An Integrated Genetic and Physical Map for the Malaria Vector *Anopheles funestus*

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ABSTRACT

We have constructed a genetic map of the major African malaria vector, *Anopheles funestus*, using genetic markers segregating in F₂ progeny from crosses between two strains colonized from different field sites. Genotyping was performed on 174 progeny from three families using 33 microsatellite markers, a single RFLP, and 15 single nucleotide polymorphism (SNP) loci. Four linkage groups were resolved and these were anchored to chromosomes X and 2 and chromosomal arms 3R and 3L by comparison with a physical map of this species. Five markers were linked to the X chromosome, 16 markers to chromosome 2, and 10 and 11 markers to chromosomal arms 3R and 3L, respectively. This significantly increases the number of chromosomally defined genetic markers for this species and will facilitate the identification of genes controlling epidemiologically important traits such as resistance to insecticides or vector competence.

THE mosquito *Anopheles funestus* is a major vector of malaria throughout much of sub-Saharan Africa, but because it is a relatively intractable species to work with, it has only recently started to receive the scientific attention such an important public health pest deserves. Knowledge of the population structure of this species is needed to improve malaria vector control strategies in Africa. Resistance to insecticides is already widespread in *A. funestus* in southern Mozambique (BROOKE *et al.* 2001) and, unless barriers to reproduction occur, there are fears that this resistance front could spread very rapidly, possibly affecting the efficacy of the many pyrethroid-based malaria control programs. Several studies have shown discrete populations of *A. funestus* with restricted gene flow between them, but as yet it is not clear whether this is simply a product of geographic distance and limited dispersal of *A. funestus* or whether it is indicative of incipient speciation (BRAGINETS *et al.* 2003; TEMU *et al.* 2004). Some evidence, based on chromosomal inversion frequencies, suggests the presence of reproductively isolated sympatric populations of *A. funestus* in West Africa (COSTANTINI *et al.* 1999; DIA *et al.* 2000). However, studies using microsatellite markers have so far given conflicting results. COHUET

et al. (2004) found no evidence for reproductive isolation in *A. funestus* in West Africa but recent results from MICHEL *et al.* (2005) suggest that the Kiribina and Folonzo chromosomal forms are indeed emerging species of *A. funestus*.

The relative paucity of studies on *A. funestus* can partially be attributed to the lack of genetic tools for this species. In contrast to *A. gambiae*, another major malaria vector in Africa, there is no large-scale EST or genomic sequence database for *A. funestus*, and relatively few genetic markers have been identified (SINKINS *et al.* 2000; COHUET *et al.* 2002; GARROS *et al.* 2004). In addition, difficulties in colonizing *A. funestus* have impeded research in this species. Indeed, as far as we are aware, stable laboratory colonies of *A. funestus* to date have been maintained in only one laboratory [National Institute for Communicable Diseases (NICD), Johannesburg, South Africa]. NICD has colonized two separate strains of *A. funestus*, FUMOZ and FANG, derived from field collections from Mozambique and Angola (HUNT *et al.* 2005), respectively, and this achievement has greatly facilitated genetic analysis of this species. We have mated these two strains and reared multiple isofemale lines. The F₂ progeny from these crosses have been genotyped for a wide range of genetic markers, some of which have been described previously and others of which are described for the first time in this report, and the data have been used to create the first genetic map for this species. By

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including a subset of markers that have been physically mapped to the *A. funestus* chromosomes, we have anchored each of the linkage groups to chromosomal arms, resulting in a low-resolution genetic and physical map. The utility of this map for future studies on *A. funestus* is discussed.

