Heliyon 10 (2024) e23693

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Aeroplane wing, a new recessive autosomal phenotypic marker in the malaria vector, *Anopheles stephensi* Liston

Chaitali Ghosh [a,*], M. Soumya [a], Naveen Kumar [a], Chethan Kumar R [a], Soumya Gopal Joshi [a], Sampath Kumar [a], Suresh Subramani [b], Sunita Swain [a]

[a] *Tata Institute for Genetics and Society, Centre at inStem – GKVK Campus, Bellary Road, Bangalore 560065, India*
[b] *University of California San Diego, La Jolla, CA 92093, USA*

ARTICLE INFO

Keywords:
Anopheles stephensi
Aeroplane wings
mutant
Crossbreeding
Autosomal
Recessive
Monogenic trait
Chromosomal inversion

ABSTRACT

A novel and distinct mutant with a phenotype, aeroplane wing (*ae*) is reported for the first time in the urban malaria vector *Anopheles stephensi*. The main aim of this study was to establish the mode of inheritance of the *ae* gene performing genetic crossings between the mutants and wild types. These mutants show extended open wings that are visible to naked eyes in both the sexes. Mutants were first noticed in a nutritionally stressed isofemale colony. Strategic genetic crosses revealed that the *ae* gene is a recessive, autosomal, and monogenic trait having full penetrance with uniform expression in its adult stage. Egg morphometric analysis confirmed that these mutants were intermediate variant. No significant differences were observed in the wing venation and size of *ae* mutants compared to their control parental lines. Further cytogenetic analysis on the ovarian polytene chromosome of *ae* mutant showed an inversion (3L*i*) on the 3L arm like its parental line. This *ae* mutant would be a prominent marker and could be useful to study the functions of related specific genes within its genome.

1. Introduction

Mutant phenotypic markers are well described in model organisms and have played an important role in characterizing their genomic information. These mutations have helped geneticists in designing many genetic crossing experiments to understand the inheritance pattern that can be used in translational research. The first phenotypic marker in *Drosophila melanogaster*, was observed through experimental mutagenesis by T. Morgan and he established the first genetic linkage maps of *Drosophila* chromosomes [1]. Phenotypic markers are visual indicators of characters, such as colour, shape, size, and such observations, dating back over 100 years, aided the discovery of the arrangement and linkage of genes [2,3]. Even to this day, morphological markers are very useful tools in understanding genetics, breeding practices and act as a vital bridge between classical and molecular genetics. Though several such milestones have been achieved in model organisms, other insect vectors and pests have largely remained elusive. Studies on such markers in other insect pests and vectors will unravel many scientific information.

Vector-borne diseases are causing huge public health problems in tropical and subtropical regions including India. *An. stephensi* is one of the potential vectors of malaria in Southeast Asia and the Arabian Peninsula. In the last decade, this species has invaded Africa and Sri Lanka, and seems to be spreading, given new reports of its detection [4–6]. Mathematical modelling suggests that over 126

* Corresponding author.
E-mail addresses: chaitali.ghosh@tigs.res.in, chaitali2008@gmail.com (C. Ghosh).

https://doi.org/10.1016/j.heliyon.2023.e23693
Received 25 June 2023; Received in revised form 30 November 2023; Accepted 9 December 2023
Available online 14 December 2023

million people may be at risk for *An. stephensi*-transmitted malaria across Africa [7]. There are three biological variants in *An. stephensi*: type, *mysorensis* and intermediate depending on the egg-float ridge number [8]. Such biological forms result from evolution for local adaptation. New mutations occur through the evolution process which cause genetic variations in organisms that impact on phenotypic traits by altering gene activity or protein function. Most of these mutations are recognized because of the changes in the phenotype. Over time, as generations of individuals with the trait continue, the advantageous traits become common and establish in the population.

Though limited, there are few reports of such mutants in mosquito vectors occurring spontaneously at the larval and adult stages of *An. stephensi*. These include stripe [9] and hairless antenna in adults [10]. Apart from these, mutants of eye-colour in *An. stephensi*, such as rosy [11], maroon [12], chestnut [13], ruby [14], and yellow body larvae [15] have also been reported. Tests for allelism were also reported using different larval colour mutants, such as grey and greenish black in *An. stephensi* [16]. Linkage studies have been reported in crosses of the colour mutant green thorax (*gt*) with ruby eye (*ru*) [17]. In the current study, we describe the isolation and genetic analysis of a novel aeroplane-winged (*ae*) mutant in *An. stephensi*. The uniqueness of this mutant line is the visually distinct phenotype of open wings while resting. We have characterized the genetic basis of *ae* genes by crossing experiments. The aeroplane (*ae*) mutant has been isolated from an intermediate variant during nutrition challenge experiments under laboratory conditions. Further, the cytogenetic studies on this mutant line have provided valuable insights on inversions and vector evolution. The present study was aimed towards the isolation and establishment of the *ae* mutant, its phenotypic characterization, and the inheritance pattern of the mutant gene (*ae*) in *An. stephensi.*

2. Materials and methods

Establishment, genetic crossings, morphometric and cytogenetic studies of wild and *ae* mutants were carried out in the TIGS (Tata Institute for Genetics and Society) insectary. The genetic crosses were performed between wild (w) and mutant (*ae*) strains for characterizing the genetic mode of inheritance of *ae* gene. The larvae and adults are reared in the insectary as per the protocol described earlier [18] maintaining the temperature at 27 ± 1 °C with relative humidity (RH) at 75 ± 5 % and photoperiod of 12h:12h (light:dark).

2.1. An. stephensi strains

The strains of TIGS-2 (T_2), TIGS-6 (T_6), T_6 isofemale, and mutants were used in this study.

2.1.1. Wild strains

T_2 strain: The strain was collected from Anna Nagar, Chennai (13.018410°N, 80.223068°E), Tamil Nadu, India. About 60 gravid females resting on concrete houses were collected between 6 and 8 a.m. using aspirators. The gravid adults were brought to the insectary and kept inside an adult cage with 10 % sugar solution. An ovicup lined with filter paper filling with 1/3-part water was kept for egg laying. The eggs were collected and kept for hatching. Larvae from eggs were transferred to the trays with little amount of larval food. After 9–10 days, larvae transformed to pupae, which were transferred to the adult cage for emergence into adults. These adults were used for developing the colony and the strain is being maintained in our insectary for over 63 generations since 2018.

T_6 strain: The strain was collected from Sriramanahalli village (12.972442°N, 77.580643°E), Bangalore rural, Karnataka, India. About 150 larvae were collected from the water stored in a cement tank from a cattle shed. The collected larvae along with water from their natural habitat were brought to the insectary. They were allowed to develop into adults and kept inside the cage with 10 % sugar solution. Blood feeding was carried out through artificial membrane feeding. The blood-fed gravid females were allowed to lay eggs in an ovicup within 3–4 days and the eggs were allowed to be hatched within 2–3 days after oviposition. During the rearing of mosquitoes, the insectary conditions were made to mimic the natural conditions as far as possible (in terms of photoperiods, temperature, humidity, nutrition, etc.). This strain is being maintained in the insectary for over 65 generations since 2018.

2.1.2. 2.1.2. T6 isofemale strain

The T6 isofemale line was established from a single blood-fed female selected from the T6 colony and maintained over generations. In every generation, 5 to 10 fully engorged blood-fed females were separated individually in each ovicup for oviposition. Eggs in each cup were allowed to hatch and cultured separately as isofemale lines. The isofemale lines were developed and maintained as per the method published earlier [19].

