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Parentage Assignment Using Microsatellites Reveals Multiple Mating in *Aedes aegypti* (Diptera: Culicidae): Implications for Mating Dynamics

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Abstract

The mosquito *Aedes aegypti* is the primary vector of the dengue, yellow fever, and chikungunya viruses. Evidence shows that *Ae. aegypti* males are polyandrous whereas *Ae. aegypti* females are monandrous in mating. However, the degree to which *Ae. aegypti* males and females can mate with different partners has not been rigorously tested. Therefore, this study examined the rates of polyandry via parentage assignment in three sets of competitive mating experiments using wild-type male and female *Ae. aegypti*. Parentage assignment was monitored using nine microsatellite DNA markers. All *Ae. aegypti* offspring were successfully assigned to parents with 80% or 95% confidence using CERVUS software. The results showed that both male and female *Ae. aegypti* mated with up to 3–4 different partners. Adults contributed differentially to the emergent offspring, with reproductive outputs ranging from 1 to 25 viable progeny. This study demonstrates a new perspective on the capabilities of male and female *Ae. aegypti* in mating. These findings are significant because successful deployment of reproductive control methods using genetic modification or sterile *Ae. aegypti* must consider the following criteria regarding their mating fitness: 1) choosing *Ae. aegypti* males that can mate with many different females; 2) testing how transformed *Ae. aegypti* male perform with polyandrous females; and 3) prioritizing the selection of polyandrous males and/or females *Ae. aegypti* that have the most offspring.

Key words: *Aedes aegypti*, CERVUS, Malaysia, mating behavior, microsatellite

In recent years, *Aedes* (Stegomyia) *aegypti* (L.) has emerged as a species of major medical concern due to its involvement in the spread of Zika, yellow fever, and dengue viruses (Agudelo et al. 2021). Moreover, health management of dengue hemorrhagic fever presents many challenges because an effective vaccine against this *Ae. aegypti* borne disease has yet to be developed (Degner and Harrington 2016). In Malaysia, *Ae. aegypti* is a vector responsible for the increasing numbers of dengue patients; the cumulative total of reported dengue cases is 26, 365 nationwide as of 1 January 2022 (Nellis et al. 2021, WHO 2022). Further challenges arising from multiple-insecticide resistant mosquito populations have restricted the choice of insecticides that can be used in interventions targeted at killing adult mosquitoes. Hence, Malaysia urgently needs to control dengue more effectively by using noninsecticidal controls such as genetically modified mosquitoes (GMM) and sterile insect techniques (SIT) to reduce mosquito density (Rasli et al. 2021). While knowledge of dengue vector control is increasing, mosquito-mating behavior remains poorly understood (Qureshi et al. 2019, Degner and Harrington 2016). The success of new innovative tools (i.e., GMM and SIT) would benefit greatly from a better understanding of the

basic mating biology of *Ae. aegypti*. For instance, knowing that both male and female *Ae. aegypti* can mate with multiple partners would warrant that dengue-engineered males must be reproductively competitive and mate with multiple females.

Field experiments on *Ae. aegypti* and *Ae. albopictus* mosquitoes show that a single male mosquito is capable of inseminating multiple females even though females quickly become refractory post-mating (Boyer et al. 2012; Helinski et al. 2012a, b; Degner and Harrington 2016). Nonetheless, under the right circumstances, *Ae. aegypti* females may mate with up to three males in field and laboratory settings (Boyer et al. 2012, Helinski et al. 2012b, Richardson et al. 2015). The *Ae. aegypti* males are aggressive in mating and can perform as many as 50 copulation attempts within one hour in conspecific mating (Dieng et al. 2016). In interspecific mating, sexually aggressive *Ae. aegypti* males perform a higher number of mating pairs with *Ae. albopictus* females compared to the *Ae. albopictus* males (Marcela et al. 2015). Nevertheless, there are contradictory findings about the sexual aggressiveness of *Ae. aegypti* and *Ae. albopictus*. Some studies report that *Ae. aegypti* males are most aggressive in mating (Thomas and Yap 1973, Black et al. 1989, Harper and Paulson 1994, Marcela et al. 2015), whereas others suggest that *Ae. albopictus* males are most aggressive in mating (e.g., Nasci et al. 1989, Bargielowski et al. 2013). However, insect breeding systems such as polygyny and polyandry are often difficult to quantify reliably just via direct observation (Richardson et al. 2015, Thomas and Yap 1973, Ridley 1988, Choochote et al. 2001). For instance, *Ae. aegypti* females can exhibit pseudocopulation, coupling repeatedly without being inseminated (Jones 1973, Helinski et al. 2012a). To address such problems, one needs to apply molecular-based methods to determine true family relationships between parents and offspring and better show the genetic benefits of different mating strategies (Boyer et al. 2012, Blouin et al. 1996, Jennions and Petrie 2000, Blouin 2003).

