An Integrated Chromosome Map of Microsatellite Markers and Inversion Breakpoints for an Asian Malaria Mosquito, *Anopheles stephensi*

Maryam Kamali, Maria V. Sharakhova, Elina Baricheva, Dmitrii Karagodin, Zhijian Tu, and Igor V. Sharakhov

From the Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061 (Kamali, Sharakhova, and Sharakhov); Laboratory of Evolutionary Cell Biology, the Institute of Cytology and Genetics, The Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia (Baricheva and Karagodin); and the Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA (Tu).

Address correspondence to Dr Igor V. Sharakhov at the address above, or e-mail: igor@vt.edu.

Abstract

Anopheles stephensi is one of the major vectors of malaria in the Middle East and Indo-Pakistan subcontinent. Understanding the population genetic structure of malaria mosquitoes is important for developing adequate and successful vector control strategies. Commonly used markers for inferring anopheline taxonomic and population status include microsatellites and chromosomal inversions. Knowledge about chromosomal locations of microsatellite markers with respect to polymorphic inversions could be useful for better understanding a genetic structure of natural populations. However, fragments with microsatellites used in population genetic studies are usually too short for successful labeling and hybridization with chromosomes. We designed new primers for amplification of microsatellite loci identified in the *A. stephensi* genome sequenced with next-generation technologies. Twelve microsatellites were mapped to polytene chromosomes from ovarian nurse cells of *A. stephensi* using fluorescent *in situ* hybridization. All microsatellites hybridized to unique locations on autosomes, and 7 of them localized to the largest arm 2R. Ten microsatellites were mapped inside the previously described polymorphic chromosomal inversions, including 4 loci located inside the widespread inversion *2Rb*. We analyzed microsatellite-based population genetic data available for *A. stephensi* in light of our mapping results. This study demonstrates that the chromosomal position of microsatellites may affect estimates of population genetic parameters and highlights the importance of developing physical maps for nonmodel organisms.

Key words: genome sequence, malaria vector, polymorphic inversion, polytene chromosome, population structure

Anopheles stephensi Liston (Diptera: Culicidae) is an important malaria vector in the Persian Gulf and South Iran (Manouchehri et al. 1976; Vatandoost et al. 2006), in urban areas of the Indian subcontinent (Pant et al. 1981; Hati 1997), as well as in rural areas of North Pakistan and East Afghanistan (Rowland et al. 2002). This species is also an outstanding laboratory model system for malaria parasite transmission studies (Abraham et al. 2004; Baton and Ranford-Cartwright 2007; Bass et al. 2008). Three morpho-ecological variants have been identified within *A. stephensi* populations—"type, intermediate, and mysorensis"—which can be identified by the number of ridges on the egg (Sweet and Rao 1937; Rao et al. 1938; Subbarao et al. 1987). The type form is mainly an urban mosquito that breeds in temporary water pools, whereas the mysorensis and intermediate variants occupy predominantly rural areas (Nagpal et al. 2003). Although mysorensis is considered mostly zoophilic and is a poor vector of malaria in India (Subbarao et al. 1987), it was found to be a major malaria vector in the southern part of Iran (Manouchehri et al. 1976). In spite of the importance of bionomic differentiation for efficient malaria transmission, the genetic structure of the biological forms and populations of *A. stephensi* remains unclear, as tools are insufficient to precisely characterize the genetic variation. A study of PCR-based RFLP (PCR-RFLP) of 1512 bp of mitochondrial DNA cytochrome oxidase subunit I and II (COI-COII) and partial sequences of COI and COII genes has found extensive gene flow among the different forms of *A. stephensi* in Iran

(Oshaghi et al. 2006). Analysis of the ribosomal DNA internal transcribed spacer 2 (rDNA-ITS2) and random amplification of polymorphic DNA loci in different populations of *A. stephensi* has demonstrated very little genetic variation among different populations suggesting that *A. stephensi* in Iran is a single species with different ecological forms in different zoogeographical zones (Djadid et al. 2006). Sequencing of the rDNA-ITS2 and domain-3 (D3) of rDNA loci of the *A. stephensi* type and mysorensis in India did not find any intraspecies sequence variation (Alam et al. 2008).

Microsatellites are informative markers for inferring population and taxonomic status of various organisms (Bruford and Wayne 1993). These markers have high levels of polymorphism and tend to evolve neutrally. They have been successfully used to study gene flow in natural populations of *A. gambiae*, *A. funestus*, and *A. nili* (Lehmann et al. 1996; Cohuet et al. 2005; Ndo et al. 2010). A set of 16 microsatellite markers has been developed for *A. stephensi* (Verardi et al. 2002). A study using 7 of these microsatellite markers has revealed high levels of genetic diversity within populations but not among geographically isolated populations in Pakistan. Deviation from Hardy–Weinberg expectations has been observed for 2 microsatellite loci in 21 tests (Ali et al. 2007). Another study of genetic variation at 8 of the 16 microsatellite loci observed a significant differentiation and a low level of gene flow among 3 ecological variants of *A. stephensi* in India (Vipin and Gakhar 2010). The study demonstrated that some microsatellites were in significant linkage disequilibrium, whereas other loci had a heterozygote deficit. Reduced recombination and selection can influence loci within polymorphic chromosomal inversions or near inversion breakpoints, resulting in estimates of gene flow that may depart significantly from those based on loci elsewhere in the genome (Lanzaro et al. 1998; Tripet et al. 2005). However, locations of the microsatellites on the chromosomes of *A. stephensi* were unknown, and interpretation of these differences among the loci was difficult.

