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High-Resolution Cytogenetic Map for the African Malaria Vector *Anopheles gambiae*

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Abstract

Cytogenetic and physical maps are indispensable for precise assembly of genome sequences, functional characterization of chromosomal regions, and population genetic and taxonomic studies. We have created a new cytogenetic map for *Anopheles gambiae* by using a high-pressure squash technique that increases overall band clarity. To link chromosomal regions to the genome sequence, we attached genome coordinates, based on 302 markers of BAC, cDNA clones, and PCR-amplified gene fragments, to the chromosomal bands and interbands at approximately a 0.5-1 Mb interval. In addition, we placed the breakpoints of seven common polymorphic inversions on the map and described the chromosomal landmarks for the arm and inversion identification. The map's improved resolution can be used to further enhance physical mapping, improve genome assembly, and stimulate epigenomic studies of malaria vectors.

Keywords

Anopheles gambiae; physical map; polytene chromosomes; heterochromatin

Introduction

Anopheles gambiae is a prominent vector in the transmission of the malaria parasite *Plasmodium falciparum* in Africa, responsible for more than one million deaths annually (Tolle 2009). Understanding the genetics of mosquito may explicate why *An. gambiae* is such a proficient vector of the disease. Cytogenetic maps, which depict chromosome structures and banding patterns, provide a basis for many types of genome analysis including: physical mapping, synteny investigation, whole genome sequence assembling, and positional cloning (Lewin et al. 2009). Anopheline mosquitoes, as other Diptera, have giant polytene chromosomes that undergo endoreplication and can be found in various tissues (Zhimulev 1996). These chromosomes are characterized by various levels of compaction that are evident from light and dark bands, as well as by diffuse puffs and specific structures of heterochromatic regions. Banding patterns are mostly consistent within a species and, in some cases, somewhat consistent between closely related species. Drawn and photo chromosomal maps have been developed for about 50 species from the genus *Anopheles* (Sharakhov and Sharakhova 2008). During the last decade, cytogenetic photomaps based on digital imaging were created for the polytene chromosomes of *An. albimanus* (Cornel and Collins 2000), *An. funestus* (Sharakhov et al. 2001), and *An. stephensi* (Sharakhova et al. 2006).

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The major malaria vector *An. gambiae* has karyotype $2n=6$. Because of homologous chromosome pairing, the polytene chromosome complement includes 5 arms: X, 2R, 2L, 3R, and 3L. The first preliminary cytogenetic map for *An. gambiae* polytene chromosomes from larval salivary glands was developed in 1956 (Frizzi and Holstein 1956). Later, more detailed drawn chromosomal maps were created for *An. gambiae* and for the X chromosome of *An. arabiensis*, known at that time as species B (Coluzzi and Montalenti 1966). The fixed inversions identified based on the banding pattern became a key for the species diagnostics. Finding polytene chromosomes in ovarian nurse cells significantly improved the quality of the chromosomal images and simplified the procedure of slide preparation (Coluzzi 1968). In the 1970's and 1980's, chromosomal maps became the major tool for investigating the mosquito's genetics and evolution. Cytogenetic analysis of chromosomal inversions helped to identify species within the *An. gambiae* complex (Coluzzi and Sabatini 1967; Coluzzi and Sabatini 1968; Coluzzi and Sabatini 1969) and led to the discovery of chromosomal forms within *An. gambiae* s.s. (Bryan et al. 1982; Coluzzi et al. 1985).

In 2002, a newly drawn high-quality map of polytene chromosomes from ovarian nurse cells was published (Coluzzi et al. 2002). This map, together with unassembled digital photo images of chromosomes (VectorBase.org), was utilized for the physical mapping and assembly of the *An. gambiae* genome (Holt et al. 2002; Sharakhova et al. 2007). The positions of ~2000 BAC clones were assigned to the chromosomal locations. However, the current genome assembly suffers from a number of physical gaps, incorrect scaffold orientation, and the presence of 42 Mb of unmapped sequences. These deficiencies hinder further accurate annotation and functional characterization of the *An. gambiae* genome. The majority of the gaps are located in gene-poor repeat-rich heterochromatic regions. More detailed physical mapping is needed to improve the genome assembly and to link heterochromatin to the genome sequence. A high-resolution cytogenetic photomap should be useful for precise physical mapping and for studying epigenetic chromatin modifications and their relationships with the genome sequence.

