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No evidence for successful interspecific cross-mating of transgenic *Aedes aegypti* (L.) and wild type *Aedes albopictus* Skuse

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Abstract. The natural and artificial mating of laboratory bred *Aedes albopictus* and transgenic *Aedes aegypti* RIDL-513A-Malaysian strain was conducted. The experiment consisted of cross-mating of homologous *Ae. aegypti* RIDL ♀ X *Ae. aegypti* RIDL ♂ and reciprocal *Ae. aegypti* RIDL ♀ X *Ae. albopictus* WT ♂. The other set comprised homologous *Ae. albopictus* WT ♀ X *Ae. albopictus* WT ♂ and reciprocal *Ae. albopictus* WT ♀ X *Ae. aegypti* RIDL ♂. This study demonstrated that reproductive barriers exist between these two species. Cross insemination occurred between *A. albopictus* male and *Ae. aegypti* female and their reciprocals. There was 26.67% and 33.33% insemination rate in *Ae. aegypti* RIDL female cross-mating with *A. albopictus* WT male and *Ae. albopictus* female cross-mating with *Ae. aegypti* RIDL male, respectively. There was 0% hatchability in both directions of the reciprocals. There was also no embryonation of these eggs which were bleached. Although none of the female *Ae. albopictus* WT was inseminated in the cross-mating with *Ae. albopictus* WT ♀ X *Ae. aegypti* RIDL ♂, a total of 573 eggs were obtained. The homologous mating was very productive resulting in both high insemination rate and hatchability rates. Generally there was a significantly higher insemination rate with artificial mating insemination of homologous than with artificial mating of reciprocal crosses. Interspecific mating between *Ae. aegypti* RIDL and *Ae. albopictus* wild type was not productive and no hybrid was obtained, indicating absence of horizontal transfer of introduced RIDL gene in *Ae. aegypti* to *Ae. albopictus*.

INTRODUCTION

Aedes aegypti is the main vector of dengue which has been reported in over 100 countries. More than 2.5 billion people live in the areas where dengue is endemic and 50-100 million cases of dengue occur every year. About 250,000-500,000 cases of dengue are officially notified annually (WHO, 2007). In the absence of effective vaccines and specific anti-viral treatment, the control of dengue is dependent on suppression of the mosquito vectors. Presently, aerially-sprayed chemical insecticides are widely used as the main control agents attempting to interrupt dengue transmission by destroying the infected adult mosquitoes. Although effective to some extent if used appropriately, nevertheless insecticides are insufficient to prevent and contain dengue outbreak, as exemplified by the rapid spread of dengue globally. Other alternative control measures, such as genetic control need to be studied. Genetic control in the form of SIT (Sterile Insect Technique) employing radiation has long been used to control other insects. Because of the deleterious effects of radiation on mosquitoes, SIT is not suitable for use in mosquito control. Recently, a new genetic control tool, Release

of Insect Carrying Dominant Lethal (RIDL) technique was successfully used to transform *Aedes aegypti*. RIDL is based on the development of transgenic insect with a genetic construct that confers repressible dominant lethality (Alphey, 2000). In its simplest form, RIDL operates in a non–sex specific manner, killing all insects carrying at least one copy of the construct unless these insect are provided with a specific repressor of the lethality. The principle is simple: if the released insects are homozygous for a dominant lethal and mate wild insects, all the progeny are heterozygous for a dominant lethal gene and so they die (Alphey & Andreasen, 2002).

A RIDL strain of *Ae. aegypti* has now been established in our insectarium. Prior to further experiments using this strain, its bionomics need to be studied in details in order to ascertain its suitability for field release experiment in future. Since *Aedes albopictus* and *Ae. aegypti* are sympatric species that occupy similar ecological niches (Klowden, 1993), it is important to determine if interspecific mating could occur between the 2 species since it is foreseeable that RIDL *Ae. aegypti* males will be released into areas with *Ae albopictus* as well. Hence this study was conducted to evaluate the possibility of cross-mating in laboratory-bred *Ae. aegypti* carrying a dominant lethal gene (RIDL) with wild type *Ae. albopictus* under laboratory conditions.