Geographical and seasonal changes in chromosomal inversion frequencies have been interpreted as a signature of natural selection (Hoffmann et al. 2004; Balanya et al. 2006; Hoffmann and Rieseberg 2008). Therefore, polymorphic inversions are useful markers for studying ecological adaptations of various species, including malaria mosquitoes (Ayala et al. 2011; Sharakhova et al. 2011). An informative approach to determine the role of inversions in a population structure and species environmental adaptation is to study the genetic variation of molecular markers inside and outside of chromosomal rearrangements (Navarro and Barton 2003). In Diptera, contrasting patterns of polymorphism at microsatellite loci and inversions have been revealed. For example, some microsatellite loci located within known inversions did not display clinal profiles even if inversions had clinal profiles (Onyabe and Conn 2001; Kennington et al. 2003; Cohuet et al. 2005; Michel et al. 2005; Ayala et al. 2011). The number of genes under selection within inversions and the inversion age can be responsible for the observed patterns.

Polymorphic inversions have been employed to investigate the population structure and gene flow between ecological forms of *A. stephensi*. Breakpoints of at least 24 paracentric inversions have been mapped to polytene chromosomes of *A. stephensi* (Mahmood and Sakai 1984; Gayathri Devi and Shetty 1992). Seven inversions are located on 2R, 4 inversions on 2L, 3 inversions on 3R, and 10 inversions on 3L. No polymorphic inversions have been identified on the X chromosome. The *2Rb* inversion was found to be highly polymorphic and widespread in natural populations of *A. stephensi*. Population studies provide support for the variation in inversion polymorphism according to geographic distribution of *A. stephensi* especially with respect to the *2Rb* inversion (Coluzzi et al. 1973a; Mahmood and Sakai 1984; Gayathri Devi and Shetty 1992). For example, a chromosomal study revealed striking differences in the kinds and frequencies of paracentric inversions between the urban population in Karachi city and rural populations in Lahore and Kasur districts in Pakistan (Mahmood and Sakai 1984). The type and mysorensis forms showed significant differences in frequencies of inversion *2Rb* (Coluzzi et al. 1973b). However, another study did not find any correlation between the inversion and the number of ridges on the egg (Suguna 1981).

In this study, we determined the locations of 12 microsatellite markers on a cytogenetic map of *A. stephensi*. All microsatellites hybridized to unique locations on autosomes both inside and outside polymorphic inversions. The chromosomal map of microsatellite markers and inversion breakpoints helps to understand better the genetic variation and differentiation in natural populations of *A. stephensi*.

Materials and Methods

Mosquito Strain and Chromosome Preparation

The Indian wild-type laboratory strain of *A. stephensi* was used in this study. To obtain the polytene chromosomes, ovaries were taken from half-gravid females and placed in Carnoy's fixative solution (3 parts of ethanol: 1 part of glacial acetic acid by volume). Ovaries were kept at room temperature overnight before being stored at −20 °C. To obtain chromosomal slides, follicles of ovaries were separated in 50% propionic acid. Then, a cover slip was used to squash the follicles. The quality of slides and the banding pattern of polytene chromosomes were analyzed using an Olympus CX-41 phase contrast microscope (Olympus America Inc., Melville, NY). Slides then were dipped into liquid nitrogen, cover slips were removed, and slides were dehydrated in 50%, 70%, 90%, and 100% ethanol. Slides were air dried and used for further experiments.

Probe Preparation

Three approaches were utilized for the microsatellite probe preparation. First, microsatellites were directly amplified

Table I Primers designed for the microsatellite loci using the *Anopheles stephensi* genome sequence