The use of microsatellite markers is now a preferred method in ecological studies as these systems are abundant and highly polymorphic. When compared to amplified fragment length polymorphism (AFLP) analysis, microsatellites can generate more highly discriminating data (Behura 2006). In contrast to microsatellite analysis, the AFLP method requires high quality DNA for enzymes to bind correctly, and it is often difficult to distinguish heterozygotes from homozygotes (Tello and Forneck 2019). Microsatellites have been widely used to examine population genetics and breeding structures for *Ae. aegypti*, but there have only been relatively limited studies that assess parentage assignment in *Ae. aegypti* (Rahman et al. 2021, Richardson et al. 2015). Accurate examination of *Ae. aegypti* parentage analysis using the microsatellite approach is crucial for developing effective dengue control strategies (Wong et al. 2012).

Previous studies examined the reproductive performance of *Ae. aegypti* by documenting numbers of mating pairs, numbers of eggs produced, female refractoriness and insemination rates (Bargielowski et al. 2015, Marcela et al. 2015, Degner and Harrington 2016, Carrasquilla et al. 2019, Agudelo et al. 2021;). Pedigree assignment was not conducted to determine how *Ae. aegypti* mating ability contributes towards production of fertile offspring. One related study (Wong et al. 2012) extracted DNA from *Ae. aegypti* hind legs and used nine microsatellite loci to track their egg-laying behavior. The same authors reported a successful microsatellite analysis, but they neither assessed polyandrous behavior in *Ae. aegypti* nor reported on how mated individual *Ae. aegypti* parents contributed towards offspring density. Hence, full parentage assignment studies are necessary to validate multiple mating in *Ae. aegypti* as recorded through direct observation and to see how this behavior impacts offspring production.

In light of insecticide-resistant mosquito populations, the use of GMM and SIT offer very promising means for the control of dengue by reducing *Ae. aegypti* populations (Oliva et al. 2012, Arham et al. 2021, Oliva et al. 2021, Rasli et al. 2021). In GMM, when wild *Ae. aegypti* females mate with genetically modified males, their offspring will not survive larval or pupal stage in the absence of tetracycline (Lacroix et al. 2012, Arham et al. 2021). In contrast, SIT releases sterile *Ae. aegypti* males that can mate with wild *Ae. aegypti* females, resulting in inseminations that do not produce progeny (Oliva et al. 2021, Rasli et al. 2021). To maximize the impact of such strategies, the sterile males need to compete effectively with the wild males when mating with the wild females leading to the highest possible level of infertile egg production by the mated females (de Valdez et al. 2011, WHO 2009). Therefore, one important future measure is to find ways of increasing the mating ability and effectiveness of genetically engineered *Ae. aegypti* mosquitoes (Pates and Curtis 2005, Massonnet-Bruneel et al. 2013, Oliva et al. 2021). In mosquitoes, fitness is related to the relative success of an individual in passing its genes to the next generation as estimated by measures of reproductive success such as fecundity, fertility, and mating competitiveness (Massonnet-Bruneel et al. 2013). Most *Ae. aegypti* females (76%) became refractory to a second mating within 2 hr of their first mating (Degner and Harrington 2016). Moreover, genetic transformation in mosquitoes can cause declines in fitness and mating competitiveness (Catteruccia et al. 2003, Irvin et al. 2004). However, to date, only little is known regarding the mating fitness of *Ae. aegypti* (Catteruccia et al. 2003, Irvin et al. 2004, Degner and Harrington 2016).

Furthermore, recent changes in behavior, such as breeding and blood-feeding, displayed by *Ae. aegypti* mosquitoes warrant the need for detailed analyses beyond simple field-based behavior observations (Dieng et al. 2010, Saifur et al. 2012, Agudelo et al. 2021). Considering the knowledge gaps concerning mating fitness and refractory periods, our goal in this study was to examine whether multiple mating would result in the universal transfer of genes from wild adults of *Ae. aegypti* to their first generation (F_1) offspring monitored by genetic analysis using ten microsatellite markers. To our knowledge, this is the first report of parentage assignment in *Ae. aegypti* conducted in Malaysia.

Materials and Methods

Mosquito Rearing

Larvae and pupae of *Ae. aegypti* were collected at water holding containers in households in Sungai Batu, Penang, Malaysia (S°17′14.1 N, 100°14′23.2 E). Household water containers, such as plant pots, trays under the plant pots, plastic pails, vases, earthen plates, discarded tires, and bottles, were inspected as potential sources of *Ae. aegypti* immature stages. A colony of *Ae. aegypti* was established at the insectarium of School of Biological Sciences, Universiti Sains Malaysia, Penang. Larvae were routinely reared on a diet of 0.1 g of larval food. The larval food was prepared using a powdered mixture of 2:1:1:1 of dog pellets, milk powder, dried cow liver, and yeast (Vector Control Research Unit, Penang). Emergent adults were examined and sorted according to species and gender using taxonomic keys (Darsie and Samanidou-Voyadjoglou 1997, Rueda 2004). The adult mosquitoes were fed with a 10% sucrose solution supplemented with a vitamin B complex. Three-day-old female mosquitoes were blood-fed on restrained mice. Engorged virgin females were used for the mating experiments. The insectarium was maintained at a temperature of 29 ± 3°C and 75 ± 10% relative humidity, with a light regime at 12:12 hr photoperiod (L:D) including

1 hr dusk and 1 hr dawn from 60 or 25-W incandescent bulbs to simulate light levels for day and dawn/dusk respectively.