2.1.3. Mutant strain

Mutants (*ae*) were isolated from an experimental nutrition-deprived stressed colony of the T_6 isofemale line. Few mosquitoes from the T_6 isofemale line (at generation 32) were collected for stress experiments. The ae mutants (4 females and 6 males) were first noticed at the 5th generation of the stress experiment. All the adult mutants were pooled together (at generation 1) and inbred over few successive generations to establish the pure mutant line, which was obtained at the 12th generation with 90 % frequency. The mutants were identified based on their wing phenotypic characters. The experiment was designed as follows. In the experimental set, a low amount of larval food (i.e., 0.033 ± 0.001g (33 mg)) was added to the tray containing nearly 100 larvae in 750 ml of RO water along with a control set provided with adequate larval food of 0.230 ± 0.01 g (230 mg, i.e. 7 times more food) with same number of larvae and same amount of water (unpublished data).

2.2. Crossbreeding experiment

Crosses were made between freshly emerged males and females of wild strains (T_2) and DW *ae* mutant adults. To ensure the virginity of male and female adults, individual pupae from both the strains were kept in perforated 1.5 ml micro-centrifuge tubes and allowed them to emerge into adults, which were screened individually for their sex and released into separate Bugdorm cages (L31 x W31 x H9 cm) for mating. A total of 14 crossing sets were designed for the study [16]. Twenty males and 10 females (2:1 ratio) taken from each line (wild and *ae* mutants) were used for each crossing experiment. Parental crosses were made only between wild types (cross 1) and pure mutants (cross 2). Reciprocal crosses were performed between the parents of wild types and pure mutants (crosses 3 and 4) to generate F_1 hybrids. Few mosquitoes from F_1 hybrids were inbred to produce F_2 generation (crosses 13 and 14) and the remaining adults were backcrossed to both parental mutants and wild types. Crosses 5 to 8 and 13 of the F_1 progeny were obtained from the male outcross (cross 3) and crosses 9 to 12 and 14 were from the female outcross (cross 4). Fecundity, % hatchability and number of males and females of both wild and mutant individuals were recorded from every crossing. Chi-square tests were calculated to compare the significance level ($P > 0.05$).

Further, F_2 hybrids were inbred *inter se* to produce F_3, F_4 and the process was continued till F_{15} generation. Screening was done to observe the frequency of mutant mosquitoes and scoring of mutants was done from every filial generation.

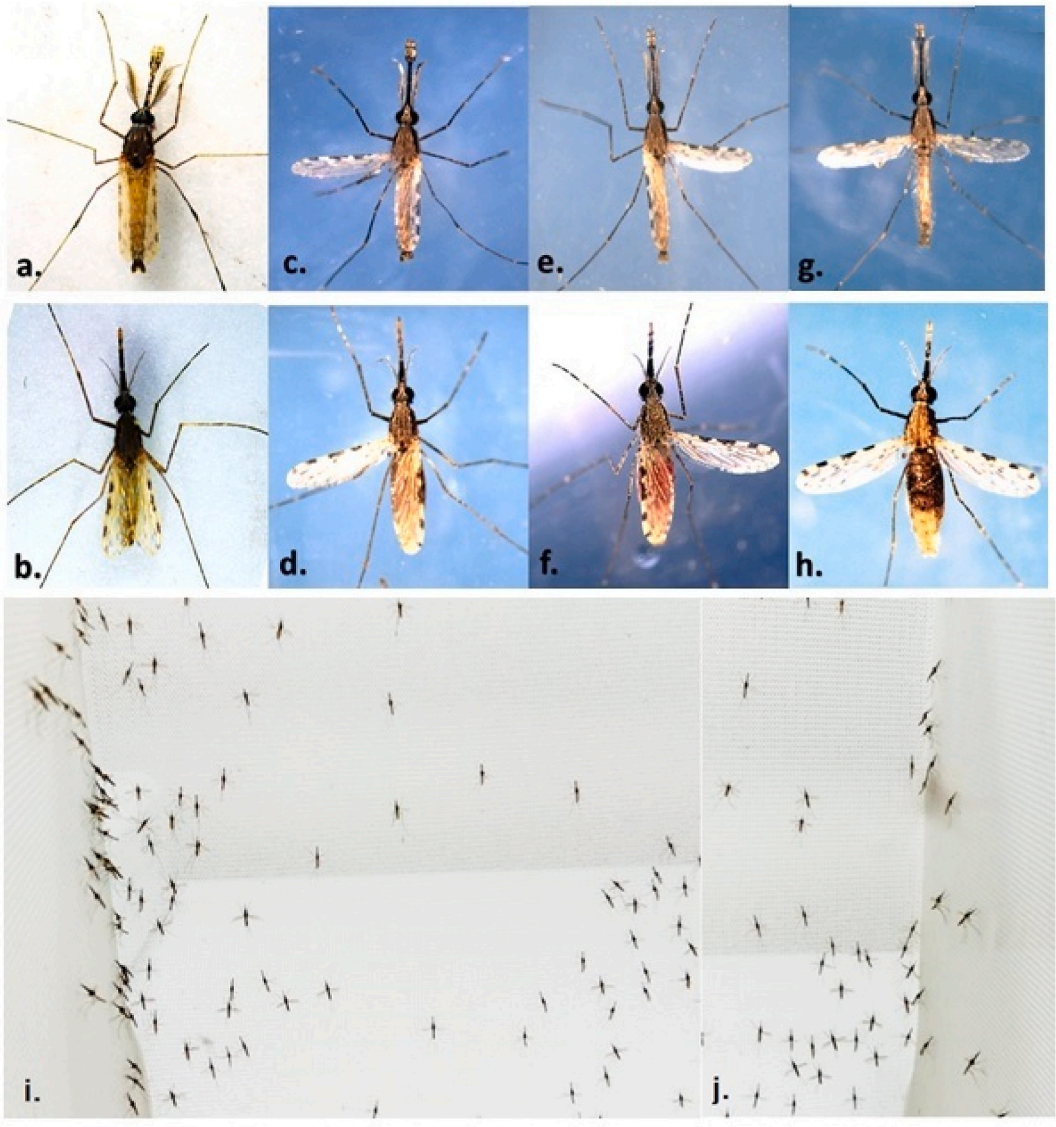

Fig. 1. Variations of wing position in *ae* mutants. (a, b) Wild male and female mosquitoes. (c, d) LW male and female mutants. (e, f) RW male and female mutants. (g, h) DW male and female mutants. (i, j) Mutant mosquitoes in colony cages. (LW – left wing; RW – right wing; DW – double wings).

2.3. Morphometric analyses of eggs

A total of 20 individual eggs were randomly selected from mutant colonies. Eggs were taken on a moist filter paper and observed under microscope using an ocular micrometre (UNILAB model GE-34, Binocular Research Microscope, Haryana, India). The measurement of different parameters such as eggs' shape and size, float length and width, float ridge numbers, etc. were documented.