Locus	Forward primer	Reverse primer	Size of the PCR product (bp)
E12	GCGAGAGCGAGAGAGTGAGT	GGCGTTCAGTTCTGTGTGAA	503
B1	GCATGGGTATGAGCCAAGTT	ATGAGTGTCGTCGTCCGTTT	510
D11	ACCAGGGGTTCACAAATTCA	TGTACACAGGAGATAACGTGCAT	493
C1	CACAGGAGATAACGTGCATTT	ACGCTCACACCACAAAACC	505
E7T	ACGAAGGAGCTGTCCGAGTA	GTCGTGTGGGGATAGTTGCT	584
B2	CAGGAAAAGCGAGTGAAAGG	TGACAGCTGTGGAAGATTCG	531
H2ii	TCGATTCGAGGCATCTTTTC	CCTTCAATGTCCGTCACCTT	564
F10	ATCCCACTACTGCACCCACT	CATCGCATGCTGATTGTTCT	580
A7	CAGTTTTGCGCAGTAGTTGG	TTTCGCCTTTCATTCCTACG	549
A10	CACGCAAGTAGGCTTTGACA	TTGAAATCGCTTCACACGAC	513
G1	CAAGCGATTTTGGGGTAGAA	TACACCACCCACCCATAACC	514
B2N	CGCTCGTAGCTATTACGGATG	CAGGGGAAAATTGCTTTCAA	570

from the genomic DNA using previously designed primers (Verardi et al. 2002). Genomic DNA of *A. stephensi* was prepared using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Science, Germantown, MD). Approximately 80–200 bp long fragments were amplified. Second, 4 microsatellites were ligated and cloned as a cluster of 2 or 3 repeats in the head-to-tail orientation in the plasmid pBluescript SK(+) (Agilent Technologies, Inc., Santa Clara, CA). Amplicons prepared in the first approach were treated with T4 DNA polymerase (SibEnzyme Ltd., Novosibirsk, Russia) for blunting the ends. Resulting fragments were treated with T4 DNA ligase (SibEnzyme Ltd.) at 14 °C for 1–2 h. The mixture was added to the dephosphorylated plasmid digested with restriction enzyme EcoRV (SibEnzyme Ltd.). Ligation was performed overnight. A ligation mixture was used to transform the XL1-Blue *Escherichia coli* strain (Agilent Technologies, Inc.). The blue-and-white screening revealed colonies containing insertions. The PCR screening revealed colonies containing a cluster of several repeats. Plasmids containing such clusters were sequenced. In this approach, 500–850 bp long fragments were amplified from the plasmid DNA using standard T7 and T6 primers (Fermentas, Inc., Glen Burnie, MD) and were labeled for *in situ* hybridization. Third, PCR products were obtained for 12 microsatellites using primers designed with the Primer3 program (Rozen and Skaletsky 2000) based on sequences identified by BLASTN in the genome assembly of *A. stephensi* (accession numbers: HQ328840–HQ328851). The genome assembly for *A. stephensi* was obtained using ×16 coverage of 454 shotgun and pair-end sequences at the Core Laboratory Facility of the Virginia Bioinformatics Institute of Virginia Tech. The size of these fragments was from 493 to 584 bp. PCR conditions were as follows: 94 °C for 5 min; 45 cycles of 94 °C for 45 s and of 50 °C for 45 s; 72 °C for 30 s; and 72 °C for 5 min. DNA was purified using the GE healthcare illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Ltd., Buckinghamshire, UK). Probes were labeled using Cy3-AP3-dUTP or Cy5-AP3-dUTP (GE Healthcare UK Ltd.) fluorophores by a Random Primer DNA Labeling System (Invitrogen Corporation, Carlsbad, CA).

Fluorescence *In Situ* Hybridization and Mapping

Labeled probes were hybridized at 42 °C to *A. stephensi* polytene chromosome slides overnight. Then, slides were washed in 0.2 × Saline Sodium citrate (SSC, 0.03 M sodium chloride, and 0.03 M sodium citrate) at 42 °C and room temperature. Chromosomes were stained using YOYO-1 (Invitrogen Corporation), and slides were mounted in 1,4-Diazabicyclo[2.2.2]octane (DABCO) antifade solution. A Zeiss LSM 510 Laser Scanning Microscope (Carl Zeiss Micro-Imaging, Inc., Thornwood, NY) was used to detect fluorescent signals. Microscopic images were taken with a digital camera, and the locations of signals were determined using a standard cytogenetic photomap of *A. stephensi* (Sharakhova et al. 2006).