Mating Experiments

These were conducted using *Ae. aegypti* adults derived from wild-caught larvae and pupae. Ten female adults (see above) were released into a standard cage (30 × 30 × 30 cm). Subsequently, 10 male adults were introduced into the cage and the mosquitoes were allowed to mate ad lib for three consecutive days. Then the males were transferred into a new cage and provided with 10% (w/v) of sucrose solution supplemented with vitamin B complex. The females were allowed to deposit eggs for six days inside the cage. Oviposition sites were prepared using 90 mm filter papers (Whatman #1, Whatman International, Maidstone, UK) folded into a cone shape, placed into one disposable plastic cup (9 × 11.5 cm), and filled with 25 ml of aged rainwater. There was one oviposition site in the cage, and it was changed every day. The egg collection method was standardized across the three mating replicates (i.e., Aed1, Aed2, and Aed3). For hatching, eggs representing the F_1 offspring were dried and then placed into enamel trays (20 × 16 × 6 cm), filled with 1 liter of aged rainwater.

Three replicate trials were conducted; Aed1 (*Aedes* cross 1), Aed2, and Aed3. The replicates were crucial to validate the occurrence of multiple mating of *Ae. aegypti*. Parentage analysis was carried out by genotyping the original 20 adults (10 males and 10 females) and 40 randomly chosen individuals from the pool of their offspring from each cross (except for Aed1, $N = 42$). Before genotyping spermathecae from each female were dissected to determine their insemination status. Three parameters were recorded, such as (1) total number of eggs laid, (2) total number of eggs hatched, and (3) insemination status of each female.

DNA Extraction and Genotyping

Genomic DNA from *Ae. aegypti* adults ($N = 60$) and their F_1 offspring ($N = 122$ overall) of were extracted using a CTAB reagent (96:4 chloroform-isoamyl alcohol: 1M Tris HCl pH 8.0, EDTA pH 8.0, 3M NaCl, and premixed 2% CTAB) described by Lardeux et al. (2008) with minor modifications. The concentration and purity of DNA extracts were determined using a micro-volume UV spectrophotometer (Quawell Technology Inc., San Jose, CA).

This study utilized ten forward and reverse pairs of PCR primers, which were first developed by Slotman et al. (2006) and by Chambers et al. (2007) with polymorphism levels as reported by Wong et al. (2012) and listed in Supp Table 1 (online only). Each PCR reaction (20 µl) was pipetted into a 96-well clear PCR plate, which was then sealed with 0.2 ml clear flat PCR 8-strip caps (Axygen Scientific, Union City, CA). Thermal cycling for all primers was performed using Mastercycler thermocyclers (Eppendorf) under the following conditions: initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 sec at 57°C, extension for 30 sec at 72°C with a final extension step of 5 min at 72°C. Each 25 µl PCR reaction contained a 5X colorless Go-Taq Flexi Buffer (Promega, US), 25 mM of $MgCl_2$, 10 mM of dNTPs, 10 mM of each primer, 0.15 µl of Go-Taq DNA polymerase (5 U/µl) and 1.6 µl of genomic DNA (50 µg/µl). The PCR products were size fractionated by electrophoresis using a 3% agarose gel (Major Science, Way Saratoga, CA) based on the following conditions: 120 mA, 90 V for 35 min. The gels were stained using ethidium bromide (Sigma-Aldrich, St. Louis, MO) for at least 15 minutes and visualized using a UV light illuminator (Syngene GeneFlash, Frederick, MD).

Three multiplex reactions (Supp Table 1 [online only]) incorporating four fluorescent-labeled forward primers: 5'FAM, 5'TAMRA, 5'ROX, and 5'HEX were designed by Macrogen Corporation (Korea). Two multiplexes had four primer sets assigned according to their expected product length in base pairs, annealing temperature and specificity. A third multiplex consisting of just two primer pairs were selected because they produced low peaks if they were mixed in with the previous multiplexes. Primers whose products are of similar sizes were dye-labeled using different colors to avoid overlap during fragment analysis.

The PCR products were sent to Macrogen Incorporation Company (Korea) for fragment analysis, using a capillary electrophoresis analyzer (ABI Model 3730XL). Genotype data (FSA format) of each sample ($N = 122$) was obtained from three *Aedes* replicates and combined for subsequent analysis. Scoring of allele peaks in electrophoretograms was performed according to Arif et al. (2010). The study involved 182 DNA samples; 60 *Ae. aegypti* parents and 122 offspring. This method employs more *Ae. aegypti* parents than Wong et al. (2012, $N = 20$) and tests a higher number of offspring compared with Richardson et al. (2015, $N = 12$). The fragment analysis was conducted twice for each DNA sample, giving a total of 364 data points for fragment analysis. The purpose of repeating the analysis for each sample was to validate the results.

