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Source: Journal of Medical Entomology, 59(2) : 545-553

Published By: Entomological Society of America

URL: https://doi.org/10.1093/jme/tjab194

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Journal of Medical Entomology, 59(2), 2022, 545–553
https://doi.org/10.1093/jme/tjab194
Advance Access Publication Date: 4 February 2022
Research

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Novel and Broadly Applicable Microsatellite Markers in Identified Chromosomes of the Philippine Dengue Mosquitoes, *Aedes aegypti* (Diptera: Culicidae)

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Subject Editor: David Severson

Received 13 July 2021; Editorial decision 27 October 2021

Abstract

Dengue is the leading arboviral infection in the Philippines. Its endemicity in the country is due to the presence of its primary mosquito vector, *Aedes aegypti* (L.). This species has limited microsatellite markers. This study characterized microsatellite markers screened in silico from intergenic regions of the updated reference genome of *Ae. aegypti* from Liverpool, U.K. Criteria for good markers are: polymorphic, inherited in a Mendelian codominant manner, no null alleles, selectively neutral, randomly associated, and broadly applicable across different regions. Genotypes were scored using ABI Peak Scanner and were screened for the presence of null alleles. Hardy-Weinberg equilibrium, linkage disequilibrium, and robustness of the markers were determined by GENEPOP using *Ae. aegypti* samples from selected highland and lowland sites ($n = 30$ each) in the Philippines and outgroups (Thailand and Vietnam). Mendelian codominant inheritance was examined using F1 offspring of *Ae. aegypti* family ($n = 30$ each) derived from samples collected from Cebu city highlands and Maramag, Bukidnon. From the 63 randomly selected markers, nine were polymorphic. Two markers (Aaeg1-3D of chromosome 1 and Aaeg3-4C of chromosome 3) satisfied all criteria, hence, are good broadly useful microsatellite markers. Two other markers (Aaeg2-2E of chromosome 2 and Aaeg3-2A of chromosome 3) met all criteria but deviated from Mendelian codominant inheritance. These new markers of the Philippine *Ae. aegypti* with their chromosomal locations relative to the other published markers are presented, and will ultimately be useful in a variety of population genetic studies of *Ae. aegypti* to protect the public health.

Key words: dengue, microsatellite marker, simple sequence repeat, *Aedes aegypti*, Philippines

Aedes mosquitoes (Diptera: Culicidae) are known vectors of yellow fever, dengue, Zika, and chikungunya viruses. Dengue in particular is a rapidly emerging arboviral infection worldwide (WHO 2017). The Philippines has the primary (*Aedes aegypti* L.) and secondary (*Aedes albopictus* Skuse) dengue vectors and four dengue virus (DENV) serotypes. *Ae. aegypti* has been primarily studied on its evolution with humans owing to its public health consequences (Brown et al. 2011). Due to rapid geographic spreading of *Aedes* vectors brought about by climate change (Liu-Helmersson et al. 2016), the global community is now facing increased public health concerns and expanded socioeconomic burden caused by their associated diseases (Paixao et al. 2018, Liu et al. 2020).

Rapid advances in molecular biology have paved the way to the development of DNA-based marker systems circumventing limitations in morphological, physiological, and cytological markers in assessing population dynamics and genetic diversity. Several DNA markers have been developed to scale up studies and augment limitations of morphological and protein markers. Of prime interest is the microsatellite DNA marker, otherwise known as simple sequence repeat (SSR). Microsatellites are stretches of DNA repeated several times consisting of mono-, di-, tri-, tetra-, and penta-nucleotide units that are dispersed in eukaryotic genomes (Mittal and Dubey 2009). Microsatellites have been specifically used as genetic markers

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for a number of arthropod vectors for human diseases such as the African malaria vector, *Anopheles gambiae* (Giles) (Diptera: Culicidae) (Zheng et al. 1993, Zheng et al. 1996), West Nile virus vector, *Culex pipiens* (L.) (Diptera: Culicidae) (Fonseca et al. 1998, Keyghobadi et al. 2004, Edillo et al. 2007) and the secondary dengue vector, *Ae. albopictus* (Manni et al. 2015, Medley et al. 2015). Not many microsatellite markers of *Ae. aegypti* have been characterized (Slotman et al. 2007, Lovin et al. 2009, Brown et al. 2011) although genetic structure studies of *Ae. aegypti* have been reported elsewhere (Monteiro et al. 2014, Gloria-Soria et al. 2016, Kotsakiozi et al. 2017, Maitra et al. 2019) and in the Philippines (Fonzi et al. 2015, Sayson et al. 2015, Carvajal et al. 2020), to mention a few. Thus, additional microsatellite markers particularly those isolated from the tropics would greatly enhance a variety of studies, including the population genetic structure of this medically important species.

National anti-dengue programs in the Philippines include massive dengue information campaigns, distribution, and training for the use of ovicidal/larvicidal (O/L) traps in schools (DOST 2013) and an enhanced *4S* strategy. The latter stands for search and destroy mosquito-breeding sites, secure self-protection measures, seek early consultation, and support fogging or spraying in hotspot areas (DOH 2019). Despite the extensive efforts, dengue outbreaks are still prevalent amidst climate change. There is a need for a deeper understanding of the genetic structure of *Ae. aegypti* populations in the country to determine strategic and timely vector control measures, however, such studies have been hampered by a limited number of good broadly applicable microsatellite markers.