MATERIALS AND METHODS

Mosquito strains

RIDL-513A-Myla *Ae. aegypti* and Wild Type (WT) *Ae. albopictus* were maintained in the insectarium at the Institute for Medical Research (IMR). Both strains were bred in isolation. The original RIDL *Ae. aegypti* was designated as RIDL-513A which was generated with a Rockefeller strain genetic background. The present strain coded as RIDL-513A-Myla was generated using a laboratory strain of Malaysian origin. It was generated using 12 homozygous female founder parents. The *Ae. albopictus* strain originated from adults collected in Selangor in 2006.

Mosquito rearing & maintenance

All experimental mosquitoes were bred in the insectarium at a temperature of 26±2ºC with 65±10% relative humidity and a 12:12 hours light and darkness. The RIDL strain was maintained in the Arthropod Containment Level-2 (ACL-2) room. Larvae were fed with liver powder at 1st and 2nd instar and later provided with partially cooked cow liver at 3rd and 4th instar. RIDL *Ae. aegypti* larvae were provided with tetracycline (30 mg/L) to suppress the lethal effect of the gene and to ensure successful completion of the life cycle. Both adults were fed on 10% sugar solution with vitamin B complex. At pupal stage, size was used to distinguish between male and female. Pupae were kept individually in glass vials to ensure virginity in newly emerged adults. Adult mosquitoes were kept as per strain in different cages of females and males. Careful sexing was done on adults to ensure that no males were present in female cages. If a male was found, then all the mosquitoes of that cage were discarded. Prior to commencement of the experiment, all the female mosquitoes were given a blood meal by feeding on white mice.

Artificial mating

Five days old female and male mosquitoes were used. The crosses for the reciprocal as well as the homologous control were carried out using an artificial mating technique as described by Ow Yang *et al.* (1963). Ten virgin male mosquitoes were placed into a flask that contained anaesthetic ether. The male mosquitoes were removed and laid on a filter paper. The thoraxes of the male mosquitoes were impaled with tip rods before the artificial mating was conducted. While waiting for the males to regain consciousness, 10 virgin females were also anaesthetized and placed upside-down on a white sheet of paper.

The genitalia of the male and female were brought together at an appropriate angle for genital contact. When the male and female were in the process of copulation,

they were left to remain so for sometime until the two sets of genitalia separated naturally. After artificial mating, individual female was introduced singly into a 0.028m^3 (1 cubic foot) screened cage with a nylon stockenette sleeve that provided access to the interior of the cage. A 10% sucrose solution with vitamin B complex was provided as a maintenance diet. The blood-fed female mosquitoes were allowed to oviposit after 3 days by placing a damp cone-shaped filter paper, placed inside a white bowl with minimum amount of water. They were allowed to oviposit for a period of 5 days. The experiment was repeated with 10 individual females and 10 individual males for all the combination crosses. Eggs oviposited by individual females were counted under a dissecting microscope. The eggs were air-dried to ensure complete embryonation. The filter papers were then immersed in dechlorinated water and eggs were observed for hatching for a period of 10 days. Eggs that did not hatch were then bleached using full strength sodium hypochlorite solution and re-examined under a dissecting microscope.

Natural cage mating experiment

In natural mating three replicates were set up with 30 females and 60 males in each reciprocal and homologous cage experiments. Thirty 4-5 days old females and sixty males were placed in each of the 4 breeding cages in four combinations consisting of 2 reciprocal cross combination and 2 homologous control populations. In the screened cages a 10% sucrose with vitamin B complex solution was provided as maintenance diet. The mosquitoes were allowed to copulate naturally in the 4 different combination cages for 2 days. The female mosquitoes were set for egg lying for a period of 5 days on a damp cone-shaped filter paper that was placed inside a white bowl with minimum amount of water. The eggs on the filter paper were removed and counted under a dissecting microscope. The eggs were air-dried to ensure complete embryonation. The filter papers were then immersed in water and eggs were later observed for hatching for a period of 10 days. Eggs that did not hatch were bleached using full strength sodium hypochlorite solution.