Parentage Analysis

Chromatograms of the fragments were analyzed using Peak Scanner Software version 1 (Applied Biosystems, Bedford, MA). DNA genotyping was conducted by comparing the resulted fluorescent peaks with the GS500LIZ size standard (Macrogen Inc., Korea). Whenever PCR irregularities were encountered, each sample was rescored and repeated. Parentage analysis was conducted using Cervus version 3.0 (Kalinowski et al. 2007, Jones and Wang 2010). Cervus uses genetic markers to assign parents to their offspring even when some genotypes are incomplete, incorrect, or missing (Marshall et al. 1998). It calculates a likelihood ratio using Delta and LOD values. The likelihood ratio is the likelihood that the candidate parent is the true parent divided by the likelihood that the candidate parent is not the true parent. This study uses Delta values as a criterion for assignment of parentage. The Delta value is the difference in LOD scores between the most likely candidate parent and the second most likely candidate parent. The derived LOD score is the natural log of the overall likelihood ratio. Delta values are especially useful when multiple candidate parents have positive LOD scores (Kalinowski et al. 2007). Cervus also produces a critical value by simulating parentage analysis using the actual data obtained in the experiments. The critical value is useful to assess the confidence of each assignment. Those Delta scores that exceed the critical value are assigned with 95% confidence. Observed (*Ho*) and expected heterozygosity (*He*) were calculated using Genetic Data Analysis version 1.0 (GDA) (Lewis and Zaykin 2001). The presence of null alleles was assessed using MicroChecker version 2.2 (van Oosterhout et al. 2004). Chi-square test was conducted to determine if the contribution levels of offspring were different among *Ae. aegypti* adults.

Results

Egg hatchability and female insemination rates were examined across the three *Ae. aegypti* mating groups: Aed1, Aed2, and Aed3. The proportions of eggs that hatched ranged between 79.3 and

85.9% (Table 1). These results show how variable mating outcomes can be because some eggs did not hatch. Moreover, one or two *Ae. aegypti* females in each group were not inseminated, even though they had three days to do so and abundant male partners to choose from.

Target loci were successfully amplified using genomic DNA extracted from individual *Ae. aegypti* as templates in each PCR reaction. Test PCR amplifications using multiplex and single-locus protocols yielded products ranging in size from 100 bp to 300 bp (Supp Fig. 1 [online only]). Our earliest multiplex reactions using five pairs of fluorescently labeled primers showed the formation of primer dimers. After PCR optimization, the concentration of $MgCl_2$ included per reaction was reduced from 25 mM to 20 mM and the multiplex group size was limited to four pairs of primers to better identify the PCR products corresponding to each of the individual microsatellite loci.

Using ten polymorphic loci for parentage analysis, a total of 182 mosquitoes comprised 60 *Ae. aegypti* adults and 122 of their F_1 offspring from the three cross-mating trials were tested. Among these individuals, 2–7 alleles were found per locus, and observed heterozygosity (*Ho*) ranged from 0.401 to 0.989 (Table 2). Analyses using MicroChecker software showed the absence of null alleles at all loci and no evidence of scoring errors due to stuttering or allelic dropout. Hardy-Weinberg equilibrium tests with Bonferroni correction were not done as they are included in CERVUS and no deviations were detected. Fragment analysis was conducted twice to confirm the allelic distributions and for manual checking of multiple mating. After examination of candidate parental and offspring genotypes from three crosses, the AC5 locus was eliminated from subsequent parentage analyses because it gave an unexpectedly high proportion of homozygotes in some pools of offspring (18 out of 122 total).

In this study, critical Delta values were calculated for parentage assignment of *Ae. aegypti* with >80% and >95% confidence, respectively, as follows: (1) Aed1: > 0.00 and 1.35; (2) Aed2: > 0.00 and > 1.36; (3) Aed3: > 0.01 and 1.37. A zero value of Delta indicates that the discriminating power of the markers is high. A minimum value of zero was set when the simulation could not find a lower bound for the critical Delta.

Parent-offspring assignments using CERVUS revealed significant multiple mating that produced fertile offspring across the three trial groups of *Ae. aegypti* (Table 3). Under the >80% confidence level, 86% (36/42) of F_1 offspring of *Ae. aegypti* were successfully assigned to their respective parents in the family Aed1. In contrast, when using a >95% strict confidence level, only 19% (8/42) of F_1 progeny were successfully assigned in Aed1 etc. Overall, the numbers of unassigned offspring were 6, 8, and 9, respectively, in each group. However, the CERVUS program was able to indicate the most likely parents for all of the offspring, albeit with 70% confidence (i.e., under a relaxed criterion). The unassigned offspring (N = 23) were then matched to their most likely parents and the results were checked manually to confirm these assignments (Supp Tables 2–4 [online only]). Manual checking involves counting the number of parental alleles among the progeny of a single female parent. For instance, if we counted 4 alleles, then we inferred that there were at least two male parents, and if we counted five or six, then, there were at least three male parents. Manual checking confirms polyandry (i.e., mating with more than 3 different partners) in seven *Ae. aegypti* females (23%) and seven males (23%). After 182 DNA samples were genotyped, we checked the number of alleles present at each locus.