A novel high-pressure method of polytene chromosome preparation was developed and tested on *Drosophila melanogaster* (Novikov et al. 2007). In our study, a modified high-pressure method was applied to the chromosomes of the malaria mosquito *An. gambiae*. By increasing the overall resolution of the cytogenetic map and chromosome squashes in general, we were able to better determine specific gene locations along the chromosome. The new map will make it possible to study how different levels of chromatin compaction affect functionality of chromosomal regions. Therefore, the improved resolution can be used to further enhance physical mapping and epigenomic studies of malaria vectors.

Results

Development of the high-resolution map

Here we present a high-resolution cytogenetic photomap of ovarian nurse cell polytene chromosomes for the major malaria vector *An. gambiae* (Fig. 1). The map was developed using a modified version of a novel high-pressure technique (Novikov et al. 2007) that applies ~150 kg/cm² of pressure to a slide placed in a vice. Approximately 90% of slides created with this method were still intact and functional after adding mechanical pressure. The 10% failure rate was due to slide breakage from the high pressure. This pressure provided a better resolution image so that the banding pattern became clearer and many new small bands became visible (Fig. 2A). The high-pressure method does not damage or change most of the chromosome structure. It flattens banded chromosomes, and thus, reveals hidden bands. The diffuse structure of the heterochromatin was more evident through the addition of high-pressure flattening. However, often the heterochromatin and other chromosomal regions ended up broken and unusable for mapping. Therefore, several regions on the map

were taken from images using squash procedures with less pressure. Chromosomes from ten slides were used to create this map. The chromosome map includes images for five chromosomal arms and mostly retains previously described divisions and subdivisions (Coluzzi et al. 2002). For the convenience of physical mapping, region 6 on chromosome X was additionally subdivided into three lettered subdivisions -- A, B, and C, -- which are absent from the 2002 map. However, this region included four lettered subdivisions on the original drawn map (published in 1967) for the salivary gland chromosomes (Coluzzi and Sabatini 1967). To make viewing easier, chromosomal images were straightened and shaped similarly to those on the drawn map (Coluzzi et al. 2002) as shown in Figure 2B.

Landmarks for recognition of chromosomal arm

Table 1 provides the description of the landmarks routinely used in our laboratory for chromosomal arm identification. Chromosome X is usually located separately from other arms on the chromosomal preparations. Chromosomes 2 and 3 form a diffuse chromocenter, which can be easily destroyed by squashing the slide (Sharakhov et al. 2001). Nevertheless, left and right arms of both chromosomes typically remain attached to each other. The most robust landmarks for all chromosomal arms are the length of the arm and morphology of the telomeric and centromeric regions.

Chromosome X, the shortest in the polytene complement, can be easily recognized by the flared, lightly stained telomeric end in region 1A and the diffuse granulated heterochromatic area on the centromeric end in region 6. Heterochromatin of region 6 ends with a dark round band on the very proximal end, which corresponds to the nucleolus organizing region (Sharakhova et al. 2007). The centromeric region can be asynaptic in some squashes. In addition, several specific structures can also be considered as landmarks for the X chromosome: large puffs in regions 4A, 3A, and 5A and two large dark bands in region 1D.

Chromosome 2 is the longest in the polytene complement. The 2R arm can be easily recognized by two couples of dark thick bands in region 7A, a dark granulated centromeric area in region 19E, and a dark large band in region 19D. An additional robust landmark for this arm is a very stable structure with two large bands followed by three thick bands in region 17C. The 2L chromosomal arm has always had a light flared telomeric end (28D region) and a large dark granulated centromeric end (20A-B). An additional specific landmark for 2L arm is a diffuse light granulated area in region 21A, which usually forms the attachment of the chromosome to the nuclear envelope (Sharakhov et al. 2001).

Chromosome 3 is relatively shorter than chromosome 2. Both arms of chromosome 3 have slightly flared telomeric ends; 3R has a dark band in the distal region of the telomere, and 3L has a very light telomere and a dark band located proximally in region 46D. Centromeric areas for both arms have a very similar, dark granulated morphology. Additional landmarks for chromosome 3 include a very dark block in region 35B of the 3R arm and a large granulated area in region 38C of the 3L arm.