Results

In the current study, we used 3 approaches to map the set of microsatellites to the polytene chromosomes from ovarian nurse cells of *A. stephensi*. In the first approach, microsatellites were amplified from genomic DNA using specific primers, which were developed before (Verardi et al. 2002). All microsatellites were successfully amplified from the genomic DNA. However, because of the small size of the products (between 82 and 200 bp), the probes failed to get labeled for successful *in situ* hybridization. In the second approach, the PCR products of 4 microsatellites (*A1*, *A10*, *B1*, and *C1*) were cloned in a plasmid as a cluster of 2 or 3 repeats in the head-to-tail orientation. In this approach, 500–850 bp long fragments were amplified from the plasmid DNA using standard T7 and T6 primers and were labeled for *in situ* hybridization with chromosomes. Labeling and fluorescence *in situ* hybridization (FISH) of these microsatellite clones were successful. However, the probes hybridized either to multiple sites on chromosomes or to heterochromatic regions, probably because of the increased size of repetitive microsatellite motifs. In the third approach, we used recently obtained genomic sequence assembly of *A. stephensi* to design primers for 493–584 bp long PCR products that contain microsatellites of interest (Table 1). We attempted to find sequences homologous to the previously described 16 microsatellite loci (Verardi et al.
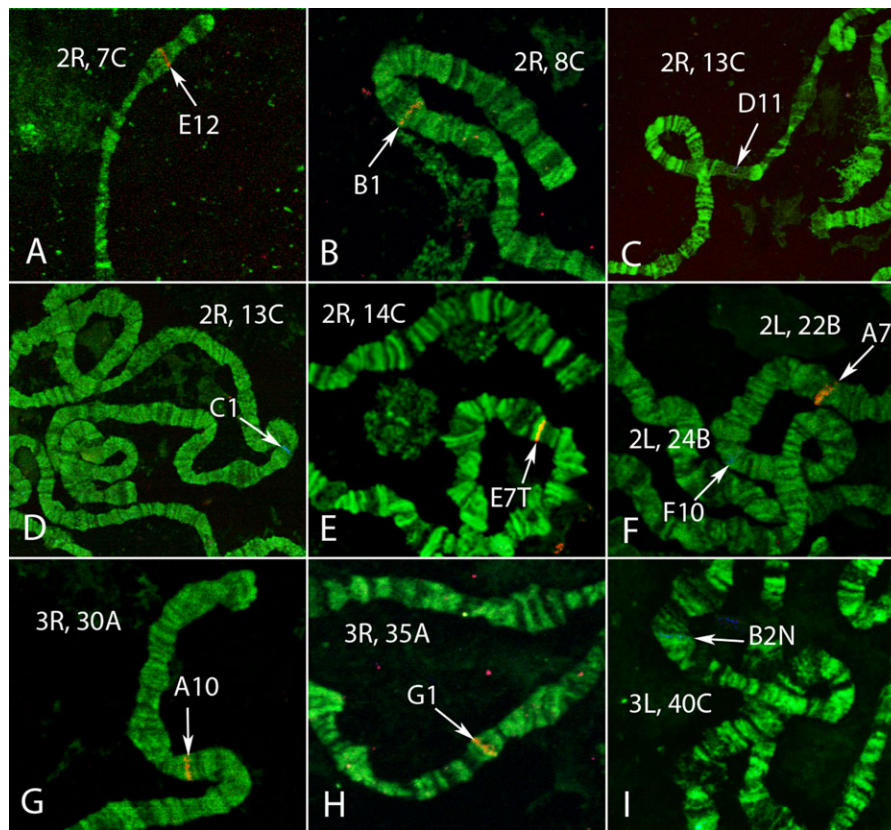
Figure 1. FISH performed on the chromosomes of *Anopheles stephensi*. Chromosomes counterstained with the fluorophore YOYO-1 and hybridized with fluorescently labeled probes Cy5 (blue) and Cy3 (red) are shown.

2002) in the *A. stephensi* genome by BLASTN. The BLASTN search did not yield either positive or unique hits for microsatellites *E7**, *A1*, and *D8T*. Primers were designed but no PCR product was obtained for microsatellite *H1*. Therefore, PCR products were obtained for 12 of 16 microsatellites using primers designed based on the *A. stephensi* genome sequences (accession numbers: HQ328840–HQ328851). We successfully hybridized these

probes to the polytene chromosomes from ovarian nurse cells of *A. stephensi* by FISH (Figure 1, Table 2).

The *A. stephensi* chromosomal complement in ovarian nurse cells consists of 5 chromosomal arms: X, 2R, 3R, 3L. All 12 microsatellites hybridized to unique locations on autosomes; no hybridization to the X chromosome was detected. We have mapped these microsatellites to the polytene chromosomes of *A. stephensi* (Figure 2). Seven of

Table 2 Locations of the *Anopheles stephensi* microsatellite markers on polytene chromosomes