This study aimed to characterize novel microsatellite markers of *Ae. aegypti* collected from the Philippines. The specific goal was to identify good, polymorphic, and broadly applicable markers of *Ae. aegypti* that will be useful for analyzing population structure studies. For a microsatellite marker to be highly useful, it should meet the following criteria: 1) polymorphic, 2) inherited in a Mendelian codominant manner, 3) absence of null alleles, 4) randomly associated, 5) selectively neutral, and 6) broadly applicable across different regions (Ardren et al. 1999, Oosterhout et al. 2006, Selkoe and Toonen 2006, Edillo et al. 2007, Slotman et al. 2007, Brown et al. 2011). Recently, Brown et al. (2011) characterized their four new microsatellite markers in addition to eight published markers (Slotman et al. 2007) (12-locus dataset) and reported similar population clustering and hierarchical relatedness between the 12-locus dataset and the 10-locus dataset excluding the two most problematic loci of Slotman et al. (2007).

Materials and Methods

Collections of *Aedes aegypti*

Highland and lowland study sites for each of the Philippine big islands (Luzon, Visayas, and Mindanao) were established (Fig. 1). These highland and lowland sites included, respectively, Baguio city (BG) (16.402°N; 120.596°E) and Quezon city (QC) (14.676°N;

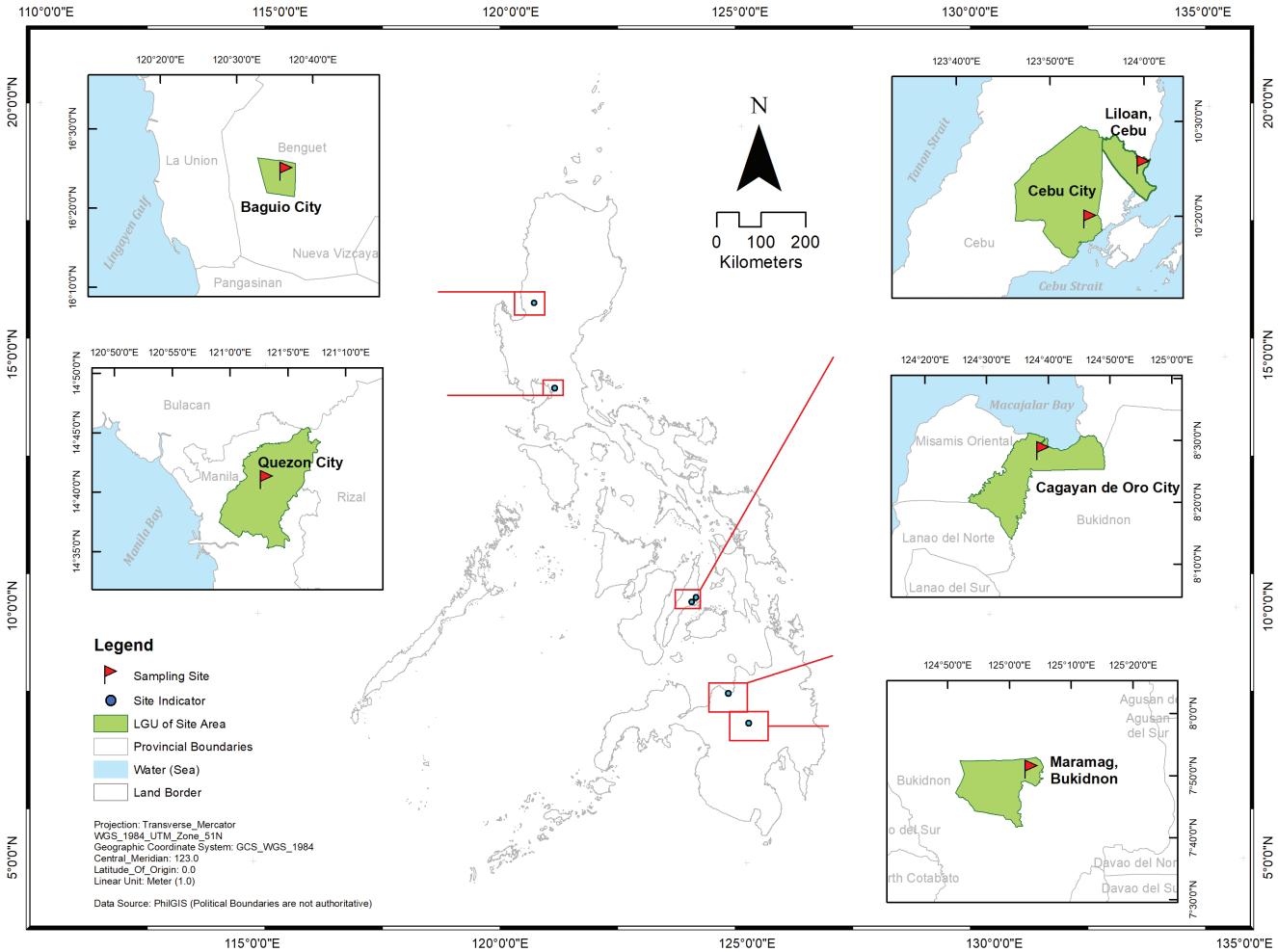


Fig. 1. Map of the Philippines highlighting the six sampling sites from Luzon, Visayas, and Mindanao.

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121.044°E) in Luzon; Cebu city (CC) mountains (10.317°N; 123.891°E) and Liloan (LIL), Cebu (10.4121°N; 123.986°E) in the Visayas; and Maramag, Bukidnon (BUK) (8.454°N; 124.632°E) and Cagayan de Oro (CDO) city (8.051°N; 124.923°E) in Mindanao. These sites were selected based on: 1) the same biotype using Corona's modified climate classification (Corporal-Lodangco and Leslie 2017), 2) high incidence of dengue cases (NDRRMC 2019, WHO WPRO 2019), 3) elevation, and 4) range of latitudes.

