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Preparation and mapping of ovarian polytene chromosome of an important malaria vector- *Anopheles Stephensi* Liston (Diptera: Culicidae)

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Abstract
Cytogenetic and physical maps of polytene chromosome are indispensible for specific assembly of genome sequences, functional description of chromosomal regions, and population genetic studies together with taxonomic analysis. The presence of polytene chromosomes in malaria mosquitoes serves a unique opportunity to develop high-resolution physical maps. Keeping this in mind a cytogenetic map for *Anopheles stephensi* ovarian polytene chromosome is prepared by using refined squash technique that increases overall band clarity. The major malaria vector *Anopheles stephensi* has karyotype 2*n* = 6. The homologous chromosome pair of the polytene chromosome complement includes 5 arms: X, 2, 3, 4 and 5.Each arm identified by specific band position and hence mapped. The physical map provides an appropriate basis for comparative genomics, which can be used for observing inversion breakpoints, duplications and also used to distinguish species complex.

Keywords: Polytene chromosome, karyotype, *Anopheles stephensi*, cytogenetic map etc.

1. Introduction
Mosquitoes in the genus Anopheles are the chief vectors of human malaria parasites and the resulting disease is one of the most noxious and costly in history (Feachemet *et al.*, 2010, White *et al.*, 2011) [8, 29]. Understanding the genetics of the mosquito may explicate why *Anopheles stephensi* is such a dexterous vector of the disease. Publication and accessibility of the *Anopheles stephensi* genome sequence accelerated research that has not only improved our basic understanding of behaviour, physiology, vector genetics, and roles in transmission, but also contributed to new strategies for fighting malaria (Holt *et al*., 2002) [18]. *Anopheles stephensi* is among approximately 60 species considered important in malaria transmission and is the key vector of urban malaria on the Indian subcontinent and the Middle East (Rafinejad *et al*., 2008, Sharma, 1999) [22, 28]. The armoury of tools available for fighting malaria is very limited so vector control still remains to be the major approach for reducing or eliminating malaria. In the 1970s and 1980s, chromosomal maps became the chief tool for investigating the mosquito's genetics behaviour and evolution. The presence of polytene chromosomes in Anopheline mosquitoes provides a great opportunity to develop high resolution physical maps. Polytene chromosomes found in Dipterans are produced by repetitive DNA replications without cell division and are characterized by huge size and characteristic banding patterns (Zhimulev, 1996) [31]. They are found in various tissues of mosquitoes including salivary glands, gut, Malpigian tubules, and ovarian nurse cells. These chromosomes are characterized by different levels of compaction that are manifest from light and dark bands, as well as by diffuse puffs and specific structures of heterochromatic areas. Banding patterns are principally consistent within a species and, in some cases, somewhat consistent between closely related species. Chromosomal maps, which depict chromosome structures and banding patterns, provide a source for many types of genome analysis including: physical mapping, whole genome sequence assembling, positional cloning and synteny investigation (Lewin *et al*., 2009) [21]. Chromosomal maps have been developed for about 50 species from the genus *Anopheles* (Sharakhov & Sharakhova, 2008) [25]. A novel high-pressure method of polytene chromosome preparation was developed and tested on *Drosophila melanogaster* (Novikov *et al*., 2007) [21]. During the last decade, cytogenetic photomaps based on digital imagining were created for the polytene chromosomes of *Anopheles albimanus* (Cornel & Collins, 2000) [7], *Anopheles funestus* (Sharakhov *et al*., 2001) [26] and *Anopheles stephensi* (Sharakhova *et al*., 2006) [27]. In 2002, a newly drawn high-quality map of polytene chromosomes from ovarian nurse cells was published (Coluzzi *et al*., 2002) [6]. The major malaria vector *Anopheles stephensi* has karyotype 2*n* = 6 with heteromorphic sex-chromosomes where males are heterogametic (XY) and females homogametic (XX) (Baker and Sakai 1979) [1]. In Anopheline cytogenetics literature, two types of designations are seen for the polytene chromosome arms: (i) the two arms of a chromosome are designated as right (R) and left (L) arms; this system is followed by *Drosophila* cytogeneticists and is followed by many Anopheline cytogeneticists; and (ii) the new nomenclature for arm designation is that suggested by Green and Hunt (1980) [14]. In this, each arm is given a separate number—2, 3, 4 and 5—for autosomal arms and the euchromatic arm of the X-chromosome seen in the polytene complement is designated as X. The cytogenetic map for *An. gambiae* polytene chromosomes from larval salivary glands was developed in 1956 (Frizzi & Holstein, 1956) [12]. Later, more detailed drawn chromosomal maps were formed for *An. gambiae* and for the X chromosome of *Anopheles arabiensis*, known at that time as species B (Coluzzi & Montalenti, 1966) [4]. Finding polytene chromosomes in ovarian nurse cells significantly improved the quality of the chromosomal images and simplified the procedure of slide preparation (Coluzzi, 1968) [5].

In our study modified method was applied for the preparation of the physical map makes it possible to study how different levels of chromatin compaction influence functionality of chromosomal parts.

2. Material and Methods

2.1 Source of mosquitoes

Adult Female Anopheles mosquitoes were collected from two different areas of Udaipur district, Rajasthan (India). Collection was made in early morning (6-8am) from different biotopes like cattle sheds and human dwellings (near to cattle shed) by using manual aspirator. *Anopheles stephensi* mosquitoes were also reared in laboratory (Insect Microbial and herbal control laboratory, Department of Zoology, MLSU, Udaipur (Raj), India. Field collected larvae were reared to adult and identified for species and then pure culture was maintained in the laboratory per WHO protocol.

Fig 1: Semi-gravid females of *Anopheles stephensi* mosquito

2.2 Polytene chromosome preparation

Ovaries were pulled out from the semi gravid females and chromosomes were prepared from the ovarian nurse cells of females followed by the method of Saifuddin *et al*. (1978) [24] and Banerjee and chatterjee (1994) [13] and specially modified by us at several stages. Ovaries of *Anopheles stephensi* mosquitoes were dissected approximately 25 h post-blood feeding at semi gravid stage of development (Fig.1). Only ovaries displaying a slightly oval shape were selected; elongated and circular ovaries were removed. Dissected ovaries were placed in fresh Carnoy's fixative for 24 h at room temperature, followed by a decrease in temperature to -20 °C until time of preparation. Fixed ovaries were divided in Carnoy's solution under a dissection microscope. Any tissue other than follicles was removed from the slide via tissue paper or fine needle. After tissue removal, the divided sections of follicles were placed onto slides containing 50% propionic acid.Usually about 6-8 slides per set of ovaries were prepared simultaneously. Follicles were then separated from each other. Finally stained with 2% lacto aceto orcein for 1hour. After proper staining 50% propionic acid were added for clarity. A cover slip was then placed on top of the ovaries and light mechanical force was created by using the tapping device specially prepared in our laboratory. The force of tapping was applied to the entire surface to express the chromosomes from the nuclei and for proper spreading. Excess liquid present on slide were removed by placing slides between the filter paper. The prepared slide then put on hot plate for 1 min so that excess propionic acid may evaporate and chromosome becomes clear. Microphotographs were taken and studied under microscope for identification of different arms. Finally karyotyping and mapping of chromosome were done.

The identification of each chromosome segment and its different arms was deduced from the specific size, characteristic shape, and banding pattern of the telomeric and centromeric ends along with certain prominent puffs and number of dark bands in different regions. Both the conventional and alternative nomenclatures for the right and left arms of the chromosome are provided in Table 1 (Green and Hunt 1980; WHO 1984) [14, 30].The ovarian polytene chromosome photomap was developed after studying the photographs and slides under the microscope. By using the Adobe Photoshop CS3 photo editing software scanned polytene chromosome photographs were used to build up a composite image without losing the optimum and minor details of characteristic banding pattern.

3. Results and Discussion

The diploid karyotype of 6 chromosomes in the genial metaphase cells of *Anopheles stephensi* comprises a pair of small acrocentic sex chromosomes (XX, female; XY, male) and a much larger autosomal arms 2,3,4 and 5.The results indicate that our modified method produces extremely clear, flat chromosomes and significantly improves structural resolution of the banding pattern. In our study we followed the designation of different chromosomal arms as x, 2, 3, 4 and 5 (Fig. 2). The arrow indicates the centromeric end. The most robust landmarks for all chromosomal arms are the length of the arm, specific banding pattern and morphology of the telomeric and centromeric regions.

Table 1: Conventional and alternative nomenclatures of the polytene chromosomes in the genus *Anopheles*.

Chromosomal arm	Conventional nomenclature	Alternative Nomenclature
X	X	X
Right arm of chromosome 2	2R	2
Left arm of chromosome 2	2L	3
Right arm of chromosome 3	3R	4
Left arm of chromosome 3	3L	5

Fig 2: The photomap of ovarian polytene chromosome of *Anopheles stephensi*

The identification and landmarks for recognition of different chromosomal arms are as follows

Chromosome X. It is the shortest in the chromosomal complement. It can be easily recognized by a prominantly stained dark band at telomeric end in region (Fig.3). At centromeric end large puff is observed which is the most charecteristic feature of this arm.

Chromosome 2(2R)**.**The right arm of chromosome 2 is the longest element among the 4 autosomal arms. The 2 arm can be easily recognized by two couples of dark thick bands at telomeric end (Fig.4). An additional robust landmark for this arm is a prominent granulated large dark band is present at centromere with small puff.

Chromosome 3(2L). This is shorter than chromosome 2.It has a very light telomere end. The number of dark bands is very less in this region. The identifying feature of this arm is the presence of doublets of dark bands especially just before centromeric end (Fig.5).

Chromosome 4(3R)**.**It is also shorter than 2 and 3 chromosome. It can be identified by the large number of puffs near centromeric end (Fig.6).

Chromosome 5(3L). It is slightly larger than X chromosome and shorter than all other. Telomeric end is characterized by fewer bands. However large number of dark bands is concentrated at the centre. Flared centromeric end have small puff (Fig.7)

Y chromosome. The high repetitive DNA content of Y chromosomes makes them difficult to assemble and thus they are often are excluded from genome sequencing and mapping

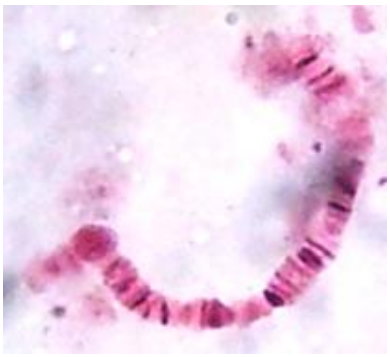

Fig 3: Chromosome X

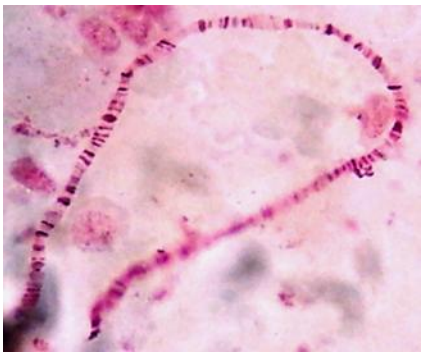

Fig 4: Chromosome2 (2R)

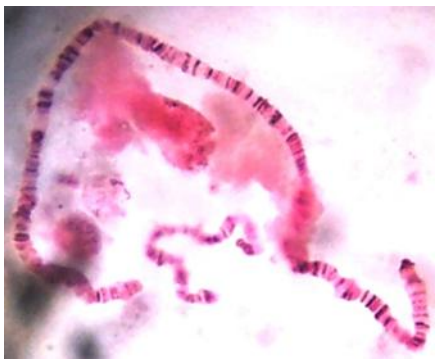

Fig 5: Chromosome 3 (2L)

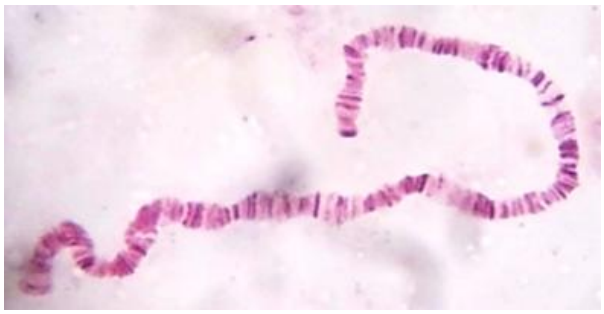

Fig 6: Chromosome4 (3R)

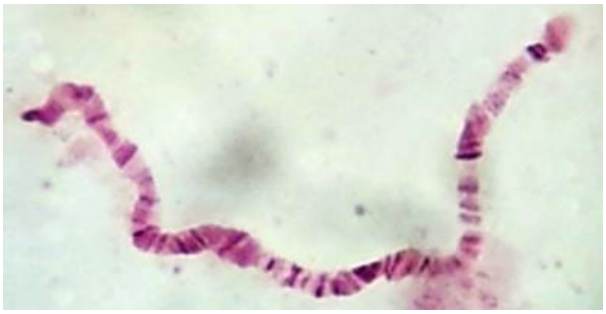

Fig 7: Chromosome 5(3L)

Physical mapping described in this article is therefore a useful approach for studying genome organization and evolution and many other purposes. This becomes possible due to the availability of polytene chromosomes in malaria mosquitoes. These chromosomes occur in various tissues of Dipteran larvae and adult. The ovarian polytene chromosome analysis described in this work provides a resource and platform for fundamental and translational research into a major urban malaria vector. Also the chromosome-based research provides exclusive perspectives on Anopheles chromosome evolution. A number of similar works has been done for different species of mosquito genera. Also for freshly isolated salivary glands of *D. melanogaster* (Novikov *et al*., 2007) [20]. George *et al* (2010) [13] provide High-resolution cytogenetic map for the African malaria vector *Anopheles gambiae.* Rishikesh (1959) has produced a map of A. stephensi, Hobbs (1962) [16] a map of A. albimanus, Frizzi & Holstein (1956) [10] a map of A. gambiae and Frizzi & Ricciardi (1955) [11] a map of A. aquasalis. This is at least a beginning. Preliminary information has been published by Frizzi & De Carli (1954) [9] and otherwork (Kitzmiller & French, 1961; Baker & Kitzmiller, 1962) [19, 2] dealing with polytene chromosomes of quadrimaculatus, freeborni, aztecus and punctipennis. Ovarian polytene chromosome from the important malaria vector *Anopheles stephensi.* Therefore by increasing the overall resolution of the cytogenetic map and chromosome squashes in general, we were able to better determine specific gene locations and banding position along the chromosome, which can be used to further enhance physical mapping and epigenomic studies of malaria vectors. Thus at the end we can conclude that the genetic study opens an exciting prospect to uncover the molecular mechanisms contributing to vectorial capacity and to identify targets potentially helpful for vector management.

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