|	Microsatellite locus	Accessions for cloned sequences (with length in bp)	Accessions for assembled sequences (with length in bp)	Location; inside/outside inversions
1.	*E12*	AF418586 (229)	HQ328840 (794)	2R, B7; *outside*
2.	*B1*	AF418596 (150)	HQ328841 (847)	2R, C8; inside
3.	*D11*	AF418581 (82)	HQ328843 (630)	2R, 13C; inside
4.	*C1*	AF418583 (114)	HQ328844 (539)	2R, 13C; inside
5.	*E7T*	AF418593 (144)	HQ328842 (980)	2R, 14C; inside
6.	*B2*	AF418588 (210)	HQ328845 (739)	2R, 16C; inside
7.	*H2ii*	AF418595 (189)	HQ328846 (619)	2R, 17A; *outside*
8.	*F10*	AF418589 (175)	HQ328847 (700)	2L, 24B; inside
9.	*A7*	AF418592 (289)	HQ328848 (985)	2L, 22B; inside
10.	*A10*	AF418591 (186)	HQ328849 (666)	3R, 30A; *outside*
11.	*G1*	AF412812 (154)	HQ328850 (702)	3R, 35A; inside
12.	*B2N*	AF418590 (134)	HQ328851 (814)	3L, 40C; inside
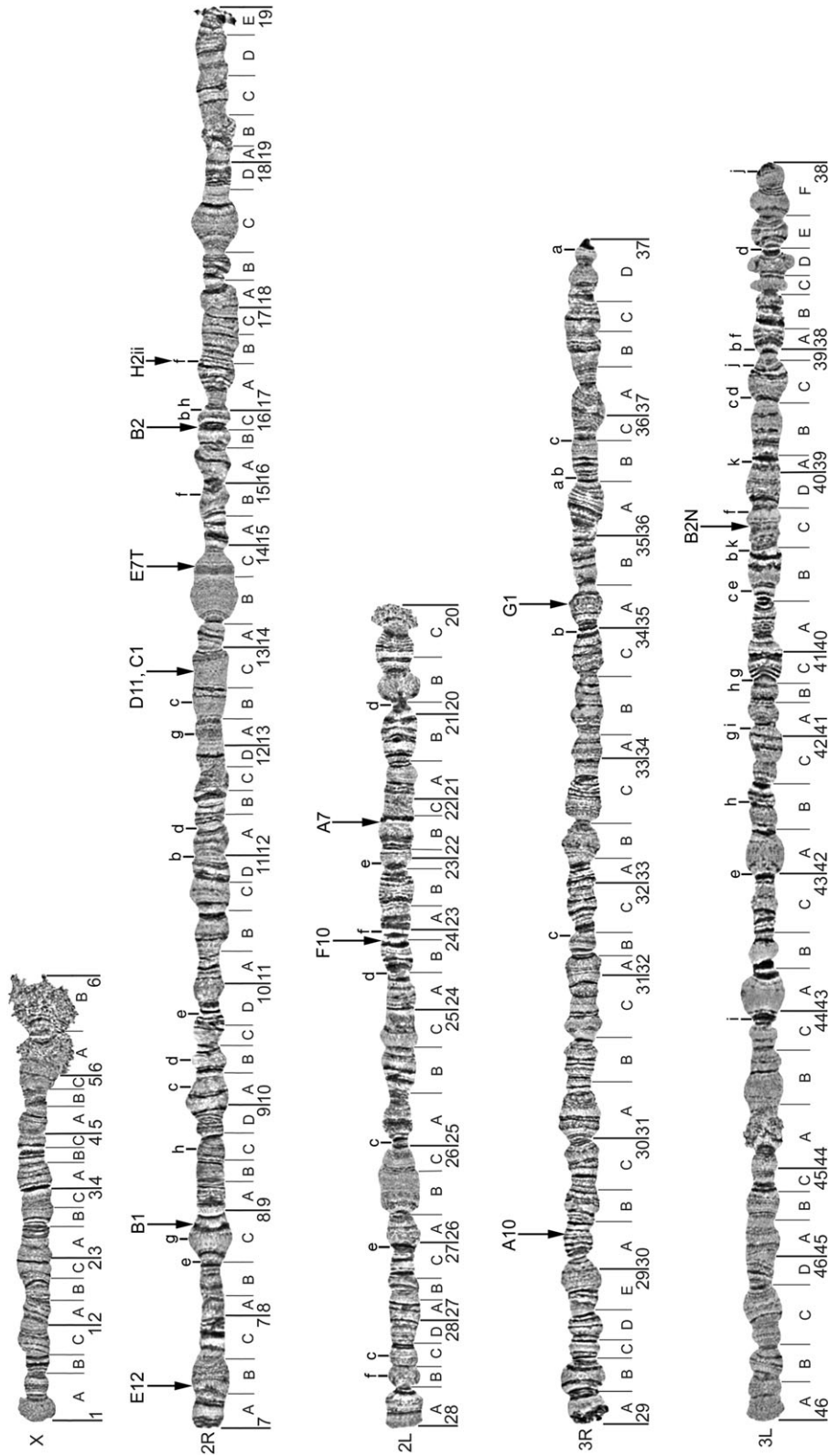
Figure 2. Physical map of 12 microsatellite markers on the *Anopheles stephensi* polytene chromosomes. Positions of inversion breakpoints are shown with lower-case letters.
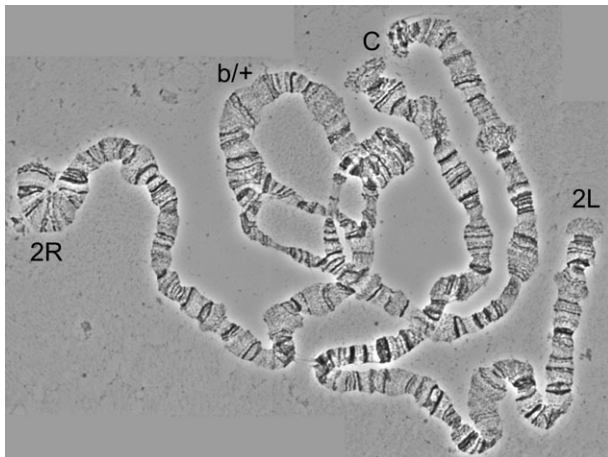
Figure 3. A photomicrograph of the polymorphic inversion *2Rb* in a heterozygote state from the Indian wild-type laboratory colony of *Anopheles stephensi*.