MATERIALS AND METHODS

Mosquito strains: The *A. funestus* FUM0Z strain was colonized from southern Mozambique in 2000 (HUNT *et al.* 2005). The original parental strain was heterozygous for resistance to permethrin. A highly resistant strain, FUM0Z-R, was derived from the parental strain by selection using standard World Health Organization procedures (WORLD HEALTH ORGANIZATION 2000). This strain now exhibits almost 100% survivorship at 1.5% permethrin exposure (B. D. BROOKE, personal communication). The FANG strain was colonized from Calueque in southern Angola in 2002. This strain shows complete susceptibility to all pyrethroids.

Genetic crosses: Reciprocal crosses using virgin FANG and FUM0Z-R females with the alternative males were set up. After two blood feeds, the females were separated and left to oviposit. Only the FANG $\times$ FUM0Z-R cross produced sufficient females that laid eggs, with 18 egg batches obtained. The F₁ progeny from each of these families were intercrossed and the females were blood fed twice. The F₂ generation progeny were reared to adults. The original female parent (F₀), six randomly selected female F₁ progeny from each family, and all of the F₂ progeny were individually stored for later DNA extraction.

Genotyping: The three largest F₂ families (families 4, 6, and 11) were selected for genotyping. DNA extractions were performed using the LIVAK method as described previously (COLLINS *et al.* 1987). Initially we used published primer sets (SINKINS *et al.* 2000; COHUET *et al.* 2002; SHARAKHOV *et al.* 2002; SOREMEKUN *et al.* 2004) to individually amplify 72 microsatellite markers from the parental and F₁ *A. funestus* genomic DNA. A tailed primer system, described by OETTING *et al.* (1998), was used to label the PCR products with a fluorescent dye to reduce the genotyping costs. PCR products were first run on 1.5% agarose gels and the products of successful PCR reactions were sized using the Beckman CEQ8000 fragment analysis software. PCR primers that failed to amplify a product of the expected size were redesigned using Primer3 software (ROZEN and SKALETSKY 2000). For informative primer pairs, the forward primer was resynthesized with a 5' fluorescent label. The microsatellite markers used in the study are listed in Table 1.

A restriction fragment length polymorphism (RFLP) marker was identified while sequencing alleles of the cytochrome P450 gene, CYP4G21. All three families were polymorphic for a *Bsr*BI restriction site within the intron of this gene. PCR primers (5' TCTTTGGCGATTCCAAGC and 5' GAACGTTTCCGGATGTGG) were designed to amplify 239 bp flanking the *Bsr*BI site. The PCR products were incubated with *Bsr*BI at 37° and the products resolved on a 2% agarose gel.

Identification of SNP loci: Single nucleotide polymorphisms (SNPs) were identified within cytochrome P450 genes or within DNA fragments that had been physically mapped to *A. funestus* polytene chromosomes (SHARAKHOV *et al.* 2002) (see Table 2 for more details). The sequences of the physically mapped cDNAs were retrieved from GenBank. Potential orthologs were identified in *A. gambiae* and the location of introns was noted. Primers were designed to flank these putative intron sites to maximize the chance of SNP identifica-

tion. Genomic DNA from seven individuals (parental female and six F₁ progeny) from each of the three families was amplified at these loci using primers designed with Primer3 software (ROZEN and SKALETSKY 2000). PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA) and directly sequenced on both strands using a Beckman CEQ 8000 automatic sequencer. Sequences were searched for polymorphisms using BioEdit and ClustalW software (THOMPSON *et al.* 1994). Putative informative SNPs were confirmed by verifying that the F₀ female parent was homozygous for each of the loci. The informative SNPs were scored in the F₂ progeny either by using the heated ligation oligonucleotide assay (HOLA) (see below) or by single-base-pair extension reaction using terminator dyes and CEQ8000 software.

Scoring of SNPs: The HOLA method involves PCR amplification of the target DNA followed by a heated oligonucleotide ligation in the presence of a detector primer labeled with biotin and a reporter primer labeled with fluorescein. The SNP is detected after a colored reaction in a plate's well between a horseradish-peroxidase-conjugated anti-fluorescein and TMB (3,3',5,5'-tetramethylbenzidine), its substrate. We followed the protocol described by W. BLACK (unpublished results) and used by LYND *et al.* (2005) for *kdr* mutation detection in *A. gambiae*. Primers used are listed in Table 3.