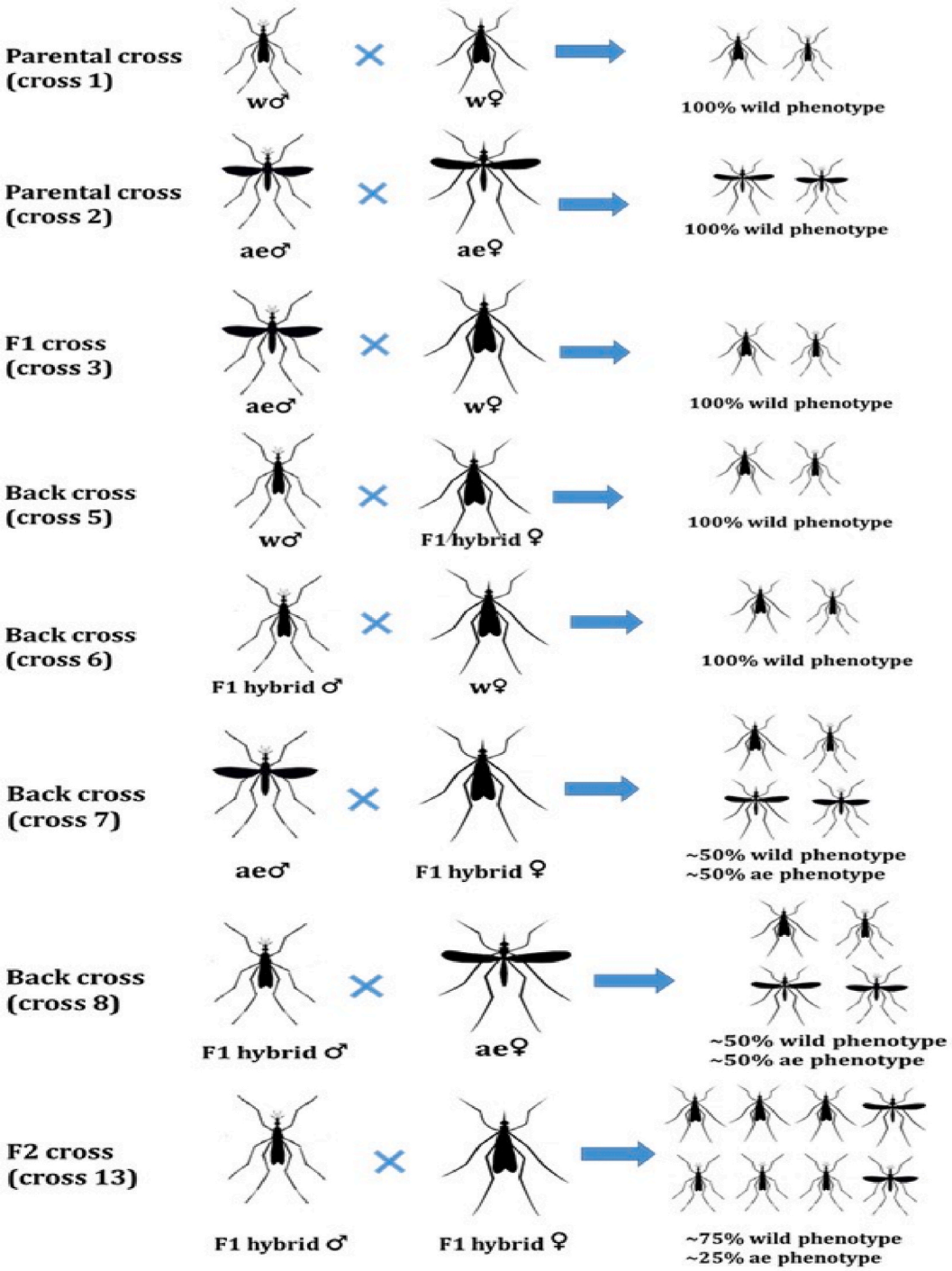

Fig. 2. Schematic illustration of different crosses between mutants (*ae*) and wild (w) types (♂- male; ♀- female).

Table 1
Inheritance pattern of aeroplane mutant (*ae*) in *An. stephensi.* [BC - backcross; ♂- male; ♀- female. *NS (non-significant; $P > 0.05$)].

Cross no.	Generation	Crosses Male (♂) Female (♀)	Total eggs	Total larvae	% H	Mutant			Wild			Grand Total	χ^2	*P* value
						♂	♀	Total	♂	♀	Total			
1	**P_1**	± x ± + + wild type wild type	246	213	86.58	–	–	–	128	66	**194**	**194**		
2	**P_1**	*ae* x *ae* *ae ae* aeroplane aeroplane	122	83	73.61	41	35	**76**	–	–	**-**	**76**		
3	**F_1**	± x *ae* + *ae* wild type aeroplane	521	392	75.23	–	–	**-**	227	135	**362**	**362**		
4	**F_1**	*ae* x ± *ae* + aeroplane wild type	449	295	79.83	–	–	**-**	156	102	**258**	**258**		
5	**BC**	± x ± *ae* + wild type wild type	197	157	79.69	–	–	**-**	78	53	**131**	**131**		
6	**BC**	± x ± + *ae* wild type wild type	198	159	80.30	–	–	**-**	72	69	**141**	**141**		
7	**BC**	± x *ae ae ae* wild type aeroplane	211	171	81.04	17	30	**47**	25	36	**61**	**108**	**0.097**	**0.757***
8	**BC**	*ae* x ± *ae ae* aeroplane wild type	189	164	71.91	22	29	**51**	26	33	**59**	**110**	**0.009**	**0.925***
9	**BC**	*ae* x ± + + wild type wild type	175	128	73.14	–	–	**-**	67	48	**115**	**115**		
10	**BC**	± x *ae* + + wild type wild type	187	154	82.35	–	–	**-**	77	55	**132**	**132**		
11	**BC**	*ae* x *ae* + *ae* wild type aeroplane	198	162	81.81	19	30	**49**	27	31	**58**	**107**	**0.376**	**0.539***
12	**BC**	*ae* x *ae* *ae* + aeroplane wild type	215	181	84.18	23	35	**58**	36	31	**67**	**125**	**1.939**	**0.164***
13	**F_2**	± x ± *ae ae* wild type wild type	218	176	80.7	25	12	**37**	69	45	**114**	**151**	**0.328**	**0.567***
14	**F_2**	*ae* x *ae* ± ± wild type wild type	225	189	84	25	16	**41**	70	55	**125**	**166**	**0.142**	**0.706***

2.4. Morphometric analyses of wings

A total of 5–6 wing samples each from both males and females of four groups (LW, RW, and DW from mutants and T_6 parental isofemale control line) were taken for analyses respectively at generation 15. Thus, ~40 wing samples were analysed. Additionally, at the 35th generation of the *ae* mutant, 10 wing samples from females and males respectively, from each of the four groups (~80 samples) were also analysed. The wings were carefully dissected on day 10 after emergence from pupae. The mosquitoes were anaesthetized using CO_2 pads and the wings were dissected using Olympus microscope (model SZX2-ILLK, Germany). The wings were measured from the alular base up to the end margin between R_1 and R_2 veins, except the fringe scale [20]. Wing images were taken using Leica stereomicroscope (model MZ10F, Germany) and the measurements of wing length and width were recorded using Leica LAS X software.

2.5. Cytogenetic studies of ae mutant and T_6 parental line

Cytogenetic studies were carried out on polytene chromosomes of ovarian nurse cells from semi-gravid females of *ae* mutant and parental T_6 isofemale lines [21]. At the 21st generation, around 56 female mosquitoes from *ae* mutants and 59 females from T_6 isofemale lines were dissected. The semi-gravid females were anaesthetized using CO_2 pad and ovaries were dissected using modified diluted Carnoy's fixative (1 part Carnoy's fixative: 19 parts distilled water). The dissected ovaries were fixed in Carnoy's fixative (3 parts methanol and 1 part glacial acetic acid) for a few minutes and was stained with a few drops of lacto-aceto-orcein (LAO) for 20–25 min. Afterwards 60 % glacial acetic acid was added to get the proper spreading of chromosome arms. A clean cover slip was kept on the sample and squashed gently. The edges of the cover slip were sealed using nail polish. The slides were screened for chromosomal inversions under 40X and 60X, respectively, using Nikon microscope (Nikon Eclipse, Model SC600). The inversion nomenclature was compared following the methods of Coluzzi et al. [22], Mahmood and Sakai [23] and Sharakhova et al. [24].

2.6. Statistical analyses

Descriptive, inferential, and predictive analyses have been performed in this study. Significance values were computed using online Java Script tests (Chi Square calculator 2x2; https://www.socscistatistics.com/tests/chisquare/). *t*-test analysis was done for wing measurement and the mean and SEM values were compared between mutant and parental strains using Vassar Stat software (http://vassarstats.net/). $P > 0.05$ was non-significant.