Ae. aegypti parental eggs were collected from natural populations in the study sites, preferably outside human dwellings, under the roof gutter with the use of modified ovicidal/larvicidal (O/L) traps. Permission from the Department of Health—Bureau of Quarantine Office, Cebu city for shipment of *Aedes* eggs was obtained beforehand. *Aedes* eggs were hatched inside transparent plastic cups (TPCs) half-filled with ascorbic acid solution (Lacour et al. 2015). Larvae were carefully pipetted into new TPCs half-filled with distilled water (DW) (~150 mL) and covered with fine-mesh cloth. Daily feeding of fish food (0.02 g) (Sakura, All Aquariums Co., Ltd., Thailand) and changing of DW every other day to avoid scum formation on the water surface were done until the larvae metamorphosed into adults. Newly emerged adults of *Ae. aegypti* were identified (Belkin 1962) and sorted out by sex, and were stored into a 1.5 mL microcentrifuge tube individually for DNA extraction.

To test for Mendelian inheritance, first generation (F1) offspring of *Ae. aegypti* were obtained by coupling the male and female reared adults from parental eggs collected from CC and BUK sites. These adult couples were put inside a TPC covered with fine-mesh cloth and with a filter paper at the bottom. They were fed with a 10% sucrose solution soaked in a cotton ball that was placed on top of the fine-mesh cloth. A 3- to 5-d mating window was given for each couple. After which, females were starved for 24 h, and were fed with chicken blood mixed with ethylenediaminetetraacetic acid (EDTA) placed on the bottom depression of plastic bottles and sealed with a thin parafilm membrane. Each plastic bottle was placed in an upright position on top of the fine-mesh cloth of PTC with coupled mosquitoes, and was frequently added with lukewarm water to simulate body heat to attract female mosquitoes to suck blood. The mosquitoes were then fed with 10% sucrose solution soaked in cotton balls that were put on top of the fine-mesh cloth of PTC. The filter paper at the bottom of the mosquito cup was moistened with DW for females to lay F1 eggs relatively 2–5 d later. F1 eggs were then hatched and reared until adult emergence.

DNA Extraction

DNA samples (n = 30 each site) were extracted from *Ae. aegypti* natural populations from BG, QC, LIL, and CDO city, and F1 siblings from CC highlands and BUK using Macherey-Nagel insect DNA extraction kit (Cat. No. 740470.50; Macherey-Nagel, Düren, Germany) following the manufacturer's protocol in a level 2 biosafety cabinet.

Selection of Isolated Microsatellite Loci of *Aedes aegypti*

Briefly, a total of 327,094 perfect SSRs were searched in the updated reference genome assembly of *Ae. aegypti* Liverpool strain (Matthews et al. 2018) using the MISA tool (Beier et al. 2017). Only 52,002 SSRs were located in chromosomes 1–3. The annotated 1,513 SSRs were filtered based on motif length (n = 2–4 bp), equal or less than 20 repeats, and located in the intergenic region. Primer BLAST was performed to generate candidate primers at the flanking sequences of the SSRs. Out of the 1,513 sequences, 21 SSRs with di-, tri-, and tetra-nucleotide motifs from each of the three *Ae. aegypti* chromosomes (total = 63 SSRs) were randomly selected for characterization considering also the logistics of this study. Their primer sequences were sent to International DNA Technologies (IDT) Inc., Singapore for synthesis.

Test on Polymorphic Microsatellite Loci

Successful amplification of the 63 SSR markers in the Philippine populations of *Ae. aegypti* was crucial in determining their presence in the genome of *Ae. aegypti* local populations. These loci were amplified using pooled DNA samples of male and female *Ae. aegypti* from each site (n = 12). An M13 sequence (5'- TCC CAG TCA CGA -3') was added to the 5' end of each forward (F) primer of the nine SSR markers. PCR reactions (final volume of 10 µL (containing 5µL of 2x Type-it multiplex PCR master mix [Qiagen Type-It microsatellite PCR kit; Cat. No. 206243], 1 µL each of 10 µM forward and reverse primers, 1 µL pooled DNA sample, 2.25 µL nuclease-free water [NFW]) were run on a Veriti 96-well Thermal Cycler (Cat. No. 4375786: Thermo Fisher Scientific, Applied Biosystems, MA, USA) with 94°C initial denaturation for 10 min, then 35 cycles of 94°C x 30 s, 55°C x 40 s, 72°C x 30 s and final extension for 5 min at 72°C (Slotman et al. 2007). Gel electrophoresis was performed using 1% agarose to determine the quality of microsatellite bands. A mixture of 2 µL loading dye and 2 µL PCR product of each candidate SSR maker was loaded into each well of an agarose gel together with AC4 locus (Slotman et al. 2007) as positive control and NFW as negative control. Bands in agarose gels were viewed through a UV trans-illuminator (Maestrogen, Nevada).

Test for Broadly Applicable Microsatellite Markers

To determine the broad applicability of these molecular markers, outgroups of *Ae. aegypti* samples from Rayong (Ry), Thailand and Ho Chi Minh (HCM), Vietnam were assessed using previously screened polymorphic microsatellite markers. Pooled DNA samples (n = 30) from each outgroup were amplified following the same concentrations of reagents and PCR profile described earlier. Amplified products were visualized on agarose gels through the UV trans-illuminator (Maestrogen, Nevada). Markers with smeared and faint bands were eliminated.

Characterization of Polymorphic Microsatellite Loci of *Aedes aegypti*

All 180 DNA extracts of Philippine *Ae. aegypti* samples together with 60 DNA samples (n = 30 each site) from Ry, Thailand and HCM, Vietnam were amplified following the PCR profile such as denaturation step at 94°C for 5 min, then 38 cycles of 94°C x 30 s, 54°C x 30 s, 72°C x 20 s and final extension for 10 min at 72°C in a Veriti thermal cycler (Cat no. 4375786; Applied Biosystems). Several optimizations (i.e., from 52°C up to 68°C with an increment of 2°C) were performed to determine the optimal annealing temperature (54 °C) of all SSR markers. PCR reactions with final volume of 10 µL (containing 5 µL of 2x Type-it multiplex PCR master mix [Qiagen Type-It microsatellite PCR kit; Cat. No. 206243], 0.2 µL of 10 µM forward [F] primer, 0.8 µL each of 10 µM reverse [R] primer and fluorescent M13, 1 µL pooled DNA sample, 2.2 µL NFW) were prepared. To get maximum peak signals, two important points were observed during the preparation of PCR reaction mixture (Schuelke 2000): 1) equimolar amounts of R primer and fluorescent-labeled M13 primer should be added to the reaction mix, and 2) the concentration of the F primer should be one-fourth that of the concentrations of R primer and M13 primer to

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facilitate the M13 primer-driven amplification reactions when the F primer was consumed.

PCR products were sent to the Philippine Genome Center (PGC), QC for fragment analyses by using ABI 3730xl DNA Genetic Analyzer (Cat no. 3730XL; Applied Biosystems) (Carlsbad, CA) and GS 500 Rox allele size standard (Cat no. 4340060A; Applied Biosystems). The two highest allele peaks of each genotype were scored using ABI peak scanner software (v.1) (Applera Corp., Norwalk, CT). The height of each peak on the y-axis was zoomed up to 28,000 relative fluorescence units (RFUs), while the allele size on the x-axis was zoomed at 90–300 bp. Capillary DNA sequencing was also performed to confirm the sequences of these microsatellite markers at the PGC, QC. Data sequences were analyzed using Chromatogram Explorer Lite of DNA Baser Assembler software (Heracle BioSoft S.R.L., Romania).

Genetic Analyses

MICROCHECKER software (v. 2.2.3) (Oosterhout et al. 2004) was used to determine the presence of null alleles. For Mendelian codominant inheritance, goodness-of-fit for Hardy-Weinberg equilibrium (H-WE) and chi-square test ($P < 0.001$) using GENEPOP software (v. 4.7.5) (Raymond and Rousset 1995) for genotypes of F1 offspring samples were performed. Other GENEPOP tests were done such as number of alleles, allele frequencies, estimates of observed (H_O) and expected heterozygosities (H_E), goodness-of-fit probability tests for H-WE to determine if the markers were selectively neutral, Fisher's exact test to detect linkage disequilibrium (LD), and pairwise estimates of Fisher's statistics (F_{ST}) between populations to assess the robustness of the SSR markers only. Sequential Bonferroni multiple comparison tests for all loci were done in H-WE and LD tests. For the latter, Markov chain parameters were set at 1,000 dememorizations, 100 batches, and 1,000 iterations. Corrections were made on the number of batches and changes were made in increments of 100.

Results

Polymorphic Microsatellite Markers

Twenty-six out of 63 primer pairs of SSR markers consistently amplified using pooled *Ae. aegypti* DNA extracts ($n = 12$) from different Philippine sampling sites, as depicted on electrophoretic gel bands. Seven out of the 26 loci were located in the first chromosome, nine in the second chromosome, and ten in the third chromosome. Three motifs were observed, namely, dinucleotide ($n = 10$), trinucleotide ($n = 12$) and tetra-nucleotide ($n = 4$). These motifs were consistent in previous studies (Slotman et al. 2007, Chambers et al. 2007, Brown et al. 2011).

Broadly Applicable Microsatellite Markers

Nine out of the 26 SSRs of *Ae. aegypti* showed promising bands on pooled DNA extracts of outgroup samples, suggesting their consistent amplification, hence, are good candidates of broadly useful microsatellite markers. Faint and smeared bands of PCR products of 26 polymorphic SSR markers that amplified samples from both outgroups (Thailand and Vietnam) on an agarose gel indicated unsuccessful amplification and were eliminated.

Screening for Null Alleles and Mendelian Inheritance

MICRO-CHECKER software (v. 2.2.3) did not detect significant evidence of null alleles in four (Aaeg1-3D, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) out of the nine SSR markers among genotyped samples from local populations (Table 1; Supplemental file 1). GENEPOP (v. 4.7.5) analysis in screening for Mendelian codominant pattern of inheritance revealed that for F1 *Ae. aegypti* belonging to CC family, four markers (Aaeg1-3D, Aaeg1-3H, Aaeg2-2E, and Aaeg3-4C) adhered to Mendelian codominant pattern (X^2 test, df = 18, $P > 0.0011$) after incorporating sequential Bonferroni correction for multiple comparison. For BUK F1 mosquito family, four markers (Aaeg1-3D, Aaeg1-3H, Aaeg2-2A, and Aaeg3-4C) also conformed to Mendelian pattern of inheritance (X^2 test, df = 18, $P > 0.0011$).

Screening for H-WE, LD, and F_{ST} Estimates

After sequential Bonferroni correction for multiple comparison, Table 1 revealed that overall, four SSR markers (Aaeg1-3D, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) conformed to H-WE ($P > 0.0011$). *Aedes aegypti* populations in BG and QC conformed to H-WE ($P > 0.0011$) in five loci (Aaeg1-3D, Aaeg1-3H, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C). LIL mosquito population had six loci (Aaeg1-3D, Aaeg1-3H, Aaeg2-2A, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) that conformed to H-WE ($P > 0.0011$). CDO population had five loci (Aaeg1-3D, Aaeg2-2A, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) that adhered to H-WE ($P > 0.0011$). LD between two loci after sequential Bonferroni multiple comparison ($P > 0.00028$) in all mosquito populations were not observed. The 9-locus dataset produced similar F_{ST} estimates as the 7-locus dataset that included the two best markers (Aaeg1-3D and Aaeg3-4C) and the rest of the SSR markers (Aaeg1-3H, Aaeg2-2A, Aaeg2-2B, Aaeg2-4D, and Aaeg3-3I) but differed in one F_{ST} estimate

Table 1. Characterization of novel and broadly applicable microsatellite markers of *Ae. aegypti* using Philippine and outgroup populations

Locus	Motif	Broadly Useful	Absence of Null Alleles	Mendelian Inheritance		Selectively Neutral	Randomly Associated	Polymorphic	Accession No.
				CC	BUK				
Aaeg1-3D	$(GTC)_5$	Yes	Yes	Yes	Yes	Yes	Yes	Yes	MW648370
Aaeg1-3H	$(CTT)_8$	Yes	No	Yes	Yes	Yes/No	Yes	Yes	MW660812
Aaeg2-2A	$(AG)_{12}$	Yes	No	No	Yes	Yes/No	Yes	Yes	MW648371
Aaeg2-2B	$(AT)_8$	Yes	No	No	No	No	Yes	Yes	MW648372
Aaeg2-2E	$(CA)_7$	Yes	Yes	Yes	No	Yes	Yes	Yes	MW648373
Aaeg2-4D	$(TTAT)_7$	Yes	No	No	No	No	Yes	Yes	MW660813
Aaeg3-2A	$(CA)_{18}$	Yes	Yes	No	No	Yes	Yes	Yes	MW660814
Aaeg3-3I	$(TTC)_6$	Yes	No	No	No	Yes/No	Yes	Yes	MW648374
Aaeg3-4C	$(CATC)_6$	Yes	Yes	Yes	Yes	Yes	Yes	Yes	MW648375

yes, conformed to criterion; no, deviated from criterion; yes/no, conformed in some populations but deviated in others; CC, Cebu city highland; BUK, Maramag, Bukidnon samples.

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Table 2. Primer sequences (F—forward, R—reverse), chromosome position based on the reference genome (Matthews et al. 2018), allele size range, total number of alleles observed, and mean H_E and H_O of the nine polymorphic SSR markers of Philippine *Aedes aegypti* (Sample size of all loci = 240)

Locus	Primer Sequence (5'-3')	Chromosome Position (Reference Name| Start| End)	Allele Size Range (bp)	Total No. of Alleles	Mean HE (HO)
Aaeg1-3D	**F:** GGACTAAAGCCAGACAAAAAT **R:** CAAAGCCGTCATAAAGAAATA	NC_035107.1|100094040|100094054	141–152	7	0.55 (0.63)
Aaeg1-3H	**F:** TCTATCGTGTTCAGCTAAAGG **R:** AGGTGCTCATAAGAACACAAA	NC_035107.1|218623319|218623342	117–199	15	0.77 (0.65)
Aaeg2-2A	**F:** ATAGGCATCTGTGTGAATAGC **R:** GGATCTTTTTGCTTCTCATTT	NC_035108.1|11685929|116865952	100–113	13	0.75 (0.57)
Aaeg2-2B	**F:** TCCTTTACGACTTTTATCTCTCA **R:** TATATAAGACGCCAATGCTCT	NC_035108.1|222239369|222239384	113–165	17	0.58 (0.13)
Aaeg2-2E	**F:** ATCCTGAAACAGTAGGTGTGA **R:** CCAACTTATTTGCGTTATGTG	NC_035108.1|204105754|204105767	141–154	9	0.59 (0.78)
Aaeg2-4D	**F:** CAGGTTGATGGACTTTCGTG **R:** GTACTTTGTTTTTGACGCATT	NC_035108.1|174180719|174180746	106–251	3	0.52 (0.26)
Aaeg3-2A	**F:** GATCAGTCCAAACACTACCAA **R:** AAAACGAGGTCAGAAAAAGTT	NC_035109.1|257409555|257409590	124–170	25	0.61 (0.78)
Aaeg3-3I	**F:** AGTCAACAATCAACAAAGAGC **R:** ATTGAACACTGAGCTGAGAAG	NC_035109.1|265852935|265852952	108–294	37	0.89 (0.70)
Aaeg3-4C	**F:** TTAACCCTCGAGTCAATCATA **R:** GTTTATCCGGGTGCAAATAG	NC_035109.1|96913182|96913205	130–173	7	0.46 (0.51)

only between Ry and BG population pairs based on the 7-locus dataset that included the two good markers but not co-dominant (Aaeg2-2E and Aaeg3-2A) and the rest of the SSR markers mentioned.

