*, 2011). Thus, fundamental control practices like elimination or larvicides treatment of breeding sites are crucial in order to control the spread of dengue and its vector instead of better vector control strategies.

Over all loci, this study revealed low genetic differentiation between *Ae. albopictus* populations from Jelutong, Batu Maung and Balik Pulau. This is in agreement with the AMOVA, where the percentage of variation among populations as low as 3.63%. These results are also supported by the results from the FCA of individual genotypes shown in Figure 3, where all the populations are clustered in one group as the separations were not absolute, which presumptively indicates that they inherit similar genetic traits. Bayesian methods implemented in STRUCTURE revealed two genetic clusters (K = 2) comprising two groups of moderate and low genetic differentiation (Table 4). However, with the evidence of FCA in our data, and no expected population structure was detected, the resulting admixed populations in STRUCTURE clusters from urban, suburban and rural area could conclude the genetic structure of *Ae. albopictus* in northern region of Peninsular Malaysia on the whole.

CONCLUSION

We found that *Ae. albopictus* populations from different developed settlements in Penang are characterized by little genetic differentiation and homogenous population structure, likely because of the range of expansion through both active (mosquito dispersal) and passive dispersal (human-aided transportation). Further research should be done by including more extensive sampling and also carrying out a detailed study on insecticide resistance genes of the vector.

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REFERENCES

Beebe, N.W., Ambrose, L., Hill, A.A., Davis, J.B., Hapgood, G., Cooper, R.D., Russell, R.C., Ritchie, S.A., Reimer, L.J., Lobo, N.F. & Syafruddin, D. (2013). Tracing the tiger: population genetics provides valuable insights into the *Aedes (Stegomyia) albopictus* invasion of the Australasian region. *PLOS Neglected Tropical Diseases* **7**(8): e2361.

Behura, S.K. (2006). Molecular marker systems in insects: current trends and future avenues. *Molecular Ecology* **15**(11): 3087-3113.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bonhomme, F. (2004). GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France).

Chen, H., Minakawa, N., Beier, J. & Yan, G. (2004). Population genetic structure of *Anopheles gambiae* mosquitoes on Lake Victoria islands, West Kenya. *Malaria Journal* **3**(1): 48.

Chistiakov, D.A., Helleman, S.B. & Volckaert, F.A.M. (2006). Microsatellites and their genomic distribution, evolution, function and application: a review with special reference to fish genetics. *Aquaculture* **255**(1-4): 1-29.

El-garj, F.M.A., Avicor, S.W., Wajidi, M.F.F. & Jaal, Z. (2015). Comparative efficacy of spatial repellents containing d-allethrin and d-trans allethrin against the major dengue vector *Aedes aegypti* (Linnaeus). *Asian Biomedicine* **9**(3): 313-320.

Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**: 47-50.

Failloux, A.-B., Fouque, F., Vazeille, M. & Rodhain, F. (2002). Isoenzyme differentiation of *Aedes aegypti* populations in French Guiana. *Medical and Veterinary Entomology* **16**(4): 456-460.

Failloux, A.B., Raymond, M., Ung, A., Chevillon, C. & Pasteur, N. (1997). Genetic differentiation associated with commercial traffic in the Polynesian mosquito, *Aedes polynesiensis* Marks 1951. *Biological Journal of the Linnean Society* **60**(1): 107-118.

Falush, D., Stephens, M. & Pritchard, J.K. (2003). Inference of population structure: extensions to linked loci and correlated allele frequencies. *Genetics* **164**(4): 1567-1587.

Fonseca, D.M., Widdel, A.K., Hutchinson, M., Spichiger, S.E. & Kramer, L.D. (2010). Fine-scale spatial and temporal population genetics of *Aedes japonicus*, a new U.S. mosquito, reveal multiple introductions. *Molecular Ecology* **19**(8): 1559-1572.

Goubert, C., Minard, G., Vieira, C. & Boulesteix, M. (2016). Population genetics of the Asian tiger mosquito *Aedes albopictus*, an invasive vector of human diseases. *Heredity* **117**(3): 125-134.

Goudet, J. (1995). FSTAT version 1.2: a computer program to calculate F-statistics. *Journal of Heredity* **86**(6): 485-486.

Gratz, N.G. (2004). Critical review of the vector status of *Aedes albopictus*. *Medical and Veterinary Entomology* **18**(3): 215-227.