In both experiments, all female mosquitoes were dissected to examine the spermathecae for the presence of spermatozoa as an indication of mating. Dissection was conducted under a dissecting microscope at 400X magnification. The whole experiment took four months to complete using 1440 male and female mosquitoes. A total number of 720 *Ae. albopictus* and the same number for RIDL *Ae. aegypti* were used for the whole experiment of natural and artificial mating that comprised 240 females and 480 males.

RESULTS

Artificial mating

Low mean number of eggs in artificial reciprocal cross-mating of *Ae. albopictus* WT ♀ with *Ae. aegypti* RIDL ♂ and *Ae. aegypti* RIDL♀ crossed with *Ae. albopictus* WT ♂ was observed. An average of 34.00 and 43.88 eggs per mated female were oviposited, respectively (Table 1). All the eggs were not viable. In homologous matings, *Ae. aegypti* RIDL ♀ laid an average of 57.22 eggs per mated female while *Ae. albopictus* WT ♂ laid 62.67 eggs per mated female with 32% and 96% hatchability, respectively.

In the reciprocal crosses of *Ae. albopictus* females with *Ae. aegypti* RIDL males, 33.33% (10) insemination rate was observed. All the eggs were not fertile with no embryo development shown after bleaching. There was 26.67% (8) insemination rate in *Ae. aegypti* female crossed with *Ae. albopictus* male. All the eggs were also non-viable but subsequent bleaching showed the presence of embryos inside the eggs after 10 days of submerging in water. Fig. 1a and 1b showed bleached embryonated eggs observed under a compound microscope which were oviposited by reciprocal forced mating of *Ae. aegypti* RIDL♀ X *Ae. albopictus* WT ♂ and Fig. 2 showed the respective female's fertilized spermathecae with spermatozoa. In homologous artificial cross-mating, the insemination rate was high at 60% and 50%, respectively.

Natural mating

There was a low egg count in natural reciprocal cross-mating of *Ae. albopictus* WT ♀ X *Ae. aegypti* RIDL ♂ and *Ae. aegypti* RIDL♀ X *Ae. albopictus* WT ♂. Although 573 eggs were obtained from *Ae. albopictus* WT ♀ X *Ae. aegypti* RIDL ♂, none of the females were inseminated. In the reciprocal cross-mating of *Ae. aegypti* RIDL♀ X *Ae. albopictus* WT ♂, 590 eggs were produced by 2 inseminated females. All the eggs were not viable. In homologous mating, a mean of 34.20 and 36.10 eggs per mated female mosquito was obtained. Eggs of *Ae. aegypti*

Table 1. Comparative egg production between reciprocals and homologous cross mating in artificial mating experiment

Cross ♀ X ♂	No of female mosquitoes used	Total No of eggs laid	No. of ♀ mated (% insemination)	Average no. of eggs per mated ♀	% hatched
Reciprocal					
Ae. albopictus WT ♀ ***X*** *Ae. aegypti RIDL* ♂	30	340	10 (33.33%)	34.00	0%
Ae. aegypti RIDL♀ ***X*** *Ae. albopictus WT* ♂	30	351	8 (26.67%)	43.88	0%
Homologous					
Ae .aegypti RIDL ♀ ***X*** *Ae. aegypti RIDL* ♂	30	1030	18 (60.00%)	57.22	32%
Ae. albopictus WT ♀ ***X*** *Ae. albopictus WT* ♂	30	940	15 (50.00%)	62.67	96%

