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Original Article

GENETICS OF OLIVE-GREEN LARVAL COLOUR MUTANT IN *ANOPHELES STEPHENSI* – A MALARIA VECTOR

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ABSTRACT

Background: Anopheles stephensi is the primary vector of urban malaria in the Indian subcontinent and the Middle East, creating an emerging threat in Africa.

Methods: A new olive-green (olg) mutant has been identified and isolated from insectary colony strains of An. stephensi. Its body colour is highly prominent in 3rd and 4th-instar larvae and persists through the pupal stage.

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Results: To determine the genetic basis of this mutant, crossing olg with wild-type mosquitoes demonstrates that the olg gene is autosomal, recessive, and controlled by a single gene. The colour expression is uniform in both sexes.

Conclusion: This mutant can be used as a genetic marker and for genetic mapping in basic and applied genetic research. The olg mutation in the urban malaria vector can be used in advanced malaria research to better understand the genetics of this species.

INTRODUCTION

Vector-borne diseases especially malaria has become a serious public health problem in the last few decades. *Anopheles stephensi* is widely prevalent across India, the Middle East, Iraq, Iran, and recently it has invaded in Africa (WHO, 2025). *Anopheles* belongs to the order Diptera under family Culicidae. The development of genetic studies on anopheline mosquitoes has been closely linked to their medical importance. Currently, vector control faces challenges due to the development of resistance and the environmental pollution for continuous application of insecticides and pesticides. It is desirable to develop an alternate strategy for the control of vector mosquitoes. Genetic control or autocidal control is one such method, which does not involve resistance among vectors. In this context, it is mandatory to have some basic genetic information necessary to carry out the genetic and cytogenetic studies (SHETTY & GHOSH, 2006). In recent years, genetics, cytogenetics, speciation, and evolutionary biology have received significant attention from researchers, particularly in the light of the demonstration of the genetic basis of insecticide resistance. Genetic analysis of mutants helps in understanding the genetic structure of this highly adaptable and invasive malaria vector.

The genetic basis of a novel aeroplane (*ae*) wing mutant in *An. stephensi* has been reported by GHOSH et al. (2024). Few naturally occurring larval colour mutants have already been reported earlier in *An. stephensi*. All such reported mutants were isolated from wild populations, and their genetic analyses revealed that these traits are either autosomal recessive or sex-linked recessive.

The present paper describes the establishment and genetic basis of inheritance of a new larval colour mutant (olive-green – *olg*) of *An. stephensi*, following the crossing technique.

MATERIAL AND METHODS

The experiment was conducted in the insectary of Tata Institute for Genetics and Society (TIGS) during March 2023 to September 2024.

(i) Mutant and wild mosquitoes

The Olive Green (*olg*) mutant was identified and isolated from the laboratory colony. The *An. stephensi* strain was collected from Mewat, Haryana, India, in September 2022. A few *olg* larvae were first detected at the 5th generation observing their body colour and started maintaining separately. In the 2nd instar stage, this mutant showed light greenish colour compared to the wild type and the colour was more prominent at the 3rd and 4th instar stages. The *olg* colony was successfully established and is being maintained in the TIGS insectary.

The wild strain of *An. stephensi* used in the crossing experiment (Mendelian Crossing) with the *olg* mutant strain was collected from Bangalore city.

(ii) Generating the homozygous *olg* mutant line

The homozygous mutant line was generated by selective inbreeding (sibling mating) over several generations and repeated screening at every generation until all offspring larvae consistently showed the same colour (GHOSH & SHETTY, 1999). After 20 generations of inbreeding, pure mutant line was developed. It showed ~100% phenotypic expressions resulting in a pure homozygous line where all offsprings appeared phenotypically identical to their parents confirming genetic uniformity and stability (Fig. 1). This homozygous line was used for the genetic crossing experiment with the wild strain.

(iii) Mosquito rearing

Mutant and wild adult mosquitoes were reared under controlled conditions in the insectary maintained at 28±1°C and 75±5% RH under a 12h:12h day–night photoperiod. They were maintained in separate adult cages (BugDorm-4S3030 – W32.5×D32.5×H32.5 cm). The adults were fed on a mixture of 8% sucrose, 2%

glucose solution mixed with 3% multivitamin syrup (Polybion LC®) (GHOSH & GHOSH, 2019). Adult females were provided with blood on day 5 or 6 to obtain eggs for continuing the next subsequent generations. After the blood meal, on day 4 the eggs were collected into an ovicup containing water lined with filter paper along the inner margin. Larvae were hatched into after 48 h of oviposition and transferred to the rearing trays (L39×B30 cm; Polylab, Catalog no. 81702). Larvae were provided with larval food prepared by mixing Brewer's yeast and dog biscuits at a ratio of 30:70. The larvae were transformed into pupae. The pupae were disinfected by washing them with 1% sodium hypochlorite (NaOCl) for 1 min before they were kept inside the adult cages. The adults emerged within 2–3 days.

(iv) Genetic crossing

Fourteen crosses were made between the wild-type and *olg* mutant for determining the mode of inheritance of *olg* mutant. A total of 14 crosses were performed for the study. For each experimental cross, 10 each virgin males and females from wild-type and *olg* mutant were selected and allowed to mate in separate adult cages. Parental crosses (P_1) were made exclusively between wild types referred as cross 1 and between homozygous mutants referred as cross 2. Reciprocal crosses were performed between the parents of wild types and *olg* mutants (crosses 3 and 4) and *vice versa* to generate F_1 hybrids. Randomly selected adult mosquitoes (10 males and 10 females) from F_1 hybrids were inbred to produce F_2 generation (crosses 13 and 14), and the remaining adults were back-crossed to both parental mutants and wild types. Crosses 5, 7, 9, 11 and 13 of the F_1 progeny were obtained from the male outcross (of cross 3) and crosses 6, 8, 10, 12 and 14 were from the female outcross (of cross 4). Scoring was done by assessing the phenotype colour of both mutant and wild strains at the 3rd and 4th instar larval stages from each crossing. Fecundity, percent hatchability and number of males and females of both wild and mutant (*olg*) individuals were recorded from every crossing.

(v) Statistical analyses

Chi-square tests were used to assess significance ($P > 0.05$). Inferential and predictive analyses have been performed in this study. Significance values were computed using on-line Java Script tests (Chi-Square calculator 2x2; https://www.socscistatistics.com/tests/chisquare/).

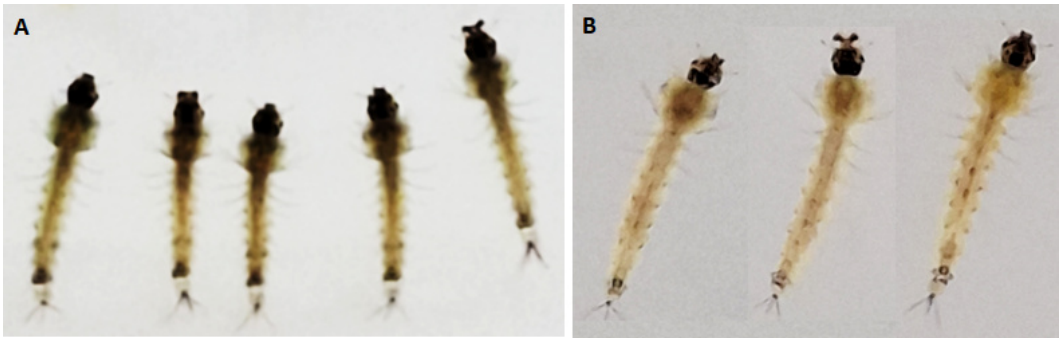

Fig. 1. Fourth instar larvae of *olg* mutant (A) and the wild strain (B) of *Anopheles stephensi*.

RESULTS

Progenies derived from each cross were analysed and the mode of inheritance of *olg* mutant was determined. Results of 14 crosses were summarized in Table 1. Parental crosses 1 and 2 confirmed the establishment and purity of the purebred mutant and wild-type, respectively. In cross 1, only wild type and in cross 2 only mutant type were obtained. In the reciprocal crosses 3 and 4, the F_1 progeny were all wild types. This indicates that the mutant gene *olg* is recessive and autosomal. In the backcross of heterozygous F_1 individuals with wild types (crosses 5, 6, 9 and 10 in Table 1), no *olg* mutant was observed among the progenies, only wild type was obtained. Conversely, the backcross of F_1 heterozygous progeny with the parental homozygous mutant types (crosses 7, 8, 11 and 12 in Table 1) resulted the expected 1:1 phenotypic expression of wild-type and *olg* mutant. The observed wild and *olg* counts were 102, 118 (cross 7), 108, 121 (cross 8), 120, 141 (cross 11) and 100, 113 (cross 12). The expected values were 110, 114.5, 130.5 and 106.5, respectively. The χ^2 values 1.16, 0.74, 1.69 and 0.79 indicates that the observed data are very close to the expected values. The *p* values were 0.28, 0.39, 0.19 and 0.37, which are much larger than the standard significance level of 0.05 and indicated non-significant differences. Adults of the F_1 progeny were inbred to yield the F_2 generation (crosses 13 and 14 in Table 1) and the wild-type and *olg* mutant were observed in expected 3:1 ratio. The observed wild and *olg* counts were 332, 95 (cross 13) and 216, 75 (cross 14), respectively. The expected data were 312.75, 104.25 (cross 13) and 218.25, 72.75 (cross 14), respectively (Table 1). The χ^2 values were 1.09 and 0.09 with *p* values of 0.29 and 0.76, respectively. This indicated the non-significant

differences between observed and expected data of wild and mutant types with a phenotypic expression of 3:1 ratio in F_2 following the Mendelian Monohybrid Cross. Crossing data of Table 1 clearly demonstrates that the inheritance of *olg* in *An. stephensi* is a single, recessive and autosomal trait.