Gubler, D.J. (2002). The global emergence/resurgence of arboviral disease as public health problems. *Archives of Medical Research* **33**(4): 330-342.

Guinand, B. (1996). Use of multivariate model using allele frequency distributions to analyze patterns of genetic differentiation among populations. *Biological Journal of the Linnean Society* **58**(2): 173-195.

Hawley, W.A. (1988). The biology of *Aedes albopictus*. *Journal of American Mosquito Control Association* **4**: 1-39.

Hoffmann, A.A., Montgomery, B.L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P.H., Muzzi, F., Greenfield, M., Durkan, M., Leong, Y.S., Dong, Y., Cook, H., Axford, J., Callahan, A.G., Kenny, N., Omodei, C., McGraw, E.A., Ryan, P.A., Ritchie, S.A., Turelli, M. & O'Neill, S.L. (2011). Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* **476**(7361): 454-457.

Huang, S., Molaei, G. & Andreadis, G. (2008). Genetic insights into the population structure of *Culex pipiens* (Diptera: Culicidae) in the Northern United States by using microsatellite analysis. *The American Journal of Tropical Medicine and Hygiene* **79**(4): 518-527.

Huber, K., Loan, L.L., Hoang, T.H., Ravel, S., Rodhain, F. & Failloux, A.B. (2002a). Genetic differentiation of the dengue vector, *Aedes aegypti* (Ho Chi Minh City, Vietnam) using microsatellite markers. *Molecular Ecology* **11**(9): 1629-1635.

Huber, K., Loan, L.L., Hoang, T.R., Tien, T.K., Rodhain, F. & Failloux, A.B. (2003). *Aedes aegypti* in South Vietnam: ecology, genetic structure, vectorial competence and resistance to insecticides. *The Southeast Asian Journal of Tropical Medicine and Public Health* **3**(1): 81-86.

Huber, K., Luu Le, L., Tran Huu, H., Tran Khanh, T., Rodhain, F. & Failloux, A.-B. (2002b). Temporal genetic variation of *Aedes aegypti* populations in Ho Chi Minh City (Vietnam). *Heredity* **89**(1): 7-14.

Ismail, N., Che Dom, N., Ismail, R., Ahmad, A.B., Zaki, A. & Camalxaman, S.N. (2015). Mitochondrial cytochrome oxidase 1 gene sequence analysis of *Aedes albopictus* in Malaysia. *Journal of the American Mosquito Control Association* **31**(4): 305-312.

Ismail, W.N.W. (2013). *Population genetics of the german cockroach, Blatella germanica L., in South-East Asia using microsatellite techniques.* [M.Sc. Thesis]. Universiti Sains Malaysia.

James, A.A., Benedict, M.Q., Christophides, G.K., Jacobs-Lorena, M. & Olson, K.E. (2006). *Evaluation of Drive Mechanisms (including transgenes and drivers) in Different Environmental Conditions and Genetic Background: Bridging Laboratory and Field Research for Genetic Control of Disease Vectors* (ed. by B. G. J. Knols & C. Louis). Dordrecht: Springer.

Jarne, P. & Lagoda, P.J. (1996). Microsatellites, from molecules to populations and back. *Trends Ecology Evolution* **11**(10): 424-429.

Kamgang, B., Brengues, C., Fontenille, D., Njiokou, F., Simard, F. & Paupy, C. (2011). Genetic structure of the tiger mosquito, *Aedes albopictus*, in Cameroon (Central Africa). *PLOS ONE* **6**(5): e20257.

Lam, S.K. (1993). Two decades of dengue in Malaysia. *Tropical Medicine*, **35**(4): 195-200.

Lehmann, T., Hawley, W.A., Kamau, L., Fontenille, D., Simard, F. & Collins, F.H. (1996). Genetic differentiation of *Anopheles gambiae* populations from eastand West Africa: comparison of microsatellite and allozyme loci. *Heredity* **77**: 192-208.

Lenormand, T. & Raymond, M. (1998). Resistance management: the stable zone strategy, *Proceedings of The Royal Society of London, Series B: Biological Sciences* **265**(1409): 1985-1990.

Lewis, M.A. & van den Driessche, P. (1993). Waves of extinction from sterile insect release. *Mathematical Bioscience* **116**(2): 221-247.

Li, L., Kamara, F., Zhou, G., Puthiyakunnon, S., Li, C., Liu, Y., Zhou, Y., Yao, L., Yan, G. & Chen, X-G. (2014). Urbanization increases *Aedes albopictus* larval habitats and accelerates mosquito development and survivorship. *PLOS ONE* **8**(11): e3301.

