A Microsatellite Map of the African Human Malaria Vector *Anopheles funestus*

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Abstract

Microsatellite markers and chromosomal inversion polymorphisms are useful genetic markers for determining population structure in Anopheline mosquitoes. In *Anopheles funestus* ($2N = 6$), only chromosome arms 2R, 3R, and 3L are known to carry polymorphic inversions. The physical location of microsatellite markers with respect to polymorphic inversions is potentially important information for interpreting population genetic structure, yet none of the available marker sets have been physically mapped in this species. Accordingly, we mapped 32 polymorphic *A. funestus* microsatellite markers to the polytene chromosomes using fluorescent in situ hybridization (FISH) and identified 16 markers outside of known polymorphic inversions. Here we provide an integrated polytene chromosome map for *A. funestus* that includes the breakpoints of all known polymorphic inversions as well as the physical locations of microsatellite loci developed to date. Based on this map, we suggest a standard set of 16 polymorphic microsatellite markers that are distributed evenly across the chromosome complement, occur predominantly outside of inversions, and amplify reliably. Adoption of this set by researchers working in different regions of Africa will facilitate metapopulation analyses of this primary malaria vector.

Anopheles gambiae, *Anopheles arabiensis*, and *Anopheles funestus* are the principal vectors of malaria in Africa. All are widely distributed from south of the Sahara Desert to northern South Africa (Gillies and De Meillon 1968). Successful vector control strategies for Africa must take into account the population genetic structure of each of these species. To date, *A. funestus* has received scant attention; the lack of molecular markers is one reason. Although understandable, the paucity of focused studies on *A. funestus* is inconsistent with its major role in malaria transmission during the dry season, when *A. gambiae* and *A. arabiensis* are less active (Fontenille et al. 1997).

Commonly used markers for inferring anopheline population structure include chromosomal inversions and microsatellites. Polytene chromosome analysis has revealed incipient speciation within *A. gambiae s.s.* and *A. funestus* (della Torre et al. 2002). Although a direct role for chromosomal inversions in the speciation process has not been established, abundant evidence suggests that particular chromosomal rearrangements are adaptive in different environments. In contrast, microsatellite markers are commonly assumed to evolve neutrally. The high polymorphism of microsatellite markers has facilitated studies at the population and infraspecific levels in these species (Donnelly et al. 2002; Kamau et al. 1999; Lehmann et al. 2003), complementing cytogenetic studies. Studies of *A. gambiae* suggest that gene flow between populations may occur at different levels across the genome (Krzywinski and Besansky 2003; Onyabe and Conn 2001). In particular, reduced recombination and
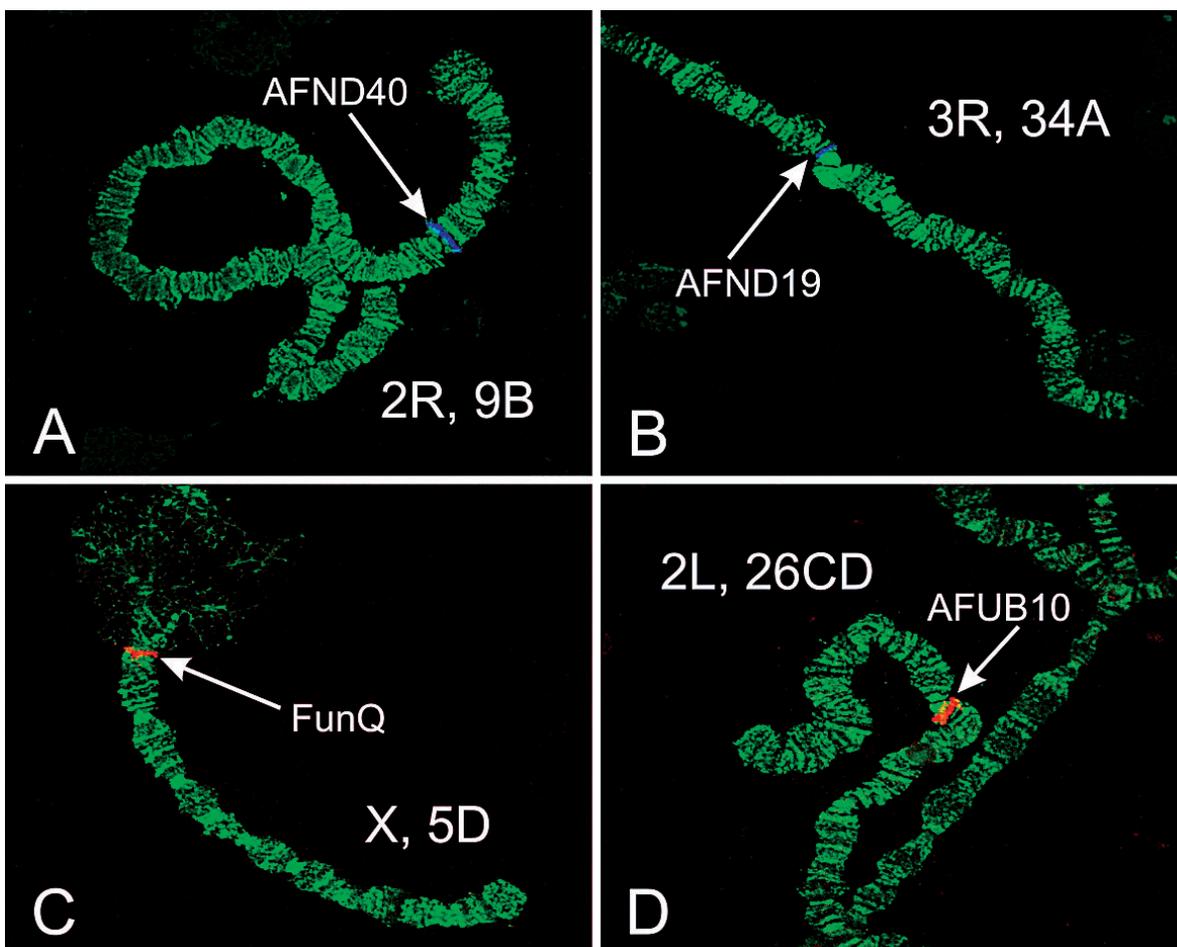
Figure 1. FISH performed on the chromosomes of *A. funestus*. Chromosomes counterstained with the fluorophore YOYO-1 and hybridized with fluorescently labeled probes Cy5 (blue) and Cy3 (red) are shown.