The single-base-pair extension method was used for SNP detection essentially as described in the Beckman CEQ SNP-primer extension kit. The template fragment was amplified by PCR with the primers used for SNP identification (Table 4). PCR templates were cleaned by adding 2 units of shrimp alkaline phosphatase (SAP) and 1 unit of exonuclease I to 6 μ l of PCR template. The mixture was incubated at 37° for 1 hr and the enzymes were inactivated by heating to 75° for 15 min. Primer extension reactions were performed in a final volume of 10 μ l containing 4 μ l of SNP-primer extension premix, 1 pmol of the SNP primer and 50 ng/ μ l of the purified DNA template. Cycling conditions were 25 cycles of 10 sec at 96°, 5 sec at 55°, and 30 sec at 72°. The SNP products obtained were purified to remove free ddNTPs by incubation for 1 hr with 1 unit of SAP at 37° and were resolved using the Beckman CEQ8000.

Statistical analysis: Chi-squared analysis was conducted on all markers to test whether they segregated according to the expected Mendelian ratio. Genetic linkage maps were generated using MAPMAKER 3.0 (LANDER *et al.* 1987). Data from individual families initially were analyzed separately and then as a pooled data set to produce a composite genetic map. Similarly, progeny of different sexes were analyzed together initially and the analysis was then repeated including only female progeny.

RESULTS

Microsatellite markers: A total of 375 progeny were obtained from the three largest families. These progeny were phenotyped for resistance to permethrin as part of a separate study to identify genes associated with insecticide resistance in *A. funestus*. The 174 progeny selected for genotyping (46, 62, and 66 progeny from families 4, 6, and 11, respectively) were balanced to contain equal progeny from the insecticide-susceptible and insecticide-resistant phenotypes.

Of the 75 microsatellite markers available, 8 were discarded either because they failed to amplify or because multiple nonspecific products were obtained (AFND1, AFND13, AFND15, AFND18, AFND22, AFND23, AFND31,

TABLE 1
Microsatellite markers used in this study

Locus	Accession no.	<i>In situ</i> location	Left primer (5'–3')	Right primer (5'–3')	Allele size range (cM)
X chromosome					
FUNE	AY6009		GACCGGTTCTGGTATCGTC	ATCGAGTCACCCAATTCTCC	136–154
FUNQ	AY6021	X 5D	GCAAACCTGCTAGTAAATGTTTCC	ACACAACGCCACCACTATGA ^a	84–98
Chromosome 2					
AFND6	AF171036	2R 12E	GCTTCTTCTCCCCTAATCTG	TCCTGCTTTTTAGTTTGTGCG	184–212
AFUB15	AY029722		GATGCCGGGAGTAATAGCAA	AGACAGCCCGTAGAACGGTA	155–191
AFND2	AF171032		ATAAACCCGTCATTCCTT	CCTATGATTTCGCTCCTGACA	131–151
AFND32	AY291367	2R 15E	GAAGCATTTTGGGTAGACTC	GCAGTTGTTTACCTTTCACCTG	103–121
AFUB14	AY029721		ATCAGTGCTCCTCCACATCC	CGTGGTTGGCAATGTTACTG	152–188
AFND17	AF171047		AAAACGCCACAAAGAGCAC	CGGGTCAAATTCTACCGTAAG	129–157
AFUB4	AY029711		CTATCAGCAGCCGCCACA	GATGCCGATGAGGAATGTTG	183–192
AFUB25	AY029723		GTGGAAACGGTGGTACTGT	CGCCATGTAGCTAGGGTTTG	212–224
AFUB10	AY029717	2L 26CD	TGTCCATGTACAACCGCAAC	TTCTCCAGCATCATCAGCