J Vector Borne Dis 46, June 2009, pp. 117–124

The genetics of green thorax, a new larval colour mutant, non-linked with ruby-eye locus in the malaria mosquito, *Anopheles stephensi* Liston

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Abstract

Background & objectives: Anopheles stephensi, an important vector of malaria continues to be distributed widely in the Indian subcontinent. The natural vigour of the species combined with its new tolerance, indeed resistance to insecticides has made it obligatory that we look for control methods involving genetic manipulation. Hence, there is an immediate need for greater understanding of the genetics of this vector species. One of the requirements for such genetic studies is the establishment of naturally occurring mutants, establishment of the genetic basis for the same and use of such mutants in the genetic transformation studies and other genetic control programme(s). This paper describes the isolation and genetic studies of a larval colour mutant, green thorax (*gt*), and linkage studies involving another autosomal recessive mutant ruby-eye (*ru*) in *An. stephensi*.

Methods: After the initial discovery, the mutant green thorax was crossed *inter se* and pure homozygous stock of the mutant was established. The stock of the mutant ruby-eye, which has been maintained as a pure stock in the laboratory. Crosses were made between the wild type and mutant, green thorax to determine the mode of inheritance of green thorax. For linkage studies, crosses were made between the mutant green thorax and another autosomal recessive mutant ruby-eye. The percentage cross-over was calculated for the genes linkage relationship for *gt* and *gt ru*.

Results: Results of crosses between mutant and wild type showed that the inheritance of green thorax (*gt*) in *An. stephensi* is monofactorial in nature. The *gt* allele is recessive to wild type and is autosomal. The linkage studies showed no linkage between *ru* and *gt*.

Interpretation & conclusion: The mutant *gt* represents an excellent marker for *An. stephensi* as it is expressed in late III instar stage of larvae and is prominent in IV instar and pupal stages with complete penetrance and high viability. The said mutant could be easily identified without the aid of a microscope. This mutant can be used extensively to conduct basic and applied research. The mutant has been maintained in two large cages in our laboratory.

Key words *Anopheles stephensi* – autosomal recessive – green thorax – independent assortment – linkage studies – ruby-eye

Introduction

The mosquito borne diseases such as malaria, lymphatic filariasis, dengue, dengue haemorrhagic fever, Japanese encephalitis, yellow fever and chikungunya are increasing day-by-day around the world. In order to combat these diseases either the parasites or the vectors should be controlled. It is generally believed that vector control is more appropriate. The control of vectors faces two important challenges, namely the development of resistance to insecticides and pesticides and the environmental pollution caused due to the constant application of insecticides and pesticides. Therefore, it is desirable to develop alter-

native strategy for the control of *Anopheles stephensi*[1,2].

Genetic studies of mosquitoes, especially of species and strains that are vectors, continue to be essential component of genetic control strategies aimed at disrupting the transmission of diseases. These alternative strategies for control require genetic characterizations of geographically isolated strains because any one control mechanism must operate throughout the range of the target mosquito species. Therefore, genetic profiles of different geographical isolates can be used to predict the potential success of laboratory altered strains intended for release into native populations of mosquito vectors. Our laboratory is currently performing genetic studies towards the eventual development of strains for genetic control of the mosquito vectors of diseases.

An. stephensi belongs to the sub-genus *Cellia* of the order Diptera. It is one of the most important vectors of malaria in Indian subcontinent. This species has developed resistance for various insecticides[3,4] and, therefore, it is mandatory that extensive cytogenetics and genetic studies of the above said species should be carried out. Isolation of naturally occurring mutant and establishing the genetic basis of the same in *An. stephensi* is one of the ongoing area of research in our laboratory. Such studies will go a long way in establishing the genetic characterization, which could be used in a genetic control programme of the said species. This paper describes the isolation and genetic studies of the spontaneously occurring larval colour mutant, green thorax (*gt*) and its linkage with another autosomal mutant, ruby-eye (*ru*) in *An. stephensi*[5].

Material & Methods

Genetic studies of green thorax (gt) mutant: The mutant colour green thorax expressed in the late III instar persisted through larval and pupal stages. In contrast to the wild type body colour, which are straw-tan coloured, the mutant has conspicuous greenish tinge on thorax region and light straw coloured abdominal region. After the initial discovery, the mutants were crossed *inter se* and pure homozygous stock of the mutant was established.

The following stocks were used for the present investigation.