12 microsatellites hybridized to the largest arm 2R; 2 microsatellites localized to each of the 2L and 3R arms, and only 1 microsatellite hybridized to the 3L arm. At least 24 paracentric inversions have been described for *A. stephensi* (Mahmood and Sakai 1984; Gayathri Devi and Shetty 1992). We placed breakpoints of these inversions on the most recently developed polytene chromosome map of *A. stephensi* (Sharakhova et al. 2006) (Figure 2). Ten microsatellites were mapped inside of the previously described polymorphic inversions. Four of them were found inside the large *2Rb* inversion, which is polymorphic in the Indian wild-type laboratory strain of *A. stephensi* (Figure 3). The locations of 2 microsatellites, *D11* and *C1*, were cytogenetically indistinguishable; they hybridized to the same band in region 13C on the 2R arm.

Discussion

Among the 3 approaches to map microsatellite markers to chromosomes, the use of the genome sequence assembly of *A. stephensi* to amplify and hybridize microsatellites by FISH was successful (Figure 1). This success resulted because the genome sequence allowed us to design primers that would amplify 493–584 bp fragments, which are suitable for effective labeling by the random primer method. The developed microsatellite map (Figure 2) can greatly improve the understanding a population genetic structure of *A. stephensi*. Three ecological forms of *A. stephensi*—type, intermediate, and mysorensis, which differ in their habitat preferences and malaria transmission, have been described (Sweet and Rao 1937; Rao et al. 1938; Subbarao et al. 1987). Population analysis using rDNA-ITS2 and mitochondrial DNA loci have demonstrated a low level of gene flow among the 3 variants (Djadid et al. 2006; Oshaghi et al. 2006). Genetic variation at 7 microsatellite loci (*F10*, *H2ii*, *E12*, *B1*, *A7*, *C1*, and *A10*) has been studied in 153

individuals belonging to 3 populations in Pakistan (Ali et al. 2007). The study has found significant deviation from Hardy–Weinberg equilibrium due to heterozygote deficit in 2 of the 21 tests (loci *E12* and *B1*). Exact tests of linkage disequilibrium showed no significant departure from equilibrium between these or any other locus pairs in any population after Bonferroni correction (Ali et al. 2007). Our study mapped both microsatellite markers, *E12* and *B1*, to the region near the telomere on the 2R arm (Figure 2) suggesting the presence of genes under selection in this chromosomal region.

Another population genetic study utilized 8 microsatellite markers (*F10*, *H2ii*, *E12*, *B1*, *G11*, *E7T*, *G1*, and *A10*) to investigate the genetic isolation among the 3 morpho-ecological variants of *A. stephensi* in India (Vipin and Gakhar 2010). These microsatellites had shown diverse patterns of genetic variation. Locus *E7T* was found to be highly differentiated between mysorensis and type forms (F_{ST} = 0.890), between intermediate and type forms (F_{ST} = 0.629), and between mysorensis and intermediate forms (F_{ST} = 0.556). Moreover all 3 ecological forms had different nonoverlapping allele sizes for *E7T* locus (Vipin and Gakhar 2010). These data suggest high genetic differentiation among the forms at the locus *E7T*. We mapped the microsatellite *E7T* inside the *2Rb* inversion (Figures 2 and 3), which is the most widespread inversion in natural populations. It has been shown that the type and mysorensis forms significantly differ in frequencies of inversion *2Rb* (Coluzzi et al. 1973b). Therefore, the presence of this microsatellite inside the *2Rb* inversion confirms a possible role of the inversion in differentiating *A. stephensi* populations. It is possible that the inversion *2Rb* acts as a barrier to gene flow among the forms. If this is the case, then *E7T* should be in linkage disequilibrium with the inversion. Other microsatellites located inside the *2Rb* inversion (*B2*, *D11*, and *C1*) have not been included in the population genetics study (Vipin and Gakhar 2010). Significant departure from Hardy–Weinberg equilibrium due to heterozygote deficits was found in intermediate and mysorensis forms across all loci, except for *G1* and *A10* in all 3 variants and *F10* in intermediate (Vipin and Gakhar 2010). Both *G1* and *A10* are located on 3R, and *F10* is located on 2L. Microsatellite *G11* has not been mapped in our study. Interestingly, the other 4 microsatellites with significant departure from Hardy–Weinberg equilibrium (*H2ii*, *E12*, *B1*, and *E7T*) were mapped on 2R arm in our study (Figure 2). We have mapped microsatellites *H2ii*, *B1*, and *E12* on the 2R arm outside of the *2Rb* inversion; *E12* and *B1* are located close to the telomere. Linkage disequilibrium was found between *H2ii* and *B1*, *E12* and *B1* in mysorensis, and *E12* and *B1* in intermediate (Vipin and Gakhar 2010). However, the Bonferroni correction for the linkage disequilibrium has not been performed.

The genome sequence assembly of *A. stephensi* can also be used to discover and develop new microsatellite markers. Future studies of these microsatellites, together with inversion polymorphisms in the natural populations, will provide a better understanding of the population structure

of *A. stephensi* and the effect of inversions on the behavior of microsatellites.

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References

Abraham EG, Islam S, Srinivasan P, Ghosh AK, Valenzuela JG, Ribeiro JM, Kafatos FC, Dimopoulos G, Jacobs-Lorena M. 2004. Analysis of the *Plasmodium* and *Anopheles* transcriptional repertoire during ookinete development and midgut invasion. J Biol Chem. 279:5573–5580.

Alam MT, Bora H, Das MK, Sharma YD. 2008. The type and mysorensis forms of the *Anopheles stephensi* (Diptera: Culicidae) in India exhibit identical ribosomal DNA ITS2 and domain-3 sequences. Parasitol Res. 103:75–80.

Ali N, Hume JC, Dadzie SK, Donnelly MJ. 2007. Molecular genetic studies of *Anopheles stephensi* in Pakistan. Med Vet Entomol. 21:265–269.

Ayala D, Fontaine MC, Cohuet A, Fontenille D, Vitalis R, Simard F. 2011. Chromosomal inversions, natural selection and adaptation in the malaria vector *Anopheles funestus*. Mol Biol Evol. 28:745–758.

Balanya J, Oller JM, Huey RB, Gilchrist GW, Serra L. 2006. Global genetic change tracks global climate warming in *Drosophila subobscura*. Science. 313:1773–1775.

Bass C, Nikou D, Blagborough AM, Vontas J, Sinden RE, Williamson MS, Field LM. 2008. PCR-based detection of *Plasmodium* in *Anopheles* mosquitoes: a comparison of a new high-throughput assay with existing methods. Malar J. 7:177.

Baton LA, Ranford-Cartwright LC. 2007. Morphological evidence for proliferative regeneration of the *Anopheles stephensi* midgut epithelium following *Plasmodium falciparum* ookinete invasion. J Invertebr Pathol. 96:244–254.

Bruford M, Wayne R. 1993. Microsatellites and their application to population genetic studies. Curr Opin Gene Dev. 3:939–943.

Cohuet A, Dia I, Simard F, Raymond M, Rousset F, Antonio-Nkondjio C, Awono-Ambene P, Wondji C, Fontenille D. 2005. Gene flow between chromosomal forms of the malaria vector *Anopheles funestus* in Cameroon, Central Africa, and its relevance in malaria fighting. Genetics. 169:301.

Coluzzi M, Di Deco M, Cancrini G. 1973a. Chromosomal inversions in *Anopheles stephensi*. Parassitologia. 15:129–136.

Coluzzi M, Di Deco M, Cancrini G. 1973b. Further observations on the egg length in *Anopheles stephensi* in relation to chromosomal polymorphism. Parassitologia. 15:213–215.

Djadid N, Gholizadeh S, Aghajari M, Zehi A, Raeisi A, Zakeri S. 2006. Genetic analysis of rDNA-ITS2 and RAPD loci in field populations of the malaria vector, *Anopheles stephensi* (Diptera: Culicidae): implications for the control program in Iran. Acta Trop. 97:65–74.

Gayathri Devi K, Shetty J. 1992. Chromosomal inversions in *Anopheles stephensi* Liston—a malaria mosquito. J Cytol Genet. 27:153–161.

Hati AK. 1997. Urban malaria vector biology. Indian J Med Res. 106:149–163.

Hoffmann AA, Rieseberg L. 2008. Revisiting the impact of inversions in evolution: from population genetic markers to drivers of adaptive shifts and speciation. Annu Rev Ecol Evol Syst. 39:21–42.

Hoffmann AA, Sgro CM, Weeks AR. 2004. Chromosomal inversion polymorphisms and adaptation. Trends Ecol Evol. 19:482–488.

Kennington WJ, Gockel J, Partridge L. 2003. Testing for asymmetrical gene flow in a *Drosophila melanogaster* body-size cline. Genetics. 165:667–673.

Lanzaro GC, Toure YT, Carnahan J, Zheng L, Dolo G, Traore S, Petrarca V, Vernick KD, Taylor CE. 1998. Complexities in the genetic structure of *Anopheles gambiae* populations in west Africa as revealed by microsatellite DNA analysis. Proc Natl Acad Sci U S A. 95:14260–14265.