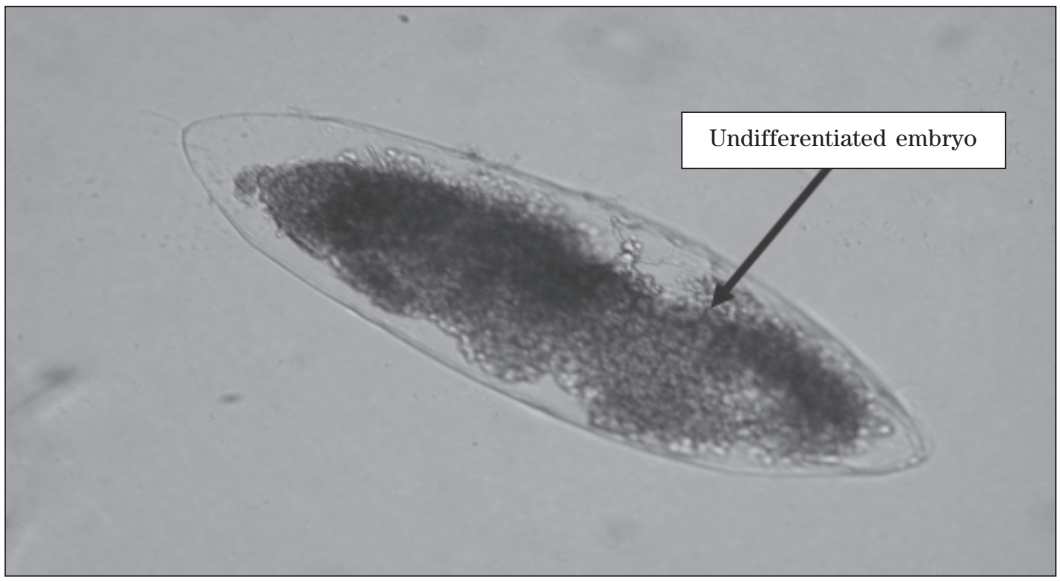

Figure 1a. Bleached embryonated eggs observed under a compound microscope oviposited by reciprocal forced mating *Ae. aegypti* RIDL♀X *Ae. albopictus* WT ♂.

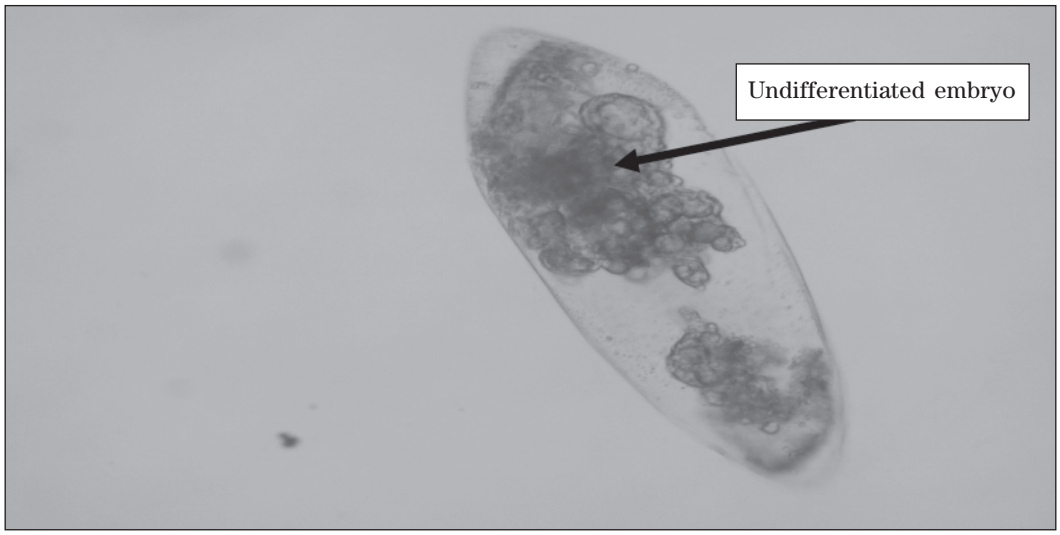

Figure 1b. Bleached embryonated eggs observed under a compound microscope oviposited by reciprocal forced mating *Ae. aegypti* RIDL♀X *Ae. albopictus* WT ♂.