The contributions of *Ae. aegypti* adults to the pool of offspring were substantially uneven, as were the numbers of mating partners per individual (see Table 4). All offspring from each trial group (Aed1, Aed2, and Aed3) were successfully assigned to their putative parents (Supp Tables 2–4 [online only]). For instance, in trial Aed1, eight males and nine females mated and produced fertile offspring; male #6 and female #9 produced more offspring (14 and 12 respectively) than other candidate parents. Two males (#3 and #7) and one female (#5) did not produce any offspring at all (Supp Table 2 [online only]). Similar findings are also apparent in the results from trial Aed2 (Supp Table 3 [online only]) including male #5 who produced

Table 1. Egg production, hatchability and insemination rates for *Ae. aegypti* females

Trial	Number of eggs laid	Number of eggs that hatched (%)	Number of females inseminated
Aed1	170	142 (83.5%)	9
Aed2	135	116 (85.9%)	9
Aed3	155	123 (79.3%)	8

Detail of *Ae. aegypti* mating outcomes and offspring summarized in Supp Tables 2–4 (online only).

Table 2. Characteristics of the ten microsatellite loci used in the parent-offspring assignment trials for *Ae. aegypti* individuals

			Heterozygosity	
Locus	Number of individuals (N)	Total number of alleles (N_A)	Observed (*Ho*)	Expected (*He*)
AC5	182	6	0.599	0.659
A10	182	4	0.429	0.415
AT1	182	6	0.665	0.712
AG5	182	6	0.753	0.706
HO8	182	2	0.401	0.402
AG1	182	3	0.489	0.463
AC1	182	6	0.797	0.664
AG4	182	4	0.989	0.594
BO7	173	7	0.410	0.631
AC2	182	3	0.659	0.660

Table 3. Parentage assignment of *Ae. aegypti* using nine microsatellite markers

	Parent pair assignment of sexes known						
	80% confidence (relaxed)		95% confidence (Strict)		Unassigned[a]		
Cross	Observed	Expected	Observed	Expected	Observed	Expected	Total off-spring
Aed1	36 (86%)	42 (100%)	8 (19%)	33 (78%)	6 (14%)	0	42
Aed2	32 (80%)	40 (100%)	17 (42%)	31 (76%)	8 (20%)	0	40
Aed3	31 (78%)	38 (94%)	13 (33%)	23 (58%)	9 (23%)	2 (6%)	40
Total (Observed)	99 (81%)		38 (31%)		23 (18%)		

[a]Unassigned offspring, $N = 23$ (18%) are assigned with confidence >70%.

Table 4. Statistical analyses for results from three *Ae. aegypti* mating groups

Ae. aegypti cross	Total mating partners (Mean ± SD)	Offspring (Mean ± SD)	Chi-square results
Aed1	M: 2.2 ± 1.3	M: 4.2 ± 4.6	M: χ^2 (7, $N = 42$) = 28.48, $P = 0.00^*$
	F: 2.2 ± 1.9	F: 4.2 ± 3.6	F: χ^2 (8, $N = 42$) = 21.00, $P = 0.01^*$
Aed2	M: 1.6 ± 1.1	M: 4.0 ± 7.5	M: χ^2 (7, $N = 40$) = 92.80, $P = 0.00^*$
	F: 1.6 ± 1.3	F: 4.0 ± 4.4	F: χ^2 (7, $N = 40$) = 26.80, $P = 0.00^*$
Aed3	M: 1.3 ± 1.2	M: 4.0 ± 4.9	M: χ^2 (7, $N = 40$) = 35.20, $P = 0.00^*$
	F: 1.4 ± 0.9	F: 4.0 ± 6.0	F: χ^2 (7, $N = 40$) = 56.80, $P = 0.00^*$

M = male, F= female;
*Significant when $P < 0.05$.
Regression analysis:
Male partner and #offspring: F (1, 28) = 17.22, $P = 0.000$.
Female partner and #offspring: F (1, 28) = 19.52, $P = 0.000$.
Both regression analyses show that *Ae. aegypti* individuals mating with most partners have the most offspring – further details are given in Supp Table 5 (online only).

the highest number of offspring (N = 25) recorded in any of the three tests. Trial Aed3 (Supp Table 4 [online only]) included female 6 who produced 19 offspring, and two males (#1 and #9) and two females (#4 and #8) who did not produce any offspring. All three cross-mating groups showed at least one male that mated with up to three or four females. Likewise, one or more *Ae. aegypti* females mated with three or more males (Table 4).