DISCUSSION

The mutant described in the present study matched with the characteristics of several other established mutants in *An. stephensi reported earlier.* The *olg* is an excellent phenotypic marker. It can be easily distinguished from the wild type with the naked eye and required no extra care for maintenance in the insectary. Controlled insectary conditions and the sample size (10 males × 10 females) reduced the risk of genetic drift and ensured the presence of mutant alleles providing a high probability of capturing and passing on heterozygous traits. Establishment of a homozygous mutant (isofemale) line was a crucial step prior conducting the genetic crossing experiment. Mutant genes were transmitted to subsequent generations in a predictable single gene patterns following Mendelian inheritance. The mutant gene has complete penetrance and expressed in homozygous recessive individuals with high viability, ensuring consistent phenotypic expression across generations. Based on the unique phenotypic colour, *olg* mutants were easily distinguished from wild-types in all the progenies of crossing experiments helped determining the genetic basis of the mutant inheritance.

Although detailed cytogenetic information is limited to a relatively small number of species compared to the vast and diverse tropical mosquito fauna, the genetic information has advanced from basic species identification to high-resolution, chromosome-level, genome assemblies, advanced genetic engineering, and gene editing addressing the need to control major disease vectors (THAKARE et al., 2022). Such advancement allows researchers to map complex genomic architectures, including chromosomal inversions that influence vector behaviour, adaptation and *Plasmodium* resistance (SRINIVASAN et al., 2022).

In our study, *olg* gene is a recessive autosomal trait and controlled by a single gene. The gene is located on a non-sex chromosome as the mutant colour appearing in both males and females (autosomal). The mutant phenotype expresses in a

homozygous condition (recessive). Likewise, a few spontaneously occurring larval colour mutants and their inheritance have been reported in *An. stephensi*. These include stripe that is controlled by a codominant gene (SAKAI et al., 1974); yellow – a recessive, autosomal gene (SHETTY et al., 1994); brown – a recessive, autosomal gene (SHETTY & CHOWDAIAH, 1994), greyish brown – a recessive autosomal gene (MADHYASTHA & SHETTY, 1999), grey and greenish black – recessive, autosomal genes (SHETTY & GHOSH, 2005). In addition, few eye-colour mutants and their genetics have been reported in *An. stephensi*. These include rosy eye – a sex-linked gene (ASLAMKHAN & GUL, 1979), maroon eye – a recessive, autosomal gene (MAHAMOOD & SAKAI, 1982), ruby eye – a recessive, autosomal gene (MADHYASTHA & SHETTY, 2002), and scarlet eye – a recessive, autosomal gene (AKHTAR & SAKAI, 1985). Moreover, larval colour mutants of greyish black – a recessive, autosomal gene (SHETTY et al., 2007), dark – a recessive, autosomal gene (HARIPRASAD & SHETTY, 2007). Green thorax gene that was reported non-linked with ruby eye (SANIL & SHETTY, 2009) in *An. stephensi*. Allelism test (SHETTY & CHOWDAIAH, 1976; SHETTY ET AL., 1995) and linkage mapping studies between two mutant strains of *An. stephensi* were carried out to determine whether the mutant genes are present at the same or different chromosomal loci (MADHYASTHA & SHETTY, 2016). To determine the specific location of the mutant *olg* gene linkage mapping and DNA sequencing study can be done in the next step.

Like *olg* these valuable phenotypic markers help in identifying qualitative or quantitative trait loci such as environmental adaptation, behavioural differences, insecticide resistance, etc. These are also used in studying insect ecology and behaviour. Despite these advantages the natural phenotypic markers are often rare, sometimes can interfere with the overall fitness of the insect making them challenging for comprehensive genetic mapping compared to DNA-based markers.

CONCLUSION

The larval colour mutant, *olg* is reported for the first time in *An. stephensi*. The *olg* mutant serves as a morphological marker, allowing for easy identification of the specific genotypes without molecular analysis or microscopic examination. This novel marker is useful for conducting fundamental genetic experiments, including the construction of linkage maps, marker identification, molecular mapping, and

biochemical studies involved in pigmentation of mutant colour. It can also be used in applied genetic research, such as isolation of chromosome translocation, synthesis of genetic sexing strain for the preferential elimination of vector females, genetic transformation studies, and synthesis of refractory strains of *An. stephensi*. Emphasizing research on mutations and advances in mosquito genetics, it is essential to develop next-generation vector control strategies to combat vector-borne diseases such as malaria, dengue, and Zika. As insecticide spraying becomes less effective due to insecticide resistance, genetic technologies will provide targeted and sustainable alternatives for vector control.

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Ethics statement

The biosafety approval for mosquito maintenance in the insectary facility and for blood feeding of mosquitoes was obtained by the TIGS through IBSC clearance (approval reference no. inStem/IEC-12-002/27E, dated 26/12/2022).

Author contributions

CG: Establishment and maintenance of the mutant isofemale and wild strains of *An. stephensi*, conceptualised and designed the experiments, performed and supervised the crossing experiments, curated and analysed data, wrote the first draft, reviewed and edited the manuscript; SM: Assisted in the experiment; SKG: Provided the field-collected strains of *An. stephensi*, and reviewed the manuscript. All the authors finalised the manuscript.

Conflict of interest

Authors declare that none of the co-authors has conflict of interest related to this manuscript.

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