Green thorax: The mutant green thorax was isolated from the Kengeri strain (Bangalore), one of the 30 strains presently maintained in our laboratory.

Wild type: Colonized from the Punjab strain. The wild type larva has straw-tan body colour.

Ruby-eye mutant: This is an autosomal recessive eye colour mutant of *An. stephensi*. The mutant was initially isolated from Pune strain of *An. stephensi*[5]. The mutant has brick-red eyes. In adults, the colour of the eyes darkens after emergence and always lack the greenish luster typical of that of the wild type. The mutant *ru* represents an excellent marker for *An. stephensi* as it expresses in all the life stages with complete penetrance and high viability. The mutant can be easily distinguished from wild type by the naked eye and requires no extra care for maintenance in the laboratory. The mutant has been maintained in our laboratory.

For linkage studies double homozygous mutant ruby-eye and green thorax (*ru gt*)/(*ru gt*) and also individual mutants ruby-eye *(ru)* and green thorax (*gt*) were used.

Mosquitoes were reared in an insectary maintained at 25 ± 1°C and 75 ± 5% relative humidity (RH). Larvae were reared in enamel pans measuring 25 x 30 cm filled to 2 cm depth with water and fed with synthetic yeast powder. Both females and males were provided with 10% sugar solution. Females were offered restrained mice for blood meal. The normal and mutant mosquitoes were maintained in large population cages. Mass mating was done for all genetic crosses. After the blood meal, the females were isolated singly in 35 ml glass vial lined with filter paper and 10 ml water was added to fill the vial to a

3 mm depth for oviposition. The resulting progeny from each female was reared as an isofamily.

Crosses were made between the wild type and mutant to determine the mode of inheritance of green thorax. Mass mating was conducted for all crosses. Some of the F_1 hybrids were inbred to obtain F_2 generations and others were backcrossed to pure-bred mutant and wild type.

Linkage studies of green thorax (gt) with ruby-eye (ru) larva: Crosses were made between the mutants green thorax (*gt*) and another autosomal recessive mutant ruby-eye *(ru)*. Both *cis* (both mutant genes on the same individual) and *trans* (mutant genes on different individual) forms of the mutants were used for linkage studies. The F_1 progeny from the individual crosses were backcrossed with double mutant and F_2 progeny were analyzed for the parental and non-parental types. The percentage crossover was calculated for the linkage relationship for the genes *gt* and *ru*. The results obtained from all the crosses were subjected to the χ^2 analysis.

Results & Discussion

Testing the mode of inheritance of green thorax (*gt*) in *An. stephensi* involved crosses between the mutant and wild type. Progeny derived from each cross was analyzed. Results of these crosses are summarized in Table 1. Crosses 1 and 2 confirmed the establishment and purity of the pure-bred wild type and green thorax respectively. None of the F_1 progeny of the reciprocal crosses 3 and 4 could be distinguished from the wild type parent. Therefore, *gt* is recessive to the wild type. In addition, the absence of mutant green thorax phenotype in the heterogametic males indicates that *gt* is autosomal. The F_1 progeny of the crosses 5, 6, 7, 8 and 9 were derived from the males out cross (cross 3 of Table 1), and the F_1 progeny of the crosses 10, 11, 12, 13 and 14 were derived from the females out cross (cross 4 of Table 1). The F_1 heterozygote progeny were backcrossed with the presumptive parental homozygote of both sexes. Results of these backcrosses (crosses 7, 8, 12 and 13 in Table 1 revealed the expected 1:1 ratio of the wild type to green thorax. The χ^2 values for these four crosses indicated non-significant deviations. In the crosses 5, 6, 10 and 11 (Table 1) involving heterozygous F_1 backcrosses to pure-bred wild type, no green thorax mutant was recovered from the progeny. Adults of the F_1 progeny were inbred to yield F_2 generation, crosses 9 and 14 (Table 1) involved heterozygous mosquitoes being mated *inter se*. Only 3:1 ratio of wild type to green thorax resulted, and no significant χ^2-values were obtained. Data in Table 1 clearly demonstrate that the inheritance of green thorax in *An. stephensi* is monofactorial in nature. Further, the *gt* allele is recessive to wild type and is autosomal.