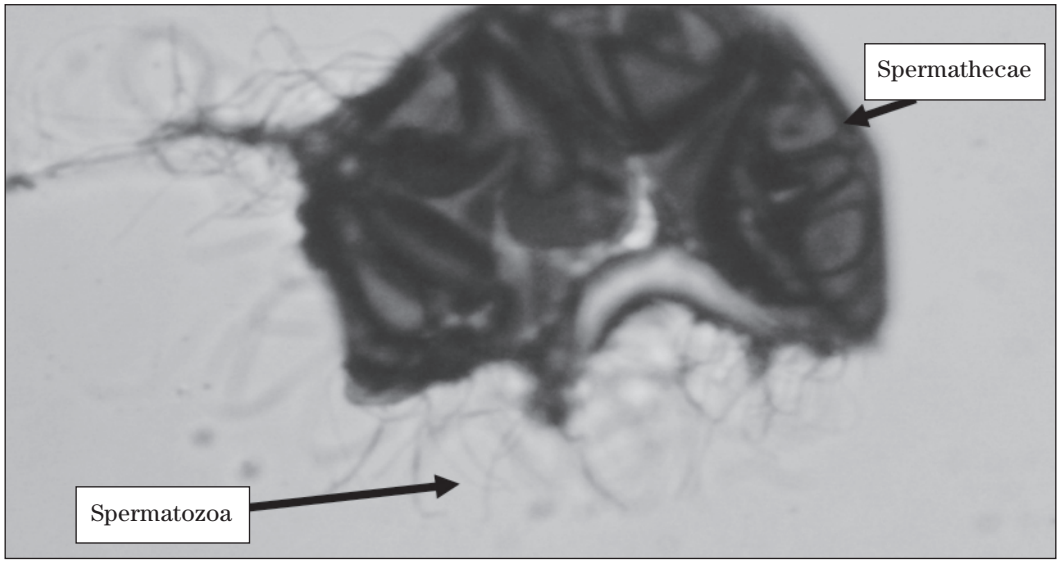

Figure 2. Fertilized spermathecae showing spermatozoa.

RIDL had 98% hatchability while for *Ae. albopictus* WT, the hatchability was 97%.

In *Ae. aegypti* RIDL female cross-mated with *Ae.albopictus* males, there was 2.22% insemination, while the reverse reciprocal mating yielded 0% insemination rate. All the eggs produced were not viable and had no embryos inside them after bleaching. In the homologous mating, insemination rate of 87.78% and 85.56% was obtained from both mating (Table 2). Generally, homologous mating was more productive than reciprocal crosses, resulting in higher insemination rate and egg hatchability.

Table 2. Comparative egg production between reciprocal and homologous cross-mating in natural mating experiment

Cross ♀ X ♂	No of female mosquitoes used	Total No of eggs laid	No. of ♀ mated (% insemination)	Average no. of eggs per mated ♀	% hatched
Reciprocal					
Ae. albopictus WT ♀ ***X*** *Ae. aegypti RIDL* ♂	90	573	0 (0%)	0	0%
Ae. aegypti RIDL ♀ ***X*** *Ae. albopictus WT* ♂	90	590	2 (2.22%)	295.00	0%
Homologous					
Ae. aegypti RIDL ♀ ***X*** *Ae. aegypti RIDL* ♂	90	2310	79 (87.78%)	34.20	98%
Ae. albopictus WT ♀ ***X*** *Ae. albopictus WT* ♂	90	2780	77 (85.56%)	36.10	97%

DISCUSSION

Cross-mating studies were initiated from time to time to study the possibility of interspecific mating of *Ae. aegypti* and *Ae. albopictus* due to their somewhat close evolutionary relationship, as both species are in the same subgenus *Stegomyia*. Most of the early investigators reported that their cross-mating were unproductive as they could not produce a progeny (Simmon *et al.*, 1930). Although cross-mating of laboratory strain was not productive, most of the authors claimed that copulation was successful as evidenced by the presence of spermatozoa in the spermathecae of female mosquitoes they used (De Buck, 1942; Bonnet, 1950; Rozeboom & Kitzmiller, 1959; Nasci *et al.*, 1989; Nazni *et al.*, 2009). However, there were other authors who claimed partial success after producing a progeny (Huang-Tich-Try, 1939; Toumanoff, 1950; Kartman, 1953; Woodhill, 1959; Thomas & Yap, 1973). Nevertheless, the progeny could not be propagated beyond the 4th generation. The offspring reportedly resembled the female parent. Some of the claimed success was attributed to contamination (Thomas & Yap, 1973), while others were attributed to parthenogenetic development of the ovum (Downes & Baker, 1949).