3. Results

3.1. Adults of ae mutants and T_6 parental strains

Three types of wing orientations were observed in both males and females of *ae* mutant lines as showed in Fig. 1. Compared to wild (Fig. 1a and b) mosquito, in left wing (LW) and right wing (RW) mutants, only the left-side (Fig. 1c and d) and right-side (Fig. 1e and f) wings are outstretched respectively, while the other wings remain in normal position like the wild types. In double wing (DW) mutants, both the left and right wings are widely extended on both sides, forming an angle to the longitudinal axis of the body (Fig. 1g and h). Fig. 1(i and j) shows the mutant mosquitoes in colony cages.

3.2. Inheritance of ae mutant gene

Testing the mode of inheritance of *ae* gene involved 14 crossings performed between the *ae* mutants and wild types. Progeny produced from each cross were analysed. Schematic illustration of different crosses is presented in Fig. 2. Results of these crosses are given in Table 1.

Crosses 1 and 2 were performed between males and females of wild-type and *ae* mutants and confirmed the establishment and purity of homozygotes of mutant and wild-type. In the reciprocal crosses (crosses 3 and 4), the F_1 progeny were wildtype. Therefore, this indicates that *ae* gene is recessive and the absence of *ae* mutants in the heterozygous F_1 progeny indicates that the gene *ae* is autosomal. Crosses 5, 6, 7, 8 and 13 were derived from the male outcross of the F_1 progeny (cross 3 of Table 1). Crosses 9, 10, 11, 12 and 14 were derived from the female outcross of the F_1 progeny (cross 4 of Table 1). When heterozygous individuals of F_1 hybrid progeny were backcrossed to pure-bred wild types (w), no mutant phenotype was observed (crosses 5, 6, 9 and 10 of Table 1); only wild individuals were noticed. However, backcrosses of F_1 heterozygous progeny with the presumptive parental homozygotes of mutant types resulted both wild and mutant phenotypes (crosses 7, 8, 11 and 12 of Table 1). Results of these backcrosses indicated the approximate 1:1 ratio of wild-type to *ae* mutant. χ^2 values of these crosses indicated non-significant deviations (for crosses 7 and 8, $\chi^2 = 0.097$, $P = 0.757$ and $\chi^2 = 0.009$, $P = 0.925$; for crosses 11 and 12, $\chi^2 = 0.376$, $P = 0.539$ and $\chi^2 = 1.939$, $P = 0.164$, respectively). Few adults from the F_1 generation were inbred to get F_2 generations (crosses 13 and 14 of Table 1). The mutant and wild type showed 3:1 ratio and no significant χ^2 values were obtained ($\chi^2 = 0.328$, $P = 0.567$ and $\chi^2 = 0.142$, $P = 0.706$). Data from Table 1 clearly demonstrate that *ae* gene is recessive and autosomal. Its inheritance is monogenic in nature (Table 1).

Results of crosses F_3 to F_{15} are summarized in Table 2 and data represents the total number of male and female mutants that appeared in each generation. In all filial generations (F_3–F_{15}), the percentage of mutant phenotypes ranged between 15 and 19 %. The lowest and highest ratios of mutant and wild mosquitoes were observed in the ratio of 0.16:1 and 0.23:1, respectively. The mean values

Table 2
The frequency of *ae* mutants over 15 generations after F_2 cross. (LW- left wing; RW- right wing; DW, double wings; ♂- male; ♀- female).

Gen	Filial	No.Eggs	No.larvae	%H	No.adults	Wild♂	Wild♀	Totalwild(♂ + ♀)	Mutant♂			Totalmutant♂	Mutant♀			Totalmutant♀	Totalmutant(♂ + ♀)	Mutant(%)	Mutant/wild(ratio)	Longevity(day)
									LW	RW	DW		LW	RW	DW					
G1	F3	131	72	54.96	52	26	16	42	2	1	2	5	1	2	2	5	10	19.23	0.19:1	~30–35
G2	F4	203	107	57.52	78	44	20	64	2	2	1	5	3	4	2	9	14	17.94	0.21:1	,
G3	F5	212	95	44.81	80	46	19	65	3	1	2	6	3	4	3	10	15	18.75	0.23:1	,
G4	F6	154	101	65.58	76	36	26	62	2	3	2	7	2	2	3	7	14	18.42	0.22:1	,
G5	F7	186	98	52.68	68	37	20	57	1	2	1	4	2	3	2	7	11	16.17	0.19:1	,
G6	F8	175	92	52.57	79	40	24	64	2	3	2	7	2	4	2	8	15	18.98	0.23:1	,
G7	F9	182	89	48.9	77	39	26	65	1	2	2	5	3	3	1	7	12	15.58	0.18:1	,
G8	F10	163	82	50.3	71	37	21	58	2	2	1	5	3	2	3	8	13	18.30	0.22:1	,
G9	F11	159	89	55.97	73	36	24	60	2	2	2	6	2	3	2	7	13	17.80	0.21:1	,
G10	F12	193	119	61.65	79	43	24	67	2	1	2	5	2	3	2	7	12	15	0.22:1	,
G11	F13	274	166	60.58	80	39	26	65	3	3	2	8	2	2	3	7	15	18.75	0.23:1	,
G12	F14	295	172	58.3	75	21	23	64	1	2	2	5	1	3	2	6	11	14.66	0.17:1	,
G13	F15	114	81	71.05	65	35	20	55	2	1	1	4	3	2	1	6	10	15.38	0.18:1	,
G14	F16	121	84	69.42	70	38	22	60	1	2	2	5	1	2	2	5	10	14.28	0.16:1	,
G15	F17	103	73	70.87	59	31	19	50	2	2	1	5	2	1	1	4	9	15.25	0.18:1	,

of fecundity and hatchability per female were ~60.33 (range 51–73; SEM = 6.91 ± 1.78) and ~57.5 (range 42–71; SEM = 9.13 ± 2.36). Data from Table 2 clearly demonstrates that the mutant gene *ae* is transferred over generations with uniform phenotypic expressions.

3.3. Morphometric analyses of eggs and wings

Morphometric analyses were carried out from eggs and wings of both *ae* mutant and parental T_6 lines and their comparative significance levels were calculated.

3.3.1. Egg stage

Egg shape: In both the lines, the eggs were black, boat shaped, concave in ventral and curved in dorsal views with blunt anterior and posterior ends, but pointed sometimes (Fig. 3).

Egg size: The maximum egg lengths of DW, RW and LW mutants were 422.5 ± 3.39, 418.5 ± 3.101 and 425 ± 2.564 μm, respectively. The maximum egg widths were 127 ± 1.791, 125 ± 1.539 and 126 ± 1.529 μm for DW, RW and LW mutants, respectively (Table 3). Overall there was no significant difference observed in the egg length and egg width when compared among DW/RW, DW/LW and RW/LW mutants ($P > 0.05$) (Supplementary Table 1).

Egg floats: The maximum egg-float lengths of DW, RW and LW mutants were 208 ± 1.716, 207 ± 1.791 and 126 ± 1.529 μm, respectively. The maximum-egg float widths were 69.5 ± 1.697, 67.5 ± 1.758 and 70 ± 1.622 μm of DW, RW and LW mutants, respectively (Table 3).

Ridges on egg float: For DW, RW and LW mutants the number of float ridges was observed 15.52 ± 0.159 (range 15–17), 15.31 ± 0.109 (range 15–16) and 15.42 ± 0.139 (range 15–17), respectively (Table 3). The difference was statistically non-significant when compared with different parameters of egg measurement of DW with its parental T_6 isofemale lines ($P > 0.05$) (Supplementary Table 2).