The results of the crosses summarized in Table 2 showed no linkage between *ru* and *gt*. The crosses 1, 2, 3 (*cis* test) and crosses 7, 8 and 9 (*trans* test) involve the F_1 progeny derived from the male out cross. Similarly, the crosses 4, 5, 6 (*cis* test) and crosses 10, 11 and 12 (*trans* test) involved the F_1 progeny derived from female out cross. The results from the crosses 3 and 6 (from *cis* test) and 9 and 12 (from *trans* test) in Table 2 clearly showed 9:3:3:1 ratio of wild type, ruby-eye, green thorax and double mutant respectively, and the expected 9:3:3:1 ratio for the above crosses has been shown in parentheses for each cross. The results from the crosses 1, 2, 4, 5, 7, 8, 10 and 11 (Table 2) show nearly 50% crossover to the parental types indicating that the two mutant genes are not linked. Hence, the two mutants are present on different autosomes and they segregate independently.

It is proposed to link the mutant green thorax (*gt*) which is being reported here and other morphological mutants such as ruby eye, greyish brown larva (*gyb*) and dark larva (*da*) which are already established and the genetic basis for the same has been studied in our laboratory[5–7], will be linked with the insecticide resistant strains, namely alphamethrin (*amr*), propoxur (*pxr*), temephos (*tr*), which are already available in our laboratory. Such integrated strains could be used in studying the linkage relationship, to prepare the linkage map, chromosome linkage correlation and applied research including

Table 1. Inheritance pattern of green thorax in *An. stephensi* Liston

Sl.	Crosses		Total eggs	Total larvae	Hatchability (%)	Wild type larva			Green thorax larva			Total	χ^2
	Male	Female				Male	Female	Total	Male	Female	Total		
1.	+ + Wild type	+ + Wild type	1242	1205	97.02	569	633	1202	–	–	–	1202	
2.	*gt* *gt* Green thorax	*gt* *gt* Green thorax	1623	1581	97.41	–	–	–	737	791	1528	1528	
3.	+ + Wild type	*gt* *gt* Green thorax	950	859	90.42	411	434	848	–	–	–	848	
4.	*gt* *gt* Green thorax	+ + Wild type	1053	1004	95.34	471	504	975	–	–	–	975	
5.	+ *gt* Wild type	+ + Wild type	764	716	93.71	302	384	686	–	–	–	686	
6.	+ + Wild type	+ *gt* Wild type	1046	964	92.54	483	432	915	–	–	–	915	
7.	+ *gt* Wild type	*gt* *gt* Green thorax	1007	967	96.02	207	226	433	181	220	401	834	1.22*
8.	*gt* *gt* Green thorax	+ *gt* Wild type	780	745	95.51	196	178	374	181	155	336	710	2.03*
9.	*gt* + Wild type	+ + Wild type	1097	965	87.96	482	451	933	–	–	–	933	
10.	+ + Wild type	*gt* + Wild type	900	74	83.77	365	371	736	–	–	–	736	
11.	*gt* + Wild type	*gt* *gt* Green thorax	829	760	91.67	204	151	359	189	190	379	738	0.542*
12.	*gt* *gt* Green thorax	*gt* + Wild type	700	639	91.28	165	134	299	161	156	317	616	0.525*
13.	+ *gt* Wild type	+ *gt* Wild type	617	564	91.41	140	266	406	56	74	130	536	0.159*
14.	*gt* + Wild type	*gt* + Wild type	561	513	91.44	142	217	359	62	53	115	475	0.137*

*Non-significant ($p<0.005$).

Table 2. Linkage studies between green thorax and ruby eye mutant larva in *An. stephensi* Liston

S.No.	Crosses		Total	*Cis* test						Cross-over (%)
	Male x Female			Parental type			Non-parental type			
				Wild type	Green thorax & Ruby eye	Total	Green thorax	Ruby	Total	
1.	*gt ru* / + + Wild type	*gt ru* / *gt ru* Green thorax & Ruby-eye	480	129	115	244	114	122	236	49.17
2.	*gt ru* / *gt ru* Green thorax & Ruby-eye	*gt ru* / + + Wild type	394	94	96	190	106	98	204	51.78
3.	*gt ru* / + + Wild type	*gt ru* / + + Wild type	485	287 (300)	47 (33.4)	334	69 (74.5)	82 (74.5)	151	30.85
4.	*gt ru* / + + Wild Type	*gt ru* / *gt ru* Green thorax & Ruby-eye	450	129	106	235	105	110	215	47.78
5.	*gt ru* / *gt ru* Green thorax & Ruby-eye	*gt ru* / + + Wild type	433	111	101	212	105	116	221	51.04
6.	*gt ru* / + + Wild type	*gt ru* / + + Wild type	470	274 (287.1)	45 (31.9)	319	82 (75.5)	69 (75.5)	151	32.13*
				Trans test						
				Non-parental type			Parental type			
				Wild type	Green thorax & Ruby eye	Total	Green thorax	Ruby	Total	
7.	± ± / *gt ru* Wild type	*gt ru* / *gt ru* Green thorax & Ruby-eye	422	117	111	228	90	104	194	45.97
8.	± ± / *gt ru* Green thorax & Ruby-eye	± ± / *gt ru* Wild type	504	131	119	250	127	127	254	50.40
9.	± ± / *gt ru* Wild type	± ± / *gt ru* Wild type	365	228 (231.3)	29 (25.7)	257	58 (54)	50 (54)	108	29.59