Landmarks for recognition of polymorphic inversions

The location of the six common polymorphic inversions on the map is indicated by brackets below the chromosomes: $+^j$, $+^b$, $+^c$, $+^u$, and $+^d$ on chromosome arm 2R and $+^a$ on chromosome arm 2L (Fig. 1). Breakpoints for the seventh common polymorphic inversion 2Rbk are indicated by lines because this inversion is based on the preexisting 2Rb inversion (Coluzzi et al. 2002). Inversion j is easily recognizable by a series of four dark bands, located in the proximal half of region 8D, and by a large, light puff in region 9A surrounded by a narrow dark band (9A distal) and two wide bands (9A proximal and 9B distal) (Table

2). Two distinct bands in region 8B followed by four lightly granulated double bands in region 8C can be utilized as additional landmarks for inversion *j*. A landmark for inversion *b* is a light-bulb-looking puff in region 12D surrounded by four dark bands on the left in subdivisions 12C-12D and one dark band on the right in region 12E. An additional landmark for the *b* inversion is a light area with four diffuse bands in region 12A which has no dark bands. Landmarks for inversion *c* are the very dark wide band followed by a narrow band in region 13A and two distinct thick bands in regions 13D-13E. Distal parts of regions 13B and 13C form two narrow zones, which make the whole structure look like a tightened asymmetrical ribbon. Inversion *u* also has a narrow zone in the middle of region 14C, one wide band in region 14A, two thick bands located close to each other in region 14B, and three distinct bands located equidistantly in regions 14D-14E. Inversion *d* is overlapped with inversion *c* in region 14. In addition to the *c* inversion landmarks, two large puffs surrounded by dark bands on both sides in regions 15 BC and 15D can be utilized as landmarks for the *d* inversion. Inversion *bk* overlaps with the *b*, *c*, *u*, and *d* inversions; therefore, the same landmarks can be used for its recognition. Inversion *a* on the 2L arm is the biggest common inversion in the *An. gambiae* genome. This inversion can be identified by two light puffs surrounded by very dark bands located distally in region 26. On the opposite proximal part of the inversion, two large puffs in regions 24A and 23C have a dark granulated structure.

Genome coordinates on the cytogenetic map

To make the cytogenetic map suitable for genomic analyses, bands and interbands were given approximate genome coordinates (Fig. 1, Table S1). Most of these coordinates represent the genome positions of BAC and cDNA clones, which were physically mapped to chromosomes (Holt et al. 2002; Sharakhova et al. 2007). Although the *An. gambiae* genome was mapped by *in situ* hybridization of ~2,000 BAC clones with polytene chromosomes (Holt et al. 2002), a large portion of BAC clones hybridized to overlapping or multiple chromosomal locations. Moreover, signals from nonfluorescent images often covered several bands and interbands. We chose markers that hybridized specifically to only one band or interband. The resolution of this physical map varies in the different locations of the chromosomes depending on the precision of the *in situ* hybridization. We attempted to provide at least one coordinate per 0.5 Mb of chromosome length. Because heterochromatic regions were not sufficiently covered with markers, we performed additional physical mapping of PCR-amplified gene fragments (Fig. 3, Table S1). The size of the mapped portion of the *An. gambiae* genome is approximately 230.5 Mb (VectorBase.org), giving the map an overall marker density of 0.76 Mb (230.5 Mb/302 markers). Individual chromosomal arm resolutions come out to be 0.76 Mb for X (24.4/32), 0.78 Mb for 2R (61.5/79), 0.71 Mb for 2L (49.4/70), 0.78 Mb for 3R (53.2/68), and 0.79 Mb for 3L (42.0/53).

Discussion

We present in this study a new cytogenetic photomap for the major malaria vector *An. gambiae*. This map is based on a modified high-pressure technique that provides greater details of chromosomes and more accurately represents the observable banding patterns (Fig. 2). This technique was first developed for freshly isolated salivary glands of *D. melanogaster* (Novikov et al. 2007). However, ovaries of mosquitoes are routinely preserved in the Carnoy's fixative solution (3 methanol: 1 glacial acetic acid by volume) before they are used for chromosomal preparations. Therefore, we modified the existing high-pressure protocol to make it suitable for the fixed ovarian nurse cell polytene chromosomes of the malaria mosquito *An. gambiae*. Several technical problems were encountered and successfully eliminated. Slide breakage was overcome by placing a second

coverslip on the opposite side of the slide as a counterbalance. Excessive bubbling was removed by using a new solution (40% propionic acid, 40% water, 20% acetic acid) instead of a 50% propionic acid solution in our original protocol. Our results indicate that the high-pressure method produces extremely flat chromosomes and significantly improves structural resolution of the banding pattern. To make a spread, we used the eraser end of a pencil to apply mechanical force to the coverslip, expressing the chromosomes from the nuclei. The high-pressure squash was achieved by placing a slide into a vice and applying $\sim 150 \text{ kg/cm}^2$ of pressure. This pressure provided a higher resolution image so that the banding pattern became clearer and many new small bands became visible (Fig. 2A). Because high pressure is applied using precision vices possessing highly parallel work surfaces, several chromosomal preparations can be made at the same time to increase throughput.

To make this map suitable for population genetics studies, we indicated chromosomal positions for seven common polymorphic inversions and described landmarks for their recognition. Inversions were shown in their standard orientation to make this map consistent with previous maps. The standard orientation of inversions in our map may not correspond to their ancestral arrangement. For example, the derived state of the standard $2L+^a$ arrangement has been demonstrated in several independent studies (Ayala and Coluzzi 2005; Sharakhov et al. 2006; Xia et al. 2008).

To facilitate further physical mapping and genome assembly of the *An. gambiae* genome, we described landmarks for chromosomal arm recognition and put approximate genome coordinates on bands, interbands, and heterochromatic regions. This photomap can now be used for various applications in cytogenetics and comparative genomics. For example, the molecular content of intercalary heterochromatin, euchromatin-heterochromatin borders, puffs, and inversions can now be determined at a higher resolution. The packaging of DNA into euchromatin and heterochromatin of *An. gambiae* could have major implications for understanding how genome sequences function and respond to *Plasmodium* infection. Further use of high-pressure chromosomal preparations for microdissection and sequencing of specific regions will directly link the chromatin structure to the genome sequence. Similarly, using high-pressure chromosomal preparations for immunostaining will improve the precision of mapping chromosomal proteins and histone modifications involved in DNA replication, gene expression, gene silencing, and inheritance.

The high-pressure technique can also be successfully applied to other species from the genus *Anopheles* and, probably, could improve the quality of chromosomal preparations from *Anopheles* salivary glands. We would also recommend using this method for species from the genus *Culex* because the *Culex* polytene chromosomes are hardly spreadable with traditional techniques (Campos et al. 2003a; McAbee et al. 2007). However, a very low yield of chromosomal preparations from salivary glands or Malpighian tubules of *Aedes aegypti* (Campos et al. 2003b) will make it more difficult to use the high-pressure method for this species.

Experimental procedures

Mosquito strain

A laboratory SUA strain of *An. gambiae* was used in this study. These mosquitoes are the M form of *An. gambiae*. Mosquitoes were reared at 28°C at 80% humidity. Mosquitoes were grown at a low density (500-750 mosquitoes per 4 liter pan) to obtain better quality chromosomes. Larvae were fed *ad libitum*. Adults were given sugar water through dampened cotton balls that were removed at least 2 hours preblood feeding to ensure that most mosquitoes had taken a blood meal. To obtain the chromosomal preparations, females were bloodfed twice with a Guinea pig.

Chromosome preparation

The experiment involved the use of fixed ovaries. Both the Carnoy's solution, 3:1 methanol: acetic acid, and 50% propionic acid were made fresh prior to slide preparation. Ovaries of *An. gambiae* SUA mosquitoes were dissected approximately 25 hours post-blood feeding at Christopher's Stage II of development. Only ovaries displaying a slightly oval shape were collected; elongated and circular ovaries were discarded. Dissected ovaries were placed in fresh Carnoy's solution for 24 hours 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. After tissue removal, the divided sections of follicles were placed onto slides containing 40% propionic acid, 40% water, 20% acetic acid (usually about four to six slides per set of ovaries, or half to one third of an ovary per slide). After approximately 5 to 10 minutes, the acid mix was replaced by a drop of 50% propionic acid to further remove any remaining tissue. Follicles were then separated from each other, and the drop of acid was replaced once again. A coverslip was lightly placed on top of the ovaries in solution, and light mechanical-force tapping created by using the eraser end of a pencil was applied to the entire surface to express the chromosomes from the nuclei. Once sufficiently spread, a mechanical vise was used to evenly apply pressure to further flatten chromosomes on the preparation. Approximately 150 kg/cm^2 of pressure was applied through the vise for a 30-second interval. Banding patterns of the chromosomes were viewed under an Olympus BX-41 phase-contrast microscope on wet slides.

Chromosome imaging and assembling

Chromosomes that showed a suitable level of polytenization were imaged by an Olympus BX-41 (Olympus America, Inc., Melville, NY, USA) with an attached Olympus Q-Color 5 camera and Q-Imaging software. Images were combined, straightened, shaped, and cropped using Adobe Photoshop. To link the chromosomal bands to genome sequences, previous images of BAC and cDNA clone *in situ* hybridizations were utilized (Lawson et al. 2009; Sharakhova et al. 2007).