3.3.2. Wing measurements

In DW mutant male, the range of wing length is 2.635–3.081 mm and width 0.471–0.591 mm; whereas in female, the range of wing length is 2.5–3.656 and width 0.603–0.852 mm (Table 4). In RW male, the range of wing length and width are 2.539–2.862 and 0.537–0.581 mm; in female wings, length and width are 2.893–3.467 and 0.727–0.846 mm, respectively. In LW male, the range of wing length and width are 2.546–3.055 and 0.445–0.505 mm. In LW female, wing length and width are 3.164–3.539 and 0.744–0.852 mm, respectively (Fig. 4a and b). In parental T_6 males, the range of wing length and width are 3.1–3.2 and 0.55–0.69 mm; while in parental T_6 females, the range of wing length and width are 3.1–3.4 and 0.726–0.841 mm, respectively (Table 5; Fig. 4c and d). Overall, no significant difference was observed in wing length measurement when compared between the mutant and parental T_6 isofemale lines ($P > 0.05$) (Supplementary Tables 3 and 4). The additional findings of wing morphometric analysis at generation 35 showed similar type of results as were observed at generation 15 (Supplementary Tables 5 and 6).

3.3.3. Cytogenetic analyses of ovarian polytene chromosome of ae mutant and T_6 isofemale parental line – 3Li inversion

In the present study, heterozygous paracentric inversions, *i*/+ were observed on 3L arm of *ae* mutants and the percent of the inversion recorded is 19.64 % (Table 6). The tentative breakpoints of the inversions on 3L arm involved 42A–44C (Fig. 5a and b). The similar 3L*i* inversion was also observed at a higher percentage (47.45 %) in its T_6 parental isofemale line.

4. Discussion

Establishment and characterization of phenotypic markers have great applications in expanding the genetic knowledge of non-

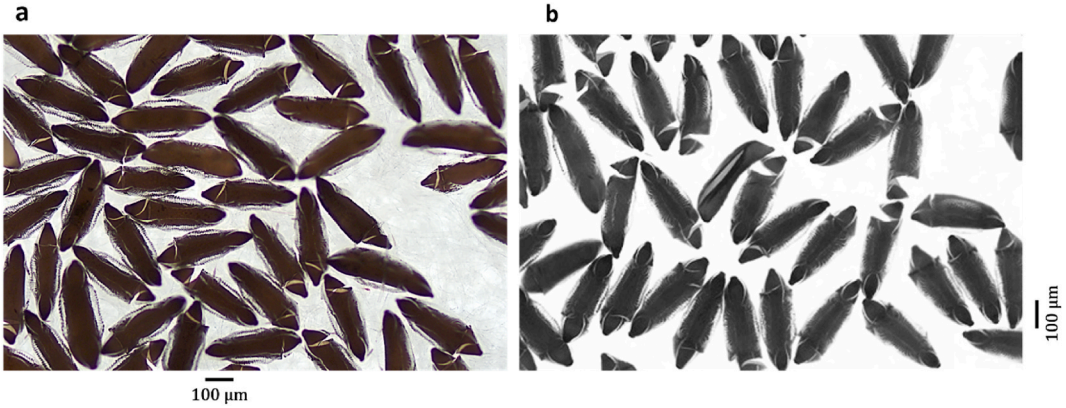

Fig. 3. Eggs of (a) T_6 isofemale parental line and (b) the *ae* mutant.

Table 3
Egg measurement of mutant strains. ER, egg float ridge; EL, egg length; EW, egg width; EFL, egg float length; EFW, egg float width among DW, RW and LW mutant eggs (DW- double wings; RW - right wing; LW - left wing).

Parameter	DW		RW		LW	
	M±SEM	SD	M±SEM	SD	M±SEM	SD
ER	15.52 ± 0.159	0.696	15.31 ± 0.109	0.477	15.42 ± 0.139	0.607
EL	422.5 ± 3.39	15.174	418.5 ± 3.101	13.869	425 ± 2.564	11.47
EW	127 ± 1.791	8.013	125 ± 1.539	6.882	126 ± 1.529	6.805
EFL	208 ± 1.716	7.677	207 ± 1.791	8.013	206.5 ± 1.817	8.127
EFW	69.5 ± 1.697	7.591	67.5 ± 1.758	7.864	70 ± 1.622	7.254

Table 4
Wing measurements of *ae* mutants (LW, left wing; RW, right wing; DW, double wings).

Mutant	Male		Female	
	Length	Width	Length	Width
LW				
Range (mm)	2.546-3.055	0.445-0.505	3.164-3.539	0.744-0.852
M±SEM	2.71 ± 0.089	0.518 ± 0.023	3.43 ± 0.068	0.792 ± 0.0194
SD	0.2007	0.053	0.152	0.0434
RW				
Range (mm)	2.539-2.862	0.537-0.581	2.893-3.467	0.727-0.846
M±SEM	2.726 ± 0.057	0.563 ± 0.008	3.163 ± 0.121	0.786 ± 0.022
SD	0.129	0.019	0.270	0.049
DW				
Range (mm)	2.635-3.081	0.471-0.591	2.5-3.656	0.603-0.852
M±SEM	2.816 ± 0.068	0.527 ± 0.018	2.991 ± 0.144	0.737 ± 0.029
SD	0.167	0.044	0.408	0.083

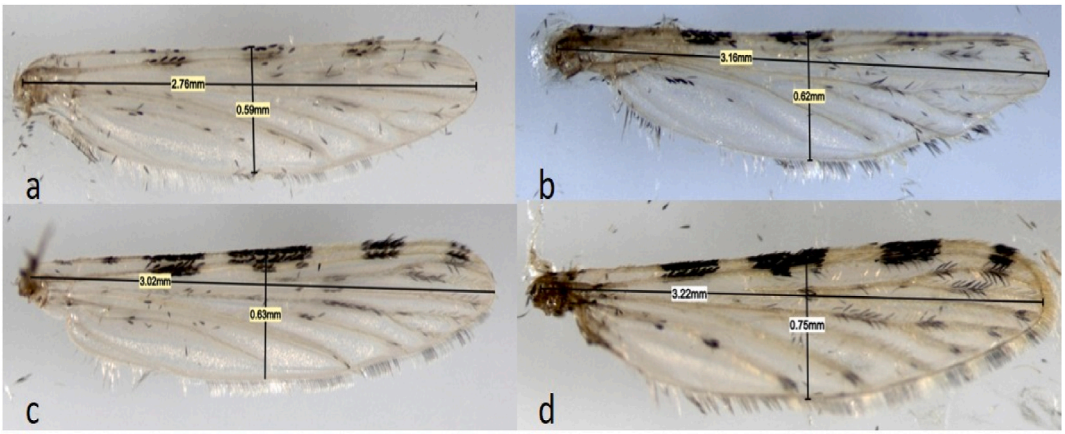

Fig. 4. Male (a) and female (b) wings of T_6 isofemale parental line, and male (c) and female (d) wings of *ae* mutants.

Table 5
Wing measurements of T_6 paternal isofemale lines.

T_6 Isofemale				
	Male		Female	
Wing measurement	Length	Width	Length	Width
Range (mm)	3.1–3.2	0.55-0.69	3.1–3.4	0.726-0.841
M±SEM	3.150 ± 0.016	0.631 ± 0.023	3.359 ± 0.03	0.783 ± 0.015
SD	0.041	0.057	0.093	0.038

Table 6
chromosomal inversions in *ae* mutant and parental T_6 isofemale strains of *An. stephensi.*

Strain	Types of chromosome inversions	Chromosome arm involved	Tentative break points	% inversion	No. of positive slides/total slides prepared
***ae* mutant**	Heterozygous	3L (*i*/+)	42A–44C	19.64 %	11/56
T_6 parental isofemale	Heterozygous	3L (*i*/+)	42A–44C	47.45 %	28/59

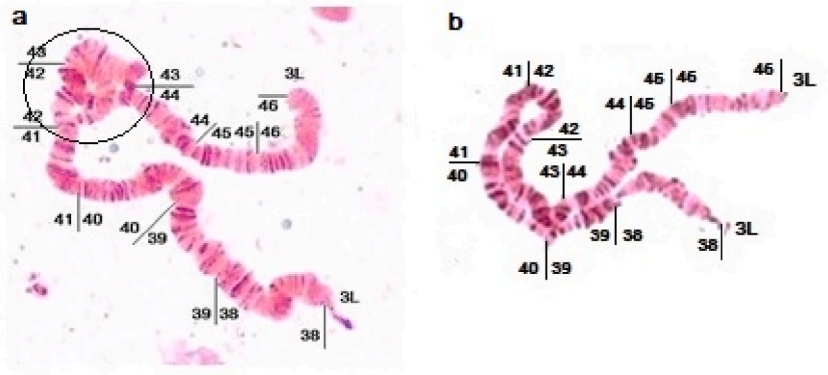

Fig. 5. Photomap of 3L polytene chromosome arm: (a) 3L*i* inversion in *ae* mutant, and (b) standard 3L arm (without inversion) in T_6 lines of *An. stephensi*.

model insect pests and vectors. These markers can be used in devising specific crosses, constructing genetic maps, and identification of loci of qualitative or quantitative traits. The phenotypic marker genes can be used as guides for insect transformation studies and to bridge classical and applied research. The study isolates and characterizes a novel phenotypic open winged mutant in *An. stephensi*. Genetic and cytogenetic studies of *An. stephensi* continue to be an important area of research for understanding the biology, behaviour and ecology to develop better, more specific, and eco-friendly means of controlling mosquito vectors.

The mutant mosquito is termed as *aeroplane* based on its abnormal wing posture. It has straightened out wings on either one or both sides with a bent abdomen, which looks exactly like an aeroplane. The bent abdomen is observed in both the sexes of males and females, especially in DW mutants. The positions of the wing in these three types of mutants (DW, LW and RW) are unique. In the typical form of DW, both wings are widely extended forming 90° angle with the longitudinal axis of the body. In LW and RW, the wings are extended towards the right side and left side of the body, respectively, while the other wings lie on the body same like wild types. The wings are of the same shape and texture as those of wild types. The wings of mutants are flat, and the venation is similar like that of wild type. There are no marked differences observed in wing structure and the halteres are also similar in shape and structure to those like wild types. The open wing character is prominently visible soon after eclosion. Mutant mosquitoes are strong, active, fertile, and comparable to their wild counterpart. They are seen flying like normal mosquitoes inside the cages and remain alive for 30–40 days. They are competent to take blood meal both from artificial blood feeding systems (Haemotek ®) and a mouse host as well. Good fecundity and hatchability were observed (75–90 and 80 %) over generations, respectively (Table 2). At the time of first detection, *ae* mutants were observed in 10 % of the nutritionally-stressed mosquito population [four females – two DW (50 %); one each from RW and LW (25 %) and six males – four DW (66.66 %); one each from RW and LW (16.66 %)] in the 5th generation. The frequency gradually increased to above 90 % [approx. 51 (56.6 %) females – 36 DW (70.58 %); eight (15.686 %) RW and seven (13.72 %) LW and approx. 39 males (43.4 %) – 27 DW (69.23 %); seven RW (17.94 %) and five LW (12.82 %) on the 12th generation. The *ae* mutant colony is currently being maintained in the TIGS insectary for over 30 generations with 95 % mutant frequency (Fig. 1 i and j).

Genetic analysis demonstrated that the *ae* gene is recessive, monogenic and autosomal. Crossing of F_3 to F_{15} progeny confirms the stability of the mutant gene in the mutant population and the wing character is unique. Precise morphometric analysis of mutant eggs shows the egg-float ridge numbers ranged between 15 and 16, which confirms the mutant strain maintained its intermediate variant like its original parental (T_6) line. However, we did not observe any differentiating phenotypic characters in larval and pupal stages in mutant lines. The mutant wing character is expressed and visible only in adult stages.

Karyotyping of ovarian and salivary polytene chromosomes is one of the common cytogenetic techniques used earlier for the genetic studies of anopheline mosquitoes. In the present study, the techniques were followed for immediate processing of polytene chromosome for laboratory-reared mosquitoes following the modified method of Ghosh and Shetty (2004), where 60 % glacial acetic acid was used [21]. However, 50 % propionic acid is used usually for long-time preserved samples fixed in Carnoy's solution from field-collected mosquito samples. In this study, the 3L*i* inversion in *ae* mutant persists as in its parental line (T_6), with a reduced frequency (19.64 % in *ae* mutants and 47.45 % in iso T_6 lines) [25]. Notably, inversion is the most prominent adaptation mechanism for new biotic and abiotic environment. Often this inversion could be developed as a specific fixed marker for that species [26]. Inversions are structural rearrangements of a chromosomal segment limiting the exchange of variation between two alternate pairs of

alleles. In many organisms, these can generate gene complexes that are adaptive and can function as supergenes. Chromosomal inversions are widespread and have been extensively associated with latitudinal and longitudinal patterns among members of the Anopheles species [24]. In An. stephensi, 16 chromosomal paracentric inversions have been reported [27,28].

The preparation of ovarian polytene chromosomes from an adult female is comparatively easier than the larval salivary gland chromosomes in anophelines. However, there are certain limitations to have correct stage samples like blood-fed adults or late 4th instar larvae from the field and the preparation is labour intensive and time consuming. Good spreading of chromosome arms is required for proper karyotyping of complex banding patterns. The standard photomap of polytene chromosome in ae mutant of *An. stephensi* provides a reference for future studies.

Recent advances in gene editing CRISPR/Cas9 to develop mutant mosquitoes heavily rely upon expression of phenotypic markers for confirming the transformation events. There have been reports of morphologically distinct mutants developed through radiation, where the phenotypes can be easily visualized in larval and adult stages. The larval colour mutants in *An. stephensi* and their genetic basis of inheritance like stripe, controlled by a codominant gene [9]; grey, an autosomal recessive gene [16]; greenish brown, an autosomal recessive gene; red eye, a recessive sex-linked gene [29]; diamond palpus, an autosomal recessive gene [30]; golden-yellow, an autosomal recessive gene [31]; white eye, a recessive sex-linked gene; yellow body larvae, an autosomal recessive gene [15]; greyish black, an autosomal recessive gene [32]; ruby-eye (*ru*), an autosomal recessive gene [10]; and dark mutant (*da*), an autosomal recessive gene [33] have been reported by several pioneer workers [34]. In *An. stephensi*, the allelism tests in certain mutant larvae have also been reported earlier. The brown mutant larvae (*br*) is allelic to green (*gr*) [35], and grey (*gy*) is allelic to greenish black (*gbl*) [16]. The linkage study between the mutants of hairless antenna (*hla*) and ruby eye (*ru*) mutant reported that the *hla* gene is suppressed by the *ru* gene in heterozygous condition [10]. The green thorax (*gt*) allele is recessive and autosomal to wild type, but there is no linkage relationship between *gt* and *ru* genes [19]. Among the adults, there are easily visualized mutant phenotypes in the wing venation, eye, and antenna in *Culex quinquefasciatus* and Culex fatigans, respectively [36,37]. Similarly, gene knockout studies targeting the flight muscle gene have been conducted in Ae. aegypti making the transformed flightless mosquitoes, phenotypically distinct from the other non-transformed mosquitoes [38]. This flightless gene was used as a phenotypic marker in the development of CRISPR-based pgSIT (precision-guided sterile insect technique) in *Ae. aegypti*. We propose that the *ae* mutant mentioned in this study could be a unique phenotypic marker of *An. stephensi* as its gene expresses with complete penetrance and high viability in its adult stages. The mutant could also be used for conducting fundamental and applied genetic research in *An. stephensi*. We hypothesize that the novel *ae* mutant phenotype in *An. stephensi* is like the aeroplane wing mutant previously reported in *D. melanogaster* [39]. In *Drosophila*, crossings between mutants and wild types produced only wild-type offsprings in F_1 progeny, but in F_2 progeny the wing character reappeared in both sexes [39]. Similar to our findings based on the experimental crosses, the gene of wing mutant in *Drosophila* was also recessive and autosomal.