contd...

Table 2. (*contd*.)

10.	± ± / *gt ru* Wild type	×	*gt ru* / *gt ru* Green thorax & Ruby-eye	457	102	103	205	128	124	252	55.14
11.	*gt ru* / *gt ru* Green thorax & Ruby-eye	×	± ± / *gt ru* Wild type	335	86	75	161	97	77	174	51.94
12.	± ± / *gt ru* Wild type	×	± ± / *gt ru* Wild type	562	307 (333)	63 (37)	370	94 (96)	98 (96)	192	34.16

*$p < 0.01$ (chi-square).

synthesis of refractory strain, genetic sexing strain for the preferential elimination of the female in *An. stephensi*. Such sterile males could be used in the control of *An. stephensi* through sterile insect technique (SIT).

Morphological and larval colour mutants and its genetic basis of inheritance in *An. stephensi* were reported by several pioneer workers. These include stripe[8], greenish brown[9], black[10], diamond palpus[11], black scale[12], golden-yellow[13], green[14], yellow[15], brown[16], pale and dark colour[17], greyish brown[6], grey[18], greyish black[19], and dark mutant[7]. Tests for allelism among certain larval colour mutants have been reported in *An. stephensi*. The mutant brown is allelic to green[16], grey is allelic to greenish black[18], dark is allelic to grey and greenish black (Hariprasad and Shetty, unpublished). A few eye colour mutants have been reported in *An. stephensi*. These include, sex linked red-eye[9], sex-linked white-eye[15], autosomal colourless-eye[20], autosomal maroon eye[21], sex linked chestnut-eye[22], creamish white-eye[23] and ruby-eye[5]. This information will go a long way in understanding the biology of insect vector of diseases and in the construction of linkage maps.

The genetic and cytological basis of insecticide resistance, and the development of resistance associated with biochemical changes for various insecticides have been established in *An. stephensi*[3,4]. Therefore, it is mandatory that alternative strategies for the control should be developed. As indicated earlier genetic control is one such strategy, which requires basic genetic characterization. We have recently reported several studies in these areas through our ongoing characterization of *An. stephensi*[24–27].

The mutant *gt* described in this study represents an excellent marker for *An. stephensi* as it expresses in late larval and pupal stage with complete penetrance and high viability. The mutant can be easily identified without the aid of a microscope. This mutant can also be used extensively for conducting basic genetic experiments including the construction of linkage maps, molecular mapping and biochemical studies involved in pigmentation of green thorax and in applied genetic research such as isolation of chromosome translocation; genetic transformation studies and creation of refractory strains in *An. stephensi*.

In the earlier studies, we have shown that the ruby-eye locus is non-linked with another autosomal mutant greyish brown in *An. stephensi*[5]. In the present study, the green thorax locus is also non-linked with ruby-eye. Like any other anopheline mosquitoes *An. stephensi* has a diploid chromosome number $2n=6$. Three pairs of chromosomes are individually recognizable and designated as I, II and III based on their length and position of the centromere. The smallest subtelocentric pair is the sex chromosome designated as I. The longer pair being designated II and the shorter as III (autosomes)[2,28]. Since both mutants,

green thorax and greyish brown locus is non-linked with ruby eye, the said mutants may be placed in the same chromosome (linkage group) of *An. stephensi*.

Acknowledgement

This work has been supported by financial assistance from University Grants Commission (UGC), New Delhi to Prof. N.J. Shetty.

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Received: 16 December 2008 *Accepted in revised form:* 18 February 2009