Discussion

In the three mating tests carried out, 122 (100%) *Ae. aegypti* offspring were successfully assigned to the parental pairs using nine polymorphic microsatellite markers and likelihood-based software. Using CERVUS parentage assignment, 31% of *Ae. aegypti* offspring are assigned at 95% while 81% of offspring are assigned at 80% confidence level. There are 18% of *Ae. aegypti* offspring assigned by relaxing the confidence limit to 70%. The present study reveals two very interesting results. First, in the mating groups, four females and two males of *Ae. aegypti* mated with three different partners. Furthermore, three females and five males of *Ae. aegypti* mated with four different partners, and possibly more (i.e., give or take undetected sperm competition). Clearly, these *Ae. aegypti* exhibited efficient mating behavior, especially those that mated with three to four different partners within three days and those that produced large numbers of offspring (i.e., 14, 16, and 25) within six days.

Aedes aegypti females are often held to be monogamous as they have been found to mate only once and are generally resistant to second insemination (Craig 1967, Spielman et al. 1967, Camargo et al. 2020). Most females (76%) become refractory to a second mating within 2 hr of their first mating, and that once female refractoriness is established, it is absolute and long lasting, so that no females having completed up to five gonotrophic cycles were re-inseminated despite having the opportunity to re-mate (Degner and Harrington 2016). The reduction in sexual receptivity is mediated by seminal fluid proteins (SFPs) produced in the accessory gland of the male reproductive tract (Craig 1967, Helinski et al. 2012a). *Aedes aegypti* males, in contrast, are polygamous and may mate three to four times consecutively before sperm depletion occurs (Helinski and Harrington 2011, Bargielowski et al. 2011). One reason for this is that *Ae. aegypti* males are aggressive in mating, i.e., they can copulate several times with different females (Thomas and Yap 1973, Marcela et al. 2015). Sexually aggressive *Ae. aegypti* males form more mating pairs with *Ae. aegypti* and *Ae. albopictus* females than *Ae. albopictus* males (Black et al. 1989, Choochote et al. 2001, Marcela et al. 2015). Also, the fact that *Ae. aegypti* males can inseminate more *Ae. aegypti* females in their lifetimes than they can do in a single day suggests that their sperm reserves (or reserves of other seminal fluid components) are depleted by successive mating and must be replenished before they can inseminate further females (Agudelo et al. 2021, Degner and Harrington 2016).

While findings on polyandry in *Ae. aegypti* males are accumulating, there is only a limited number of studies that report and/or assess the polyandrous behavior of *Ae. aegypti* females and whether all of the multiple matings contribute to inseminated spermathecae and produce viable offspring. Previous studies show that 6.25% of *Ae. aegypti* females ($N = 48$) can mate with up to three different male partners (Richardson et al. 2015), and 14% of *Ae. aegypti* females receive semen from more than one male (Helinski et al. 2012b). In comparison, our study demonstrates *Ae. aegypti* females can mate with three to four different *Ae. aegypti* males, 13 and 10% respectively, giving a total of 23% from a sample of 30 females. The discrepancies observed between the results of others and those presented here are potentially due to different methodologies being employed to assess

polyandrous mating. Richardson et al. (2015) estimated the number of *Ae. aegypti* male parents that contributed to offspring using *Ae. aegypti* maternal and offspring genotypes, but they did not genotype the males. Helinski et al. (2012b) used males with stable isotope labeled semen to assess multiple mating by examining the presence of radioactive labels in *Ae. aegypti* female spermathecae, but they did not examine DNA genotypes. Hence, our results add a further dimension to a growing body of research showing that both male and female *Ae. aegypti* are polygamous, with individuals taking up to four different partners, all of whom are capable of producing fertile offspring. This set of findings challenges the long-standing belief of some biologists that all *Ae. aegypti* females mate only once in their lifetime (e.g., see Gwadz and Craig 1968, Gwadz et al. 1971, Clements 1999, Bargielowski et al. 2011).

Our results (Supp Tables 2–4 [online only]) show individual contributions of parents to the pools of offspring were unequal across all three mating trials (for analysis see Table 4 with Chi-square tests giving $P < 0.05$). In each cross, some of the candidate parents did not contribute to offspring, most probably due to a poor physiological condition, e.g., where the males were unable to produce good quality gametes (Agudelo et al. 2021, Dieng et al. 2016). In addition, some candidate parents were very successful in competitive mating and produced relatively large numbers of offspring compared with others. However, in this study, since not all offspring were collected for the parentage analysis, it remains possible that some successful candidate parents were excluded. For instance, spermathecae dissection revealed nine inseminated females in the Aed1 and Aed2 experiments, but after assignment analysis, only eight females were shown to produce offspring in the latter.