selection can influence loci within 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). Therefore analysis of population genetic structure should be informed by the location of genetic markers with respect to polymorphic inversions. Comparison of patterns of variation among loci inside and outside of inversions may provide insight about levels of gene flow between populations as well as the role of chromosomal inversions in these populations.

Within the last few years, polymorphic microsatellite markers for *A. funestus* were developed at three institutions: the University of Notre Dame (Schemerhorn et al. 2003; Sinkins et al. 2000), State University of New York at Buffalo (Sharakhov et al. 2001a), and ISEM-Institut de Recherche pour le Développement (Cohuet et al. 2002). Application of these loci at various locales throughout Africa has revealed high levels of polymorphism within *A. funestus* samples and significant differentiation between locales more than 150 km apart (Braginets et al. 2003). Unfortunately none of the loci developed have been physically mapped. In this study we mapped 32 polymorphic *A. funestus* microsatellite markers to

the polytene chromosomes. Based on this map, we suggest 16 as a reference set whose employment by different research groups will facilitate comparison and integration of population genetic studies conducted in different parts of Africa.

Materials and Methods

Physical Mapping by Fluorescent in situ Hybridization (FISH)

To obtain polytene chromosome preparations, indoor spray catches in villages near Ouagadougou, Burkina Faso, were carried out in September 1999 and December 2001. Adults were sorted in the field under a dissecting microscope and ovaries at the appropriate stage were dissected into fresh Carnoy's solution (ethanol:glacial acetic acid, 3:1). These were stored at $-20°C$ until processing. Ovaries were gently pressed with a coverslip in 50% propionic acid, dipped in liquid nitrogen, and sequentially dehydrated in 50%, 70%, 95%, and 100% ethanol. The quality of banding pattern of polytene chromosomes was examined under an Olympus BX60 phase-contrast microscope. Probes were prepared from
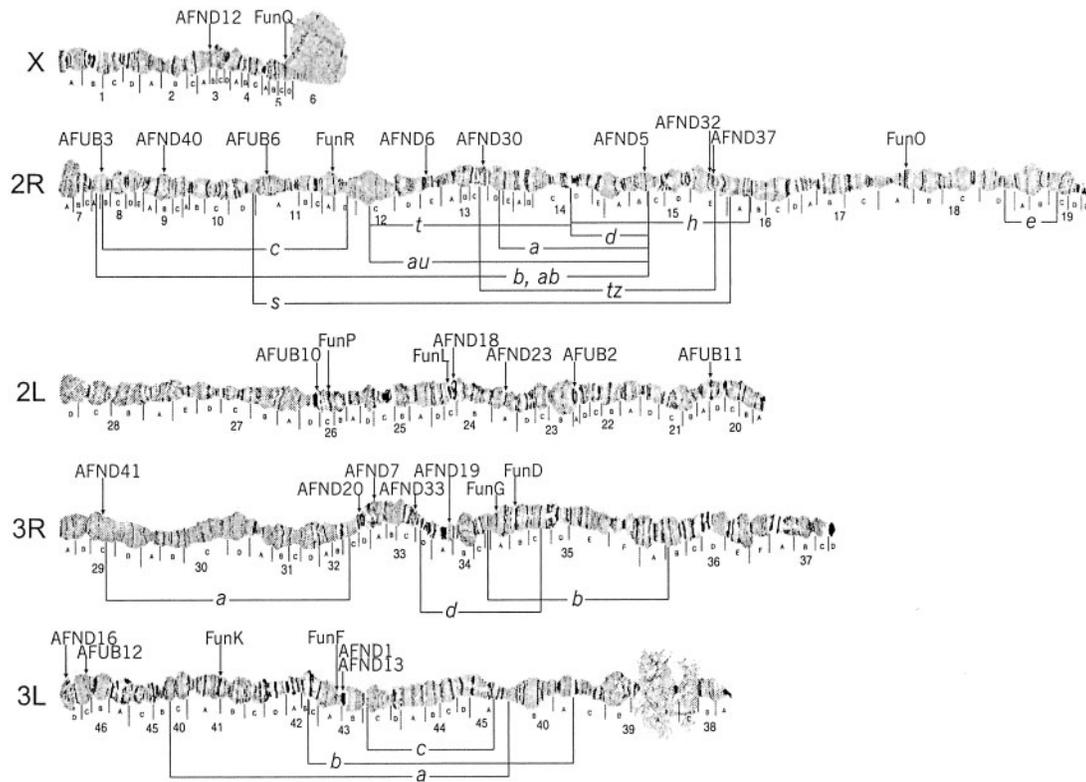
Figure 2. Locations of microsatellite loci (arrows) and inversions (brackets) with respect to the photomap of *A. funestus* polytene chromosomes (Sharakhov et al. 2002). Inversions are shown in their standard and presumed ancestral orientation (Green and Hunt 1980). Positions of microsatellites that hybridized to multiple locations (*AFND2*, *AFND3*, *AFND8*, *AFND31*, *AFUB5*, *AFUB15*, *FUN H*, *FUN J*, and *FUN N*) are not shown.

approximately 100 ng of either microsatellite-containing plasmid or polymerase chain reaction (PCR) product produced using 1 pmol each of the forward and reverse universal M13 primers. Cycling conditions were 95°C for 5 min; 25 cycles of 94°C for 30 s, 70°C for 2 min; and 68°C for 3 min. The DNA was labeled with Cy3-AP3-dUTP or Cy5-AP3-dUTP (Amersham) using the GIBCO BRL BioPrime DNA labeling system (Life Technologies) with dNTPs from the nick translation kit (Amersham), in half-volume reactions. The in situ hybridization was performed with the GIBCO BRL in situ hybridization and detection system, following the manufacturer's recommended protocol. After hybridization, chromosomes were washed in 0.2× SSC and counterstained with YOYO-1 or propidium iodide (Sigma) and mounted in DABCO antifade solution (Sigma). Fluorescent signals were detected using a Bio-Rad MRC 1024 scanning confocal (2 channel/LaserSharp 3.2 program/networked) system.

Results and Discussion

Integrated Map of Microsatellite Markers and Inversion Breakpoints

Using FISH, we mapped *A. funestus* microsatellite clones on the five arms of the polytene chromosome complement

(Figure 1). [In Figure 1 and hereafter, we adopt the naming convention of AFND# (*A. funestus*, Notre Dame, locus) to distinguish all microsatellite loci developed at the University of Notre Dame. Thus the set originally designated *AF1-22* in Sinkins et al. (2000) is now *AFND1-22*.] Incorporation of different fluorescent labels allowed us to probe simultaneously with two different microsatellite clones. Of 40 clones used as probes, 31 mapped to single chromosomal locations on the *A. funestus* cytogenetic map and 9 hybridized to multiple locations. Hybridization to multiple locations is not uncommon for microsatellite-containing clones (Zheng et al. 1996). In addition, we found that sequence flanking *AFND16* was identical to corresponding sequence from cDNA 04_D01, which mapped to a unique location (Sharakhov et al. 2002). Taken together, a total of 32 microsatellite markers could be assigned to single chromosomal locations (Figure 2). Also indicated in Figure 2 are the breakpoints of all 17 polymorphic inversions found to date in *A. funestus*. Eleven were found on arm 2R (*a, b, c, d, e,* and *h* [Green and Hunt 1980]; *s, t, ab,* and *au* [Boccolini et al. 1998]; *tz* [Lochouarn et al. 1998]), two on arm 3R (*a* and *b* [Green and Hunt 1980]), and two on arm 3L (*a* and *b* [Green and Hunt 1980]). Our ongoing cytogenetic studies of *A. funestus* in Burkina Faso revealed two new inversions, 3L*c* and 3R*d*, reported here for the first time. Precise positions of the breakpoints for
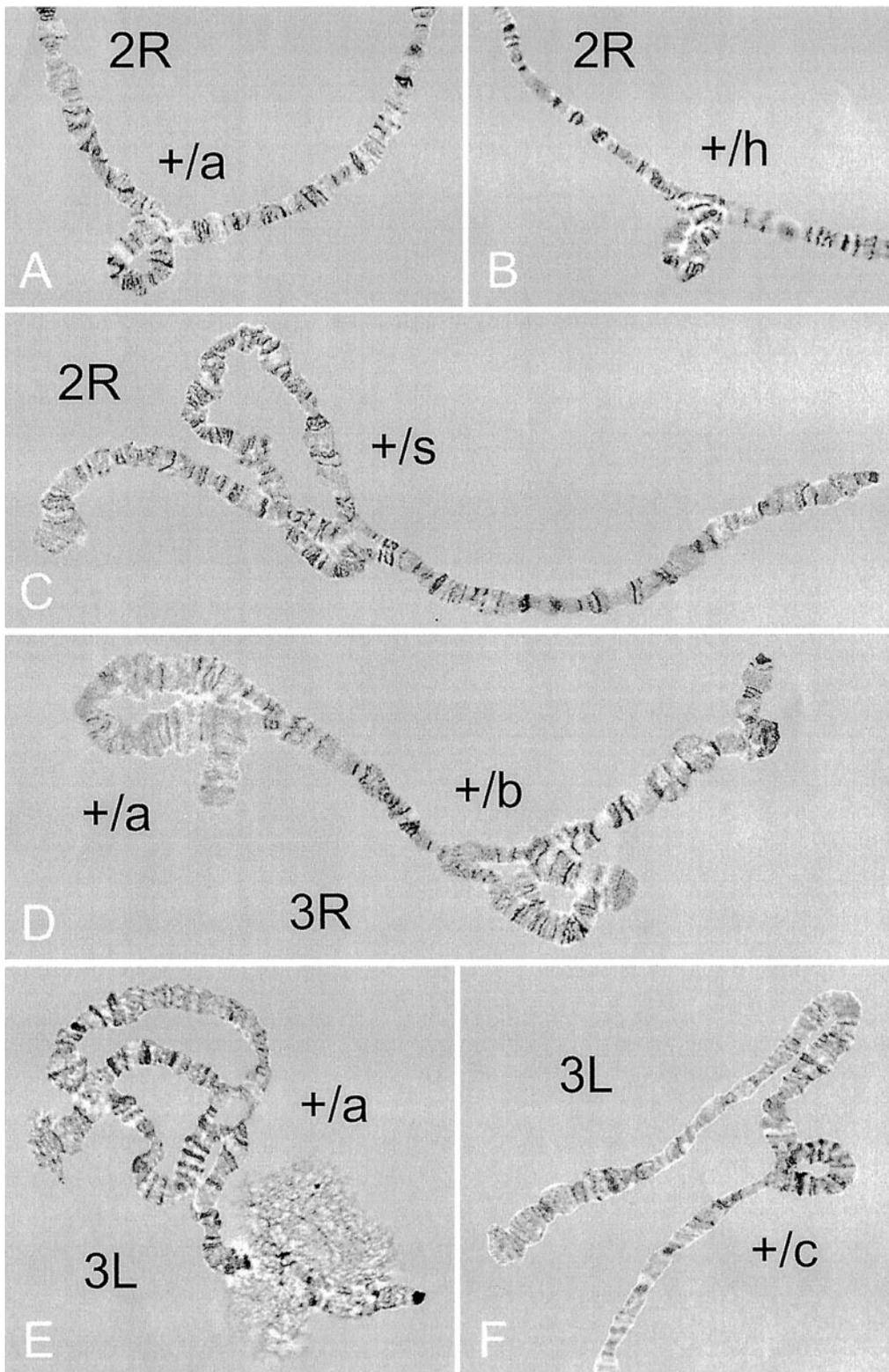
Figure 3. Photomicrographs of polymorphic inversions of *A. funestus* collected from Burkina Faso (panels A, C, D, E, F) and Kenya (panel B). Note that inversions in panels A, D, and E have been recorded in both countries; inversion 2R*h* (panel B) has not been observed in Burkina Faso, and those in panels C and F were not observed in Kenya.

inversion 2R*tz* (Figure 2) were communicated to us by I. Dia. The breakpoints for 2R*h* (Figure 2) are based on observations made from inversion homozygotes and heterozygotes from Cameroon. Note that these breakpoints may be coincident with those described previously for 2R*h* by Sharakhov et al. (2001b) from Kenya, but the rarity of this inversion in Kenya and the lack of available inversion homozygotes precludes a definitive conclusion at this time. Figure 3 shows photomicrographs of some polymorphic inversions that occur in Burkina Faso and Kenya, including inversion 2R*h*.