Lehmann T, Hawley W, Kamau L, Fontenille D, Simard F, Collins F. 1996. Genetic differentiation of *Anopheles gambiae* populations from East and West Africa: comparison of microsatellite and allozyme loci. Heredity. 77:192–200.

Mahmood F, Sakai RK. 1984. Inversion polymorphisms in natural populations of *Anopheles stephensi*. Can J Genet Cytol. 26:538–546.

Manouchehri A, Javadian E, Eshighy N, Motabar M. 1976. Ecology of *Anopheles stephensi* Liston in southern Iran. Trop Geogr Med. 28:228–232.

Michel AP, Guelbeogo WM, Grushko O, Schemerhorn BJ, Kern M, Willard MB, Sagnon N, Costantini C, Besansky NJ. 2005. Molecular differentiation between chromosomally defined incipient species of *Anopheles funestus*. Insect Mol Biol. 14:375–387.

Nagpal BN, Srivastava A, Kalra NL, Subbarao SK. 2003. Spiracular indices in *Anopheles stephensi*: a taxonomic tool to identify ecological variants. J Med Entomol. 40:747–749.

Navarro A, Barton NH. 2003. Accumulating postzygotic isolation genes in parapatry: a new twist on chromosomal speciation. Evolution. 57:447–459.

Ndo C, Antonio-Nkondjio C, Cohuet A, Ayala D, Kengne P, Morlais I, Awono-Ambene PH, Couret D, Ngassam P, Fontenille D, et al. 2010. Population genetic structure of the malaria vector *Anopheles nili* in sub-Saharan Africa. Malar J. 9:161.

Onyabe DY, Conn JE. 2001. Genetic differentiation of the malaria vector *Anopheles gambiae* across Nigeria suggests that selection limits gene flow. Heredity. 87:647–658.

Oshaghi M, Yaaghoobi F, Abaie M. 2006. Pattern of mitochondrial DNA variation between and within *Anopheles stephensi* (Diptera: Culicidae) biological forms suggests extensive gene flow. Acta Trop. 99:226–233.

Pant CP, Rishikesh N, Bang YH, Smith A. 1981. Progress in malaria vector control. Bull World Health Organ. 59:325–333.

Rao BA, Sweet WC, Subbarao AM. 1938. Ova measurements of *A. stephensi* type and *A. stephensi* var. *mysorensis*. J Malar Inst India. 1:261–266.

Rowland M, Mohammed N, Rehman H, Hewitt S, Mendis C, Ahmad M, Kamal M, Wirtz R. 2002. Anopheline vectors and malaria transmission in eastern Afghanistan. Trans R Soc Trop Med Hyg. 96:620–626.

Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 132:365–386.

Sharakhova MV, Antonio-Nkondjio C, Xia A, Ndo C, Awono-Ambene P, Simard F, Sharakhov IV. 2011. Cytogenetic map for *Anopheles nili*: Application for population genetics and comparative physical mapping. Infect Genet Evol. 11:746–754.

Sharakhova MV, Xia A, McAlister SI, Sharakhov IV. 2006. A standard cytogenetic photomap for the mosquito *Anopheles stephensi* (Diptera: Culicidae): application for physical mapping. J Med Entomol. 43: 861–866.

Subbarao S, Vasantha K, Adak T, Sharma V, Curtis C. 1987. Egg-float ridge number in *Anopheles stephensi*: ecological variation and genetic analysis. Med Vet Entomol. 1:265–271.

Suguna SG. 1981. Inversion(2)R1 in *Anopheles stephensi*, its distribution and relation to egg size. Indian J Med Res. 73(Suppl):124–128.

Sweet W, Rao B. 1937. Races of *Anopheles stephensi* Liston, 1901. Ind Med Gaz. 72:665–674.

Tripet F, Dolo G, Lanzaro GC. 2005. Multilevel analyses of genetic differentiation in *Anopheles gambiae s.s.* reveal patterns of gene flow important for malaria-fighting mosquito projects. Genetics. 169:313–324.

Vatandoost H, Oshaghi MA, Abaie MR, Shahi M, Yaaghoobi F, Baghaii M, Hanafi-Bojd AA, Zamani G, Townson H. 2006. Bionomics of *Anopheles stephensi* Liston in the malarious area of Hormozgan province, southern Iran, 2002. Acta Trop. 97:196–203.

Verardi A, Donnelly M, Rowland M, Townson H. 2002. Isolation and characterization of microsatellite loci in the mosquito *Anopheles stephensi* Liston (Diptera: Culicidae). Mol Ecol Notes. 2:488–490.

Vipin M, Gakhar S. 2010. Genetic differentiation between three ecological variants ('type','mysorensis' and 'intermediate') of malaria vector *Anopheles stephensi* (Diptera: Culicidae). Insect Sci. 17:335–343.