Fluorescent in situ hybridization

PCR probes were chosen from poorly assembled regions of the *An. gambiae* genome. Many of these probes were based on genes located near expected heterochromatin - euchromatin boundaries on each chromosome arm. Primers were designed using the Primer3 program (<http://frodo.wi.mit.edu/primer3/>). PCR products ranged from 400-600 bp in size. The *in situ* hybridization procedure was done as previously described (Sharakhova et al. 2006). PCR products were gel purified using the GeneClean kit (Qbiogene, Inc., Irvine, CA). The DNA was labeled with Cy3-AP3-dUTP (GE Healthcare UK Ltd., Buckinghamshire, England) using Random Primers DNA Labeling System (Invitrogen Corporation, Carlsbad, CA, USA). DNA probes were hybridized to the chromosomes at 39°C overnight in hybridization solution (Invitrogen Corporation, Carlsbad, CA, USA). Then the chromosomes were washed in 0.2XSSC (Saline-Sodium Citrate: 0.03M Sodium Chloride, 0.003M Sodium Citrate) counterstained with YOYO-1, and mounted in DABCO. Fluorescent signals were detected and recorded using a Zeiss LSM 510 Laser Scanning Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Cytogenetic photomap of *An. gambiae* ovarian nurse cell chromosomes. Genome coordinates are shown above X, 2R, 2L, 3R, and 3L chromosomal arms. Divisions and subdivisions are indicated by numbers and letters below the chromosomes. The location of common polymorphic inversions $+^j$, $+^b$, $+^c$, $+^{bk}$, $+^u$, $+^d$, and $+^a$ is shown in their standard orientation by brackets below the chromosomes.

Figure 2.

(A) Comparison of *An. gambiae* polytene chromosome spreads prepared using the high-pressure (top) and traditional (bottom) technique. The images cover divisions 30–34 of the 3R arm. (B) Comparison of the high-resolution photomap (top) and drawn map (Coluzzi et al. 2002) (bottom) of the X chromosome of *An. gambiae*. The vertical lines connect the same regions.

Figure 3. Fluorescent *in situ* hybridization of fragments of heterochromatic genes to the polytene chromosomes 2L and 3L of *An. gambiae*. Arrows indicate signals of hybridization in diffuse intercalary heterochromatin.

Table 1 Major landmarks of the banding pattern for the *An. gambiae* chromosomal arm recognition

Chromosome	Telomere	Centromere	Additional landmarks
X	Flared light zone	Diffuse granulated area ending with dark round band in NOR, sometimes asynaptic	Large puffs in 4A, 3A, 5A regions, two large dark bands in region 1D
2R	Two couples of dark thick bands in region 7A	Dark granulated area in region 19E with dark large band in region 19D	Two large bands followed by three thick bands in region 17C
2L	Very light flared area	Dark granulated area	Diffuse light granulated area in region 21A
3R	Dark slightly flared dark band in the distal area	Dark granulated area	Dark block in region 35B
3L	Light slightly flared zone with dark band in the proximal area	Dark granulated area	Large granulated area in region 38C

NOR – nuclear organizing region

Table 2 Chromosomal landmarks for recognizing common polymorphic inversions in *An. gambiae*

Inversion	Major landmarks	Additional landmarks
2Rj	Four dark bands located in proximal half of the region 8D; large light puff in region 9A surrounded by the narrow dark band in distal 9A; two wide bands located in regions 9A proximal - 9B distal	Two distinct bands in region 8B followed by four light granulated double bands in region 8C
2Rb	“Bulb” – a puff in region 12D surrounded by four dark bands in regions 12C-12D and one dark band in region 12E	Light area with four diffuse bands in region 12A, no dark bands around
2Rc	“Ribbon” – very dark wide band followed by narrow band in region 13A; distal parts of regions 13B and 13C form two narrow zones – the middle part of the “ribbon”	Two distinct thick bands in regions 13D-13E
2Ru	One wide band in region 14A and two thick bands located close to each other in region 14B and three distinct bands located at the same distance in regions 14D-14E	Narrow zone in the middle of region 14C
2Rd	Narrow zone in the middle of region 14C with couple of double bands in regions 14A-14B located distally and three distinct bands located proximally in regions 14D-14E	Two large puffs surrounded with dark bands on both sides in regions 15B-C and 15D
2La	Two light puffs surrounded by very dark bands located distally in the inversion in region 26	Two large granulated puffs in regions 24A and 23C with no dark bands around