Reference Set of Microsatellite Markers

To facilitate population genetic studies on *A. funestus* we suggest a reference set of 16 microsatellite markers (Table 1). The composition of this set was influenced by balancing factors including reliable amplification, easy scoring, even coverage of all chromosome arms, and location preferably outside of known polymorphic inversions. We included *AFUB3*, *AFND40*, *AFND19*, *AFND1*, and *FUN F*, despite their location inside of inversions, for the following reasons. First, inversions 2R*h*, 2R*ab*, 2R*c*, 3R*d*, and 3L*b* are relatively rare and do not occur widely in Africa (Boccolini et al. 1998; Green and Hunt 1980). Second, although inversion 3L*a* has a broad geographic distribution, *AFND1* and *FUN F* are in the middle of this relatively long inversion. Experimental data from *Drosophila* suggest that reduced levels of variation associated with inversions are confined to the vicinity of inversion breakpoints rather than to the middle of long inversions, where double crossovers and gene conversion can take place (Depaulis et al. 1999).

Application to the Study of *A. funestus* Chromosomal Forms

In parts of West Africa there is cytogenetic evidence for temporally and microspatially overlapping, but nonpanmictic populations of *A. funestus* (Costantini et al. 1999; Guelbeogo et al., unpublished). Provisionally named "Folonzo" and "Kiribina," these are defined according to the joint distribution of inversions on 2R (*a*, *s*) and 3R (*a*, *b*) (see Costantini et al. [1999] for the algorithm). Of the 15 microsatellites that mapped inside or near inversions, several were associated with these key inversions (Figure 2). Notably, *AFND5* is located near the proximal breakpoint of 2R*a* and two additional loci, *FUN D* and *FUN G*, are positioned within 3R*b* near the distal breakpoint. *AFUB6*, *AFND32*, and *AFND37* are located near the breakpoints of inversion 2R*s*. Therefore the possible correspondence between molecular and cytogenetic differentiation between the two forms can be tested using these six markers.

Conclusion

A significant problem encountered in vector population genetic studies is that various investigators use different markers or different methods which makes data comparison difficult or impossible. Therefore it is extremely important to standardize methods and sets of markers (Krzywinski and

Table 1. Chromosomal locations of the reference set of *A. funestus* microsatellite loci

	Locus[a]	Location (arm, subdivision, inside/outside inversions)
1.	*AFND12*	X, 3B; outside
2.	*FUN Q*	X, 5D; outside
3.	*AFUB3*	2R, 8B; inside of 2R*b*, *ab*
4.	*AFND40*	2R, 9B; inside of 2R*b*, *c*, *ab*
5.	*FUN O*	2R, 18A; outside
6.	*AFUB11*	2L, 20D; outside
7.	*AFND23*	2L, 24A; outside
8.	*FUN L*	2L, 24C; outside
9.	*AFUB10*	2L, 26CD; outside
10.	*AFND41*	3R, 29C; outside
11.	*AFND20*	3R, 32D; outside
12.	*AFND7*	3R, 33A; outside
13.	*AFND19*	3R, 34A; inside of 3R*d*
14.	*AFND1*	3L, 43B; inside of 3L*a*, *b*
15.	*FUN F*	3L, 43A; inside of 3L*a*, *b*
16.	*AFUB12*	3L, 46C; outside

[a] For primer sequences, molecular size, and polymorphism statistics of *AFND1–AFND41* see Sinkins et al. (2000) and Schemerhorn et al. (2003); for *AFUB2–AFUB15* see Sharakhov et al. (2001a); for *FUN D–FUN R* see Cohuet et al. (2002).

Besansky 2003; Wang et al. 1999). In this study we determined the locations of microsatellite markers with respect to the chromosomal banding pattern and the position of all known chromosomal inversions and we suggest a standard set of microsatellite loci for future applications. Using common molecular markers by various investigators in different regions will lend much-needed explanatory power to assessments of *A. funestus* population structure across Africa.

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