Mohamed, M.S.E.M. (2013). *Ecology and population genetics of the malaria vector Anopheles arabiensis Patton in the Republic of Sudan.* [Ph.D. Thesis]. Universiti Sains Malaysia.

Nelder, M., Kesavaraju, B., Farajollahi, A., Healy, S., Unlu, I., Crepeau, T., Regavendran, A., Fonseca, D. & Gaugler, R. (2010). Suppressing *Aedes albopictus*, an emerging vector of dengue and chikungunya viruses, by a novel combination of a monomolecular film and an insect-growth regulator. *The American Journal of Tropical Medicine and Hygiene* **82**(5): 831-837.

Nor Atikah, F.M. (2017). Ecological, biological and genetic structure of the dengue vector *Aedes albopictus* from different developed settlements in Penang Island. [M.Sc. Thesis]. Universiti Sains Malaysia.

Pasteur, N. & Raymond, M. (1996). Insecticide resistance genes in mosquitoes: their mutations, migration, and selection in field populations. *Journal of Heredity* **87**(6): 444-449.

Pasteur, N., Marquine, M., Rousset, F., Failloux, A.-B., Chevillon, C. & Raymond, M. (1995). The role of passive migration in the dispersal of resistance genes in *Culex pipiens quinquefasciatus* within French Polynesia. *Genetics Research* **66**: 139-146.

Paupy, C., Chantha, N., Reynes, J.-M. & Failloux, A.-B. (2005). Factors influencing the population structure of *Aedes aegypti* from the main cities in Cambodia. *Heredity* **95**(2): 144-147.

Porretta, D., Gargani, M., Bellini, R., Calvitti, M. & Urbanelli, S. (2006). Isolation of microsatellite markers in the tiger mosquito *Aedes albopictus* (Skuse). *Molecular Ecolology Notes* **6**(3): 880-881.

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* **155**(2): 945-959.

Rahman, G.M.S. (2012). *Recent ecological, physiological and protein profile of the dengue vector population in Penang Island, Malaysia*. [Ph.D. Thesis]. Universiti Sains Malaysia.

Rattanarithikul, R. & Panthusiri, P. (1994). Illustrated keys to the medically important mosquitoes of Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health* **25**(Suppl 1): 1-66.

Ravel, S., Herve, J.P., Diarrassouba, S., Kone, A. & Cuny, G. (2002). Microsatellite markers for population genetic studies in *Aedes aegypti* (Diptera: Culicidae) from Cote d'Ivoire: evidence for a microgeographic genetic differentiation of mosquitoes from Bouake. *Acta Tropica* **82**(1): 39-49.

Raymond, M. & Rousset, F. (1995). Genepop (version 1.2): population genetic software for exact tests and ecumenisms. *Journal of Heredity* **86**(3): 248-249.

Sokal, R.R. (1979). *Ecological Parameters Inferred from Spatial Correlograms In Contemporary Quantitative Ecology and Related Econometrics*. Fairland, Maryland: International Co-operative Publishing House.

Sunnucks, P. (2000). Efficient genetic markers for population biology. *Trends in Ecology and Evolution* **15**(5): 199-203.

Usmani-Brown, S., Cohnstaedt, I. & Munstermann, L.E. (2009). Population genetics of *Aedes albopictus* (Diptera: Culicidae) invading populations, using mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 5 sequences. *Annals of the Entomological Society of America* **102**(1): 144-150.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. (2004). Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Resources* **4**(3): 535-538.

Weir, B.S. & Cockerham, C.C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**(6): 1358-1370.

Zawani, M.K.N., Abu, H.A., Sazaly, A.B., Zary, S.Y. & Darlina, M.N. (2014). Population genetic structure of *Aedes albopictus* in Penang, Malaysia. *Genetics and Molecular Research* **13**(4): 8184-8196.

Zhong, D., Lo, E., Hu, R., Metzger, M.E., Cummings, R., Bonizzoni, M., Fujioka, K.K., Sorvillo, T.E., Kluh, S., Healy, S.P., Fredregill, C., Kramer, V.L., Chen, X. & Yan, G. (2013). Genetic analysis of invasive *Aedes albopictus* populations in Los Angeles County, California and its potential public health impact. *PLOS ONE* **8**(7): e685586.