Nevertheless, all studies on interspecific mating of the two *Aedes* species were conducted using wild type. With recent development of transgenic *Ae. aegypti* with the major objective of releasing males of this mosquito into the environment in a sustained manner to control the wild population, there is no doubt that concerns will be raised again on the possibility of interspecific mating of *Ae. aegypti* and *Ae. albopictus*; as the later species, which is a known dengue vector, is also found abundantly in dengue endemic areas in which *Ae. aegypti* is also present (Chen *et al.*, 2006). In addition, the possibility of transfer of the engineered RIDL gene from *Ae. aegypti* to *Ae. albopictus* is also important in view of horizontal gene transfer to non-target organisms.

In this study, an insemination rate of 33.33% (10) in *Ae. albopictus* females which were cross-mated with male RIDL *Ae. aegypti* was obtained from forced mating experiments. From the 10 mosquitoes which oviposited a mean of 34 eggs per mated female, none of these eggs were fertile. These observations were in agreement with studies conducted by Nazni *et al.* (2009) in which a mean of 26 eggs per mated female was obtained, using wild type Malaysian strains. Leahy & Craig (1967), who obtained similar results when their *Ae. albopictus*

female cross-mated with *Ae. aegypti* male had 62% insemination rate with 2 mosquitoes ovipositing an average of 4 eggs. These eggs too did not hatch. They also reportedly identified five reproductive barriers which isolated these species from each other.

In the reciprocal crosses using forced mating, *Ae. aegypti* RIDL females exhibited 26.67% insemination rate after cross-mated with *Ae. albopictus* male and had an average of 43.88 eggs per mated females which oviposited. None of the eggs hatched, although subsequent bleaching indicated presence of embryo. About 169 (48%) of the total number of eggs laid had partially developed embryos. Spermatozoa were observed in the spermathecae of the three female mosquitoes which laid the eggs. Using wild type Malaysian strains, Nazni *et al.* (2009) reported similar results, but the mean number of eggs per female was higher at 81.50 and none of these hatched.

In natural mating experiment, *Ae. aegypti* RIDL female cross-mated with *Ae. albopictus* WT males laid a mean of 295 eggs per mated female with 2.22% insemination rate. This is very similar to observation of Nazni *et al.* (2009) who obtained a mean of 271 eggs per mated female in experiments using the wild type Malaysian mosquitoes.

Although the reciprocal crosses of *Ae. albopictus* WT ♀ X *Ae. aegypti* RIDL ♂ in natural mating experiment had 0% insemination rate, a total of 573 eggs were obtained but none hatched. Subsequent bleaching showed the absence of embryos in all the unhatched eggs. These were unfertilized eggs that were nevertheless oviposited. Downes & Baker (1949) suggested that fertilization by the male of the other species was not a true fertilization, but only served to stimulate parthenogenetic development of the ovum.

In conclusion, though both *Ae. aegypti* and *Ae. albopictus* share almost the same habitat especially in urban centers and rarely in rural remote areas in Malaysia, where *Ae. albopictus* is more prevalent compared to *Ae. aegypti*, interspecific mating is unlikely. This study had clearly indicated that cross-mating between these species is not feasible regardless whether the mosquito is genetically modified or not. The interspecific compatibility between these species when sharing similar habitat in Malaysia is not significant enough to allow mating to produce a hybrid. *Aedes aegypti* RIDL males when released into the field are unlikely to transfer their genes horizontally to *Ae. albopictus*. This possibility is even more remote in other mosquito species and insects. This is due to reproductive isolation barrier that also obviously exists in genetically-modified *Ae. aegypti*.

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