Polymorphism and Heterozygosities (H_E and H_O)

Table 2 shows that nine loci were polymorphic although they varied in the number of alleles per locus (2–20 alleles) in each population. These sequence data were submitted to the GenBank database with their accession numbers (Table 2). In all populations, Aaeg3-3I locus was the most polymorphic with 37 alleles; Aaeg2-4D, the least polymorphic with only three alleles. H_E estimates were moderate to high (0.41–0.94) (Table 2). No singletons were detected in Aaeg2-2E, Aaeg2-4D, and Aaeg3-4C, whereas one singleton in Aaeg1-3D, Aaeg1-3H, and Aaeg2-2A, and ten in Aaeg3-3I.

Characteristics of Microsatellite Loci in Outgroup Populations

Rayong, Thailand

For *Ae. aegypti* samples from Ry, Thailand (n = 30), four loci (Aaeg1-3D, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) were devoid of null alleles with the use of MICROCHECKER software (Table 1). After sequential Bonferroni correction for multiple comparison, five markers (Aaeg1-3D, Aaeg2-2E, Aaeg3-2A, Aaeg3-3I, and Aaeg3-4C) conformed to H-WE (P > 0.0011). Significant deviations from LD after pairwise tests, incorporating a sequential Bonferroni correction, were not observed (Table 1) (P > 0.00028). All loci were polymorphic (2–12 alleles) although Aaeg2-4D, Aaeg1-3D, and Aaeg3-4C had only two alleles. H_E estimates were low to high (0.24–0.84).

Ho Chi Minh, Vietnam

Null alleles were not detected in five loci (Aaeg1-3D, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) screened among HCM samples (n = 30) (Table 1). Conformity to H-WE was observed in loci Aaeg1-3D, Aaeg2-2A, Aaeg2-2E, Aaeg3-2A, Aaeg3-3I, and Aaeg3-4C. All loci showed random association of alleles after pairwise tests, incorporating a sequential Bonferroni correction (P > 0.00028). All loci were polymorphic (4–11 alleles) although Aaeg2-4D had two alleles only. H_E estimates (0.38–0.87) were moderate to high in all markers. Thus, in both outgroup samples, four markers (Aaeg1-3D, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) consistently conformed to all criteria of a good microsatellite marker.

Discussion

Polymorphism and Heterozygosities

All nine loci were polymorphic. H_E estimates were moderate (0.46) to high (0.89) in populations of *Ae. aegypti* from the Philippines and outgroups (Ry, Thailand and HCM, Vietnam). Excess of homozygotes (or heterozygote deficiencies) were observed in Aaeg1-3H, Aaeg2-2A, Aaeg2-2B, Aaeg2-4D, and Aaeg3-3I, implying that homozygotes were greater than expected under H-WE (Selkoe and Toonen 2006). Several reasons include the presence of null alleles (Callen et al. 1993, Ardren et al. 1999), effects of inbreeding (Callen et al. 1993, Selkoe and Toonen 2006), and selection for or against a specific allele (Selkoe and Toonen 2006).

Null Alleles and Mendelian Inheritance

Absence of null alleles were consistently observed in four markers (Aaeg1-3D, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) in both *Ae. aegypti* populations from local and outgroups. Null alleles are caused by loss-of-function mutation (Chapuis and Estoup 2006) and may cause biases in estimating genotypic and allelic frequencies in population genetic studies (Oosterhout et al. 2006), heterozygote deficiencies (Callen et al. 1993, Ardren et al. 1999), errors in genetic parentage analysis (Dakin and Avise, 2004), and misleading analysis in population differentiation (Chapuis and Estoup 2006). Thus, it is crucial for a microsatellite marker to lack null alleles.

On the other hand, three markers (Aaeg1-3D, Aaeg1-3H, and Aaeg3-4C) consistently conformed to Mendelian codominant pattern in both F1 mosquito families from CC and BUK, and may be useful in assessing genetic connectivity of *Ae. aegypti* populations. Departures from Mendelian fashion may be due to nondisjunction during meiosis, presence of null alleles (Oosterhout et al. 2006), and mutations during sexual reproduction (Tesson et al. 2013).

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Table 3. Goodness-of-fit probability tests for Hardy-Weinberg Equilibrium of the nine microsatellite markers in local and outgroup populations of *Aedes aegypti*. *P-value* = 0.0011 incorporating sequential Bonferroni correction for multiple comparison tests; (*) deviated from H-WE

Locus	Natural Populations				Outgroups	
	Luzon		Visayas	Mindanao	Thailand	Vietnam
	BG	QC	LIL	CDO	RY	HCM
Aaeg1-3D	0.0279	0.0903	0.0720	0.0020	0.0711	0.4059
Aaeg1-3H	0.0022	0.0380	0.0537	0.0007*	0.0000*	0.0000*
Aaeg2-2A	0.0001*	0.0005*	0.0032	0.0032	0.0000*	0.0569
Aaeg2-2B	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*	0.0000*
Aaeg2-2E	0.2883	0.1680	0.0811	0.7687	0.0727	0.0014
Aaeg2-4D	0.0000*	0.0001*	0.0000*	0.0000*	0.0000*	0.0000*
Aaeg3-2A	0.9846	1.0000	0.9868	1.0000	0.3058	0.9849
Aaeg3-3I	0.0000*	0.0000*	0.0000*	0.0000*	0.4057	0.0028
Aaeg3-4C	0.3141	0.6330	0.2003	0.5983	1.0000	0.3893