Furthermore, Kelley and Bell (1999) characterized and identified an allele called aeroplane-like (ae-l) of a novel locus, which is responsible for the aeroplane (*ae*) mutant, and resulted from a transformation experiment in *D. melanogaster* [40]. Further molecular examination in *D. melanogaster* revealed that aeroplane (*ae*) gene is controlled by a regulatory allele of *tsh* (teashirt). The mutant wing posture phenotype of homozygous *ae* flies is due to a defect in the hinge region of the wing. The base of the wing is fused to the thorax in the pleural wing region [41]. Further, a molecular characterization of *ae* mutant gene in the open-winged phenotype of *An. stephensi* would be an interesting study in future.

Anopheles species has played a great role in chromosomal inversion studies. We intended to observe if any changes occurring in polytene chromosome arms of mutant mosquitoes due to nutrition stress the wild mosquitoes often encounter in the natural environment and get adapted to establish over subsequent generations. The genome of the species exhibits this adaptive process by changing the structural pattern of the chromosomes and altering its sequences [42] which affect the mosquito fitness by regulating the expression of genes located near the breakpoints of the inversions [43]. Several or even hundreds of genes are present in the inversion regions. Recombination is drastically reduced in the heterozygote conditions [44]. Chromosomal inversions have significant roles in adaptation process that has been reflected by strong associations between their frequencies and number of phenotypic traits. However, in the present study, 19.64 % inversion was observed in the *ae* mutant compared to the T_6 parental line (47.45 %). This study would be a timely contribution on the development of genetic markers and potential gene targets in the malaria-transmitting mosquito, *An. stephensi*.

5. Conclusions

Aeroplane, a new wing mutant in *An. stephensi* is reported for the first time. The genetic analyses of the above mutant demonstrate that the mutant gene, *ae* is autosomal, recessive, and might be controlled by a single gene. Three types of wings orientations are observed in *ae* mutants, i.e., DW, LW and RW and the differences in measurements among three types were observed statistically non-significant in the egg and wing morphometrics when compared with the parental lines. Cytogenetic study confirmed the persistence of the 3L*i* inversions in *ae* mutants like their parental lines. Further molecular genetics study would enlighten several novel avenues to identify target gene/s for causing mutations. It would also advance our knowledge of the evolution of mutation in vectors which might consider the effective ways to manage vector control.

6. Ethics statement

Institutional ethical approval for use of human blood for mosquito feeding [approval Ref. No. inStem/IEC-12/002, Bangalore, from Institute for Stem Cell Science and Regenerative Medicine (inStem)] and biosafety approval for mosquito maintenance facility

(approval Ref. No. TIGS 3rd IBSC March 2018) were obtained. The human blood used for mosquito feeding for colony maintenance was collected from Lion's Blood Bank, Bangalore, India.

Funding

This research is funded from the intramural grants of Tata Institute for Genetics and Society (TIGS), Bangalore, India.

Data availability statement

All data are available within this article and in supplementary files.

CRediT authorship contribution statement

Chaitali Ghosh: Writing - review & editing, Writing - original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M. Soumya:** Methodology. **Naveen Kumar:** Methodology. **Chethan Kumar R:** Methodology. **Soumya Gopal Joshi:** Methodology. **Sampath Kumar:** Writing - review & editing, Methodology. **Suresh Subramani:** Writing - review & editing. **Sunita Swain:** Writing - review & editing, Project administration.

Declaration of competing interest

All the authors have declared that there is no competing interests.

Acknowledgements

The authors are thankful to Dr. Rakesh Mishra, Director, Tata Institute for Genetics and Society (TIGS), Bangalore, India for his guidance and support. The authors thank Dr. Susanta Kumar Ghosh, Former Scientist G and Head, ICMR-NIMR (National Institute of Malaria Research), Bangalore, India for his guidance in the establishment of TIGS insectary. We are thankful to Mr. Joydeep Roy for his technical support. The authors acknowledge the NCBS/inStem Animal Care and Resource Center (ACRC) which was partially supported by the National Mouse Research Resource (NaMoR) (grant #BT/PR5981/MED/31/181/2012; 2013–2016 & 102/IFD/SAN/5003/2017–2018) from the Department of Biotechnology, GoI for providing mice for this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23693.

References

[1] T.H. Morgan, Sex-limited inheritance in *Drosophila*, Science 32 (1910) 120–122, https://doi.org/10.1126/science.32.812.120.
[2] A.H. Sturtevant, The linear arrangement of six sexlinked factors in *Drosophila*, as shown by their mode of association, J. Exp. Zool. 14 (1913) 43–59, https://doi.org/10.1002/jez.1400140104.
[3] H. Wexelsen, Linkage between quantitative and qualitative characters in barley, Hereditas (Lund) 17 (1933) 323–341.
[4] M. Seyfarth, B.A. Khaireh, A.A. Abdi, S.M. Bouh, M.K. Faulde, Five years following first detection of *Anopheles stephensi* (Diptera: Culicidae) in Djibouti, Horn of Africa: populations established-malaria emerging, Parasitol. Res. 118 (3) (2019) 725–732, https://doi.org/10.1007/s00436-019-06213-0. PMID: 30671729.
[5] F. Ishtiaq, S. Swain, S. Kumar, *Anopheles stephensi* (Asian mosquito), Trends Parasitol. 37 (6) (2021) 571–572.
[6] T. Lehmann, R. Bamou, J.W. Chapman, et al., Urban malaria may be spreading via the wind—here's why that's important, Proc. Natl. Acad. Sci. U.S.A. 120 (18) (2023), e2301666120, https://doi.org/10.1073/pnas.2301666120.
[7] M.E. Sinka, S. Pironon, C. Massey, J. Longbottom, A new malaria vector in Africa: predicting the expansion range of *Anopheles stephensi* and identifying the urban populations at risk, Proc. Natl. Acad. Sci. U.S.A. 117 (40) (2020) 24900–24908, https://doi.org/10.1073/PNAS.2003976117.
[8] S.K. Subbarao, K. Vasantha, T. Adak, V.P. Sharma, C.F. Curtis, Egg float ridge number in Anopheles stephensi: ecological variation and genetic analysis, Med. Vet. Entomol. 1 (3) (1987) 265–271, https://doi.org/10.1111/j.1365-2915.1987.tb00353.
[9] R.K. Sakai, M.P. Iqbal, R.H. Baker, The genetic of stripe, a new morphological mutant in the malaria mosquito, *Anopheles stephensi*, Can. J. Genet. Cytol. 16 (1974) 669–675, https://doi.org/10.1139/g74-073. PMID: 4447908.
[10] A.D. Madhyastha, N.J. Shetty, Genetic and linkage studies of new autosomal sex- limited mutant hairless antenna in *Anopheles stephensi* Liston, J. Insects 2016 (4) (2016) 1–8, https://doi.org/10.1155/2016/4792702.
[11] K. Akhtar, R.K. Sakai, Genetic analysis of three new eye colour mutations in the mosquito, *Anopheles stephensi*, Ann. Trop. Med. Parasitol. 79 (4) (1985) 449–455, https://doi.org/10.1080/00034983.1985.11811944. PMID: 3907559.
[12] F. Mahmood, R.K. Sakai, Genetic analysis of maroon eye in *Anopheles stephensi*, Mosq. news 42 (1982) 33–35.
[13] H.R. Rathor, S. Rashid, G. Toquir, Genetic analysis of a new sex-linked mutant 'chestnut eye' an allele of the white eye locus in the malaria vector *Anopheles stephensi*, Mosq. news 43 (1983) 209–212.
[14] A.D. Madhyastha, N.J. Shetty, Ruby-eye a new autosomal mutant in the malaria mosquito, *Anopheles stephensi* Liston, Indian J. Med. Res. 115 (2002) 194–200. PMID: 12362559.
[15] N.J. Shetty, P. Gayathri, S. K Narang, P.D. Foglesong, D.J. Joslyn, White eye and yellow larva: mutants in *Anopheles stephensi* Liston (Diptera: Culicidae), Florida Entomol 77 (4) (1994) 498–504.
[16] N.J. Shetty, C. Ghosh, Grey larvae, an allele of greenish black larvae in the malaria mosquito *Anopheles stephensi* Liston, J. Cytol. Genet. 6 (2005) 35–40.