The results of our present study agree with those reported by Wong et al. (2012). Using similar microsatellite markers developed by Slotman et al. (2006) and Chambers et al. (2007), Wong and her co-workers managed to correctly match 149 offspring ($N = 200$) to their respective parents. Therefore, the data from the present study, taken together with those of Wong et al. (2012), confirm the power of these nine microsatellite markers for genetic assessment in *Ae. aegypti*. Importantly, Wong et al. (2012) used their microsatellite markers to track egg-laying behavior, but they did not assess multiple mating behaviors of *Ae. aegypti*. The same authors amplify DNA extracted from a single hind leg and calculate parentage exclusion probabilities (i.e., the formula by Jamieson and Taylor 1997) to match offspring to parents (Wong et al. 2012). In contrast, our study amplifies DNA from whole *Ae. aegypti* bodies and uses likelihood ratio for parentage assignment. Paternities assigned with 80% confidence are more dependable than those achieved by direct observation and are also better than those obtained by a purely exclusionary approach, where confidence in the paternity of nonexcluded males is generally unknown (Marshall et al. 1998). In comparison to the Wong et al. (2012) report, our study confirms polygamous mating in both sexes of *Ae. aegypti* and demonstrates the varying mating capability of *Ae. aegypti* individuals to mate and produce offspring across three mating groups. We removed the AC5 locus from the *Ae. aegypti* parentage assignment because it shows no less than 18 offspring to be fully homozygous. This precautionary approach increases confidence in genetic parentage assignment as it reduces the incidence of false exclusion of true parents (Dakin and Avise 2004). Other study also excludes one locus AG1 due to sex-linked in some families of *Ae. aegypti* parentage assignment (Wong et al. 2012).

Previous mating studies on *Ae. aegypti* have used markers such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) to assign sibling relationships among field-collected larvae (Apostol et al. 1994, Colton et al. 2003). Although RAPD markers do not absolutely require parental information, they can still generate high misclassification rates because they segregate as dominant loci. As a result, individuals who are homozygous for a dominant allele cannot be distinguished from heterozygotes (Roderick 1996). The RFLP method requires a large starting amount of DNA because it is not PCR-based. This limits the number of loci that can be practically analyzed and creates an inability to discriminate among closely related groups (Colton et al. 2003). Wong et al. (2012) used microsatellite markers and parentage exclusion probabilities to match *Ae. aegypti* offspring to parent genotypes for tracking oviposition behavior. Since exclusion-based analysis uses the principles of Mendelian inheritance, it requires genotyping information about both potential parents. The exclusion power of this type of marker analysis is severely reduced if only a single parental genotype is known.

The parentage assignment method described in our study does not depend directly on Mendelian inheritance principles (Jones et al. 2010). Rather, it is based on estimates of population allele frequency. Likelihood values can then be used to statistically identify the most likely candidate parent pair via likelihood ratio tests (Kalinowski et al. 2007). Moreover, it also accommodates genotypic mismatches in the data due to mutations or experimental error, as well as having the capacity to detect the presence of null alleles (Jones et al. 2010). The parentage assignment framework in CERVUS is most powerful when both genotypes of all candidate parental pairs are known (Kalinowski et al. 2007). Moreover, despite the higher precision of single-nucleotide polymorphisms (SNPs), a recent study demonstrates that microsatellites can efficiently uncover population genetic processes (i.e., such as gene flow) and may be superior for parentage analysis for species with reduced genetic diversity (Hauser et al. 2021). Current study shows for 18% unassigned *Ae. aegypti* offspring, any mismatch at a single allele prevents 100% agreement between offspring and parental genotypes. Accepting some low threshold of mismatches (one or two) is common during parentage analyses (Wang 2010, Wong et al. 2012). Such mismatches can occur due to mutation (unlikely) or genotyping error (always possible) (Kalinowski et al. 2007). Morrissey and Wilson (2005) report that allowing for genotyping error produces lower rates of false paternity assignment than assuming there are no errors — even when data contain errors. Our study uses a new version of CERVUS (version 3.0) that implements a corrected likelihood equation that accommodates genotyping error, hence improving the success of parentage assignment (see details in Kalinowski et al. 2007).

Oftentimes, the mating ability of *Ae. aegypti* is assessed by documenting the number of mating pairs, female insemination, and the number of eggs produced, in genetically engineered *Ae. aegypti* (Bargielowski et al. 2011), in conspecific (Helinski et al. 2012b, Richardson et al. 2015, Degner and Harrington 2016) and interspecific mating (Bargielowski et al. 2015, Marcela et al. 2015, Carrasquilla et al. 2019). It has long been known that *Ae. aegypti* females can engage in multiple mating without being inseminated (Jones 1973) and are able to produce eggs without actually mating with *Ae. aegypti* males (Dieng et al. 2016). These facts show the pressing need to validate mating fitness through genetic parentage assessment.

This study is the first to employ CERVUS for examining multiple mating and offspring-parent assignment in *Ae. aegypti*. Although CERVUS was developed to examine parentage assignment of Rum red deer (Marshall et al. 1998), we demonstrate that CERVUS can be applied to successfully examine mosquito mating data. Other well-known software products employed for parentage analysis include

COLONY, FAMOZ, and MASTERBAYES (Flanagan et al. 2019), but these methods are yet to be tested in the pedigree analysis of *Ae. aegypti*. Each one requires different genotypic information, such as both parental genotypes, one paternal genotype, and/or whether the progeny is composed of full or half siblings (Flanagan et al. 2019). In *Ae. aegypti,* Richardson et al. (2015) used the program GERRUD to link offspring to maternal parents but recognized that their frequency of polyandry may have been underestimated because some polyandrous families were not detected. Our study is different because it includes pools of maternal, paternal, and offspring genotypes, which can only be resolved by using CERVUS, which was designed for this specific purpose. It has been successfully applied to assign offspring to parents in various insect studies such as flies, spiders, and bees (Mikát et al. 2019, Pandulli-Alonso et al. 2020, Muhwezi et al. 2020).

Genetically modified or sterile males are released to mate with wild *Ae. aegypti* females on the basis that sired offspring will die, hence reducing the *Ae. aegypti* population (Lacroix et al. 2012, Arham et al. 2021). Based on our new data, future control strategies of dengue vectors should focus on understanding the consequences of polygamous mating in both males and females of *Ae. aegypti*, particularly in GMM and SIT techniques. First, wild *Ae. aegypti* females cannot be assumed to give birth to offspring from a single father (Choochote et al. 2001, Helinski et al. 2012b). Second, the contributions of individual parents to the pool of F1 offspring appear to be exceptionally varied among individuals (Richardson et al. 2015). Therefore, the release of modified *Ae. aegypti* males (GMM and SIT) must be large-scale and prolonged to be most effective because *Ae. aegypti* females who have mated with the modified males may still go on to mate with wild type individuals (Richardson et al. 2015). Monandrous *Ae. aegypti* females will not produce offspring after mating with sterile males, but polyandrous *Ae. aegypti* females could potentially remate with nonsterile males.

Mating fitness of the males and females of *Ae. aegypti* needs to be further examined in detail. Several aspects of mosquito biology, such as age, body-size, female fecundity, and types of strain, have been reported to regulate the reproductive success of *Ae. aegypti* (Helinski and Harrington 2011, Aldersley and Cator 2019, Agudelo et al. 2021;). Large body-size *Ae. aegypti* males have greater mating capacity than small males, and *Ae. aegypti* females mated to large males have higher fecundity than otherwise (Helinski and Harrington 2011). Male age influences re-mating incidence of *Ae. aegypti*; 54.5% of *Ae. aegypti* females mated to an old male (21–22 d old) re-mate, as compared with 24% of *Ae. aegypti* females initially mated to a young male (4–5 d old) (Agudelo et al. 2021). Repeated mating and multiple inseminations of *Ae. aegypti* females ensure the transfer of adequate amounts of sperm to fertilize eggs (Choochote et al. 2001, Agudelo et al. 2021). Wild strains of *Ae. aegypti* that have access to better nutrients and live in near ideal environments in the field produce greater numbers of spermatozoa than colony insects reared in a laboratory for over 40 yr (Ponlawat and Harrington 2007). Our study uses *Ae. aegypti* adults derived from immature stages of a wild strain and reared in a standardized laboratory setting (i.e., same food type and quantity for larvae and pupae across three mating groups) to produce a similar body size (Dieng et al. 2016). Therefore, we assess the mating fitness of *Ae. aegypti* primarily based on female insemination and adult genetic contribution towards F1 offspring – we deduce that *Ae. aegypti* with high mating fitness tend to mate more with different partners and produce more offspring (Supp Tables 2–5 [online only]). Additional studies that assess the mating fitness by manipulating the four factors above followed by parent-offspring assignment of *Ae. aegypti* may present different findings.

In conclusion, our analysis demonstrates significant polygamous behavior in wild caught *Ae. aegypti* males and females. Although this study uses relatively small samples to assign the offspring to parents, it demonstrates important implications for noninsecticide control studies. Evidence suggests that GMM and sterile *Ae. aegypti* males can suppress 80% of wild-type *Ae. aegypti* populations in Grand Cayman (Harris et al. 2012) and reduce *Ae. aegypti* density by 95% in Brazil (Carvalho et al. 2015). Genetically modified *Ae. aegypti* males need to be competitive enough to outperform wild *Ae. aegypti* males and mate with wild *Ae. aegypti* females to effectively reduce the local *Ae. aegypti* population. *Aedes aegypti* females will remate if *Ae. aegypti* males are unable to induce refractoriness in the females (Bargielowski et al. 2011, Agudelo et al. 2021). Hence, the extent of multiple matings as shown in this study must be carefully incorporated into dengue vector control programs. There is an urgent need to assess the mating capacity and how the genes are transferred to viable offspring using genetic parentage assignment. The selection of modified or sterile males must consider three criteria: 1) selecting *Ae. aegypti* males that can mate with up to four different females; 2) evaluating transformed *Ae. aegypti* male performance with polyandrous females; and 3) prioritizing the selection of polyandrous *Ae. aegypti* males and/or females that produce the most offspring. Based on our current findings, we suggest future *Ae. aegypti* mating studies include all offspring-parent genotypes to increase the rigor of parentage assignment, as well as to verify *Ae. aegypti* female insemination.

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Supplementary Data

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

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