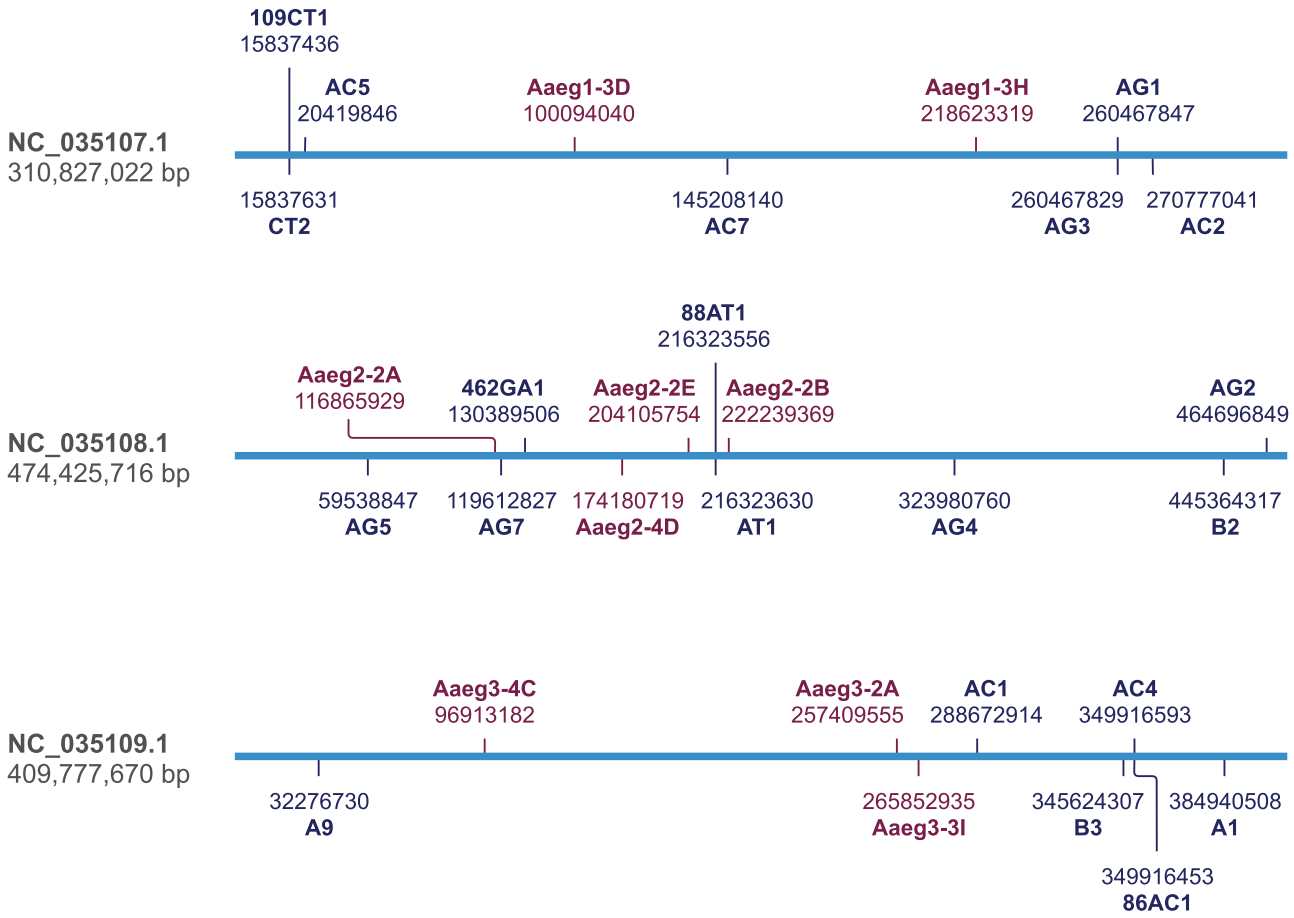


Fig. 2. Location of the previously reported and the new SSR loci in the genome of *Aedes aegypti* using the published genome assembly (Matthews et al. 2018) as the reference. Each location is marked by the name of the locus and its coordinate (in bp), in blue font for published markers (Slotman et al. [2007], Brown et al. [2011] and Lovin et al. [2009]) or in red font for the new markers (this study). The coordinates indicate the location of the first 5′ nucleotide position of the forward (F) PCR primer for the published markers or the first 5′ nucleotide position of the repeat region for the new markers (from Table 2, column 3).

Selectively Neutral and Randomly Associated SSRs

Four markers (Aaeg1-3D, Aaeg2-2E, Aaeg3-2A, and Aaeg3-4C) consistently conformed to H-WE in both local and outgroup populations of *Ae. aegypti* (Table 3). Conformity to H-WE implies that allele frequencies in a population with random mating remain constant from one generation to the next in a large population in the absence of evolutionary forces such as mutation, migration, and natural selection. Violations on any or all of these assumptions cause deviations from H-WE (Hartl 2020). Moreover, deficiency of heterozygotes generated by null alleles may also cause deviations from H-WE (Lanzaro et al. 1998, Lehmann et al. 2003). Moreover, random association between two loci were consistently observed in both local and outgroup populations, suggesting close physical genetic linkage was unlikely. Factors that influence LD include selection, mating system, genetic drift, mutation rate, population structure (Hartl 2020), inbreeding, and null alleles (Callen et al. 1993,

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Lehmann et al. 1998). LD is not expected if heterozygote deficiency is caused by null alleles (Lehmann et al. 2003).

Several studies on the population structure of *Ae. aegypti* (Brown et al. 2011, Fonzi et al. 2015, Sayson et al. 2015, Hopperstad et al. 2019) used the seven markers developed by Chambers et al. (2007), 13 markers by Slotman et al. (2007), four markers by Brown et al. (2011), and four re-designed markers by Lovin et al. (2009). Slotman et al. (2007) and Brown et al. (2011) characterized these markers by assessing deviations from H-WE, screening for null alleles, and LD, although Brown et al. (2011) utilized their four new markers with eight previously developed markers to assess the population structure of *Ae. aegypti* worldwide. Moreover, Chambers et al. (2007) included LD, classification of microsatellite repeats, and polymorphism. Lovin et al. (2009) assessed polymorphism, deviation from H-WE, genetic map location, and multiplex-ready groups of markers that were used to determine the population genetics of *Ae.* aegypti populations in Haiti. However, these studies (Chambers et al. 2007, Slotman et al. 2007, Lovin et al. 2009, Brown et al. 2011) did not determine the codominant Mendelian inheritance. Only Lovin et al. (2009) and this current study provided the chromosomal location of microsatellite markers based on the linkage map positions of the previously defined genetic loci, and on *Ae. aegypti* reference genome (Matthews et al. 2018), respectively. Moreover, we located the chromosomal locations of the current SSR markers relative to the published markers (Chambers et al. 2007, Slotman et al. 2007, Lovin et al. 2009, Brown et al. 2011) using the reference genome (Matthews et al. 2018) (Fig. 2). Thus, this current study characterized comprehensively novel SSR markers utilizing natural populations of Philippine *Ae. aegypti,* for the first time, by analyzing H-WE, LD, codominant Mendelian inheritance in F1 siblings, screening for null alleles, heterozygosities, chromosomal locations relative to other markers and broad applicability in other Asian countries.

Isolation of polymorphic microsatellite markers in *Culex pipiens* (Keyghobadi et al. 2004) has also been reported with H_E and H_O analysis only, which was utilized in a separate study by Keyghobadi et al. (2006) to determine the population structure of *Cx. pipiens* in Hawaii. Edillo et al. (2007) characterized microsatellite markers of *Cx. pipiens* by analyzing deviations from H-WE, LD, codominant Mendelian inheritance, heterozygosities (H_E and H_O), presence of null alleles, and broader applicability in other countries. Edillo et al. (2009) utilized these good markers (Edillo et al. 2007) in a separate study on the population structure of *Cx. pipiens* populations in eastern North America. In *Anopheles* (Lather et al. 2015), genetic analysis of polymorphic microsatellite markers included deviations from H-WE, LD, codominant Mendelian pattern, presence of null alleles, heterozygosities, and cross-amplification in other species. In other taxa, Masran and Majid (2018) developed seven microsatellite markers from *Cimex hemipterus* (Fabricius) (Hemiptera: Cimicidae) by analyzing results from H-WE, LD, and screening for null alleles on their natural populations. Recent SSR characterization isolated from fin whales (Tardy et al. 2020) and earthworm (Liu et al. 2020) assessed H-WE, LD, allelic diversity, heterozygosities, and cross-amplification with other species. These studies (Keyghobadi et al. 2004, Edillo et al. 2007, Slotman et al. 2007, Lather et al. 2015, Masran and Majid 2018, Liu et al. 2020, Tardy et al. 2020) did not necessarily compare between newly characterized microsatellite markers with a set of previously good published markers and those datasets of published markers with problematic loci like that of Brown et al. (2011) for *Ae. aegypti.*

Other techniques include genotyping-by-sequencing (GBS) (Elshire et al. 2011) and restriction site-associated DNA sequencing (RADseq) (Davey and Blaxter 2010). The latter has been used to develop markers of *Ae. aegypti* (Evans et al. 2015, Rasic et al. 2015). Sunde et al. (2020) noted that between RADSeq and microsatellite analysis, RADSeq is a better technique because it produces a higher number of loci.

In conclusion, this study determined two novel and broadly useful microsatellite markers of Philippine *Ae. aegypti* such as Aaeg1-3D of chromosome 1 and Aaeg3-4C of chromosome 3, that can be added to markers of this species previously developed. Moreover, two markers (Aaeg2-2E of chromosome 2 and Aaeg3-2A of chromosome 3) may still be of use since they met all criteria except that they did not conform to Mendelian codominant inheritance. These latter markers can be used with caution, i.e., these should be utilized together with Aaeg1-3D and Aaeg3-4C and other good microsatellite markers (Chambers et al. 2007, Slotman et al. 2007, Lovin et al. 2009, Brown et al. 2011). In the Philippines, since dengue outbreaks are recurring (NDRRMC 2019, WHO WPRO 2019) and currently only a few studies on the genetic structure of *Ae. aegypti* were conducted (Fonzi et al. 2015, Sayson et al. 2015, Carvajal et al. 2020), the need to conduct more studies on population structure of this species in the country, in particular, and in the tropics, in general, is very relevant. The additional markers developed in this study will be very useful in studies on the population structure of this species in the tropics, as in our separate study of the Philippine *Ae. aegypti* to promote effective and timely vector control strategies for public health. In the future, these research initiatives may lead to implementation of urgent actions to safeguard public health against dengue, Zika, chikungunya, and yellow fever that are transmitted by this vector species.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Acknowledgments

This research was funded by the Philippine Council for Health Research and Development (PCHRD) and University of San Carlos (USC), Cebu city to F.E.E. and the Accelerated Science and Technology Human Resource Development Program (ASTHRDP) of the Department of Science and Technology (DOST) to D.A.L. We also like to acknowledge JR Powell and A Gloria-Soria of Yale University, CT, USA for sharing their DNA extracts of *Ae. aegypti* from Thailand and Vietnam, and for MM Cabahug and RJ Hernandez of University of San Carlos (USC), Cebu city for their valuable contribution in sample preparations.

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