[17] D. Sanil, N.J. Shetty, The genetics of green thorax, a new larval colour mutant, non-linked with ruby – eye locus in the malaria mosquito, *Anopheles stephensi*, J. Vector Borne Dis. 46 (2) (2009) 117–124.
[18] S.K. Ghosh, C. Ghosh, New Ways to Tackle Malaria. In a Book Chapter 'Current Topics in the Epidemiology of Vector-Borne Diseases', 2019, https://doi.org/10.5772/intechopen.89467. IntechOpen, UK.
[19] C. Ghosh, N. Kumar, R.B.S. Kushwah, et al., Enrichment of phenotype among biological forms of *Anopheles stephensi* Liston through establishment of isofemale lines, Parasit. Vectors 16 (2023) 79, https://doi.org/10.1186/s13071-02305696-2.
[20] A.B. Wilke, R.O. Christe, L.C. Multini, et al., Morphometric wing characters as a tool for mosquito identification, PLoS One 11 (8) (2016), e0161643.
[21] C. Ghosh, N.J. Shetty, Tests for association of fenitrothion resistance with inversion polymorphism in the malaria vector, *Anopheles stephensi* Liston, Nucleus-Calcutta-Int. J. Cytol. 47 (2004) 164–168.
[22] M. Coluzzi, A. Sabatini, V. Petrarca, M.A. di Deco, Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex, Trans. R. Soc. Trop. Med. Hyg. 73 (1979) 483–497, https://doi.org/10.1016/0035-9203(79)90036-1.
[23] F. Mahmood, R.K. Sakai, Inversion polymorphisms in natural populations of *Anopheles stephensi*, Can. J. Genet. Cytol. 26 (1984) 538–546.
[24] I.V. Sharakhov, B.J. White, M.V. Sharakhov, et al., Breakpoint structure reveals the unique origin of an interspecifc chromosomal inversion (2La) in the *Anopheles gambiae* complex, Proc. Natl. Acad. Sci. USA 103 (2006) 6258–6262.
[25] S. Srinivasan, C. Ghosh, S. Das, et al., Identification of a TNF–TNFR-like system in malaria vectors (*Anopheles stephensi*) likely to influence *Plasmodium* resistance, Sci. Rep. 12 (2022), 19079, https://doi.org/10.1038/s41598-022-23780-y.
[26] M. Kirkpatrick, N. Barton, Chromosome inversions, local adaptation and speciation, Genetics 173 (2006) 419–434, https://doi.org/10.1534/genetics.105.047985.
[27] M. Kamali, M.V. Sharakova, E. Baricheva, et al., An integrated chromosome map of microsatellite markers and inversion breakpoints for an Asian malaria mosquito, *Anopheles stephensi*, J. Hered. 102 (2011) 719–726.
[28] A. Thakare, C. Ghosh, T. Alalamath, et al., The genome trilogy of *Anopheles stephensi*, an urban malaria vector, reveals structure of a locus associated with adaptation to environmental heterogeneity, Sci. Rep. 12 (2022) 3610.
[29] V.P. Sharma, S.K. Subbarao, M.A. Ansari, R.K. Razdan, Inheritance pattern of two new mutants red-eye and greenish brown-larva in *Anopheles stephensi*, Mosq. news 39 (1979) 655–657.
[30] S.K. Sakai, R.H. Baker, C.J. Dubash, K. Raana, The genetics of diamond palpus in *Anopheles stephensi*, Mosq. news 41 (1981) 125–128.
[31] T. Adak, S.K. Subbarao, V.P. Sharma, Genetics of golden yellow larva in *Anopheles stephensi*, J. Am. Mosq. Control Assoc. 6 (1990) 672–676.
[32] N.J. Shetty, T. Zin, M. Zu Minn, Greyish black – a new autosomal larval colour mutant in the malaria mosquito – *Anopheles stephensi*, J. Cytol. Genet. 8 (1) (2007) 21–25.
[33] T.P.N. Hariprasad, N.J. Shetty, Dark larvae – a new autosomal larval colour mutant in the malaria mosquito, *Anopheles stephensi* Liston, J. Commun. Dis. 39 (3) (2007) 147–151.
[34] K. Akhtar, R.K. Sakai, Genetic analysis of three new eye colour mutations in the mosquito, *Anopheles stephensi*, Ann. Trop. Med. Parasitol. 79 (4) (1985) 449–455, https://doi.org/10.1080/00034983.1985.11811944. PMID: 3907559.
[35] N.J. Shetty, P. Kumar, S.K. Narang, P.D. Fogelsong, D.J. Joslyn, Brown larva: an allele of the green larva mutation in the malaria mosquito *Anopheles stephensi*, J. Hered. 86 (1995) 309–311.
[36] N.J. Shetty, B.N. Chowdaiah, Tests for allelism among certain colour larval colour mutant in *Culex quinquefasciatus*, Mosq. news 36 (4) (1976) 477–482.
[37] J.B. Kitzmiller, X-ray induced mutation in the mosquito, Culex fatigans, Expt. Parasitol 7 (4) (1958) 439–462.
[38] K. Chae, C. Valentin, C. Dawson, E. Jakes, K.V. Myles, Z.N. Adelman, A knockout screen of genes expressed specifically in Ae. aegypti pupae reveals a critical role for stretchin in mosquito flight, Insect Biochem. Mol. Biol. 132 (2021), 103565.
[39] T. Quelprud, Aeroplane, a second chromosome recessive wing mutant in *Drosophila melanogaster*, Hereditas 15 (1) (1931) 97–119.
[40] K.H. Soanes, J.B. Bell, Rediscovery and further characterization of the *aeroplane* (ae) wing posture mutation in *Drosophila melanogaster*, Genome 42 (3) (1999) 403–411, https://doi.org/10.1139/g98-143.
[41] H.S. Kelley, J.B. Bell, The Drosophila aeroplane mutant is caused by an I-element insertion into a tissue-specific teashirt enhancer motif, Genome 44 (5) (2001) 919–928, https://doi.org/10.1139/g01-077.
[42] A.A. Hoffmann, C.M. Sogro, A.R. Weeks, Chromosomal inversion polymorphisms and adaptation, Trends Ecol. Evol. 19 (2004) 482–488, https://doi.org/10.1016/j.tree.2004.06.013.
[43] J.E. Pérez-Ortín, A. Querol, S. Puig, E. Barrio, Molecular characterization of achromosomal rearrangement involved in the adaptive evolution of yeast strains, Genome Res. 12 (2002) 1533–1539, https://doi.org/10.1101/gr.436602.
[44] A.D. Stump, M. Pombi, L. Goeddel, J.M.C. Ribeiro, J.A. Wilder, A. della Torre, et al., Genetic exchange in 2La inversion heterokaryotypes of *Anopheles gambiae*, Insect Mol. Biol. 16 (2007) 703–709, https://doi.org/10.1111/j.1365-2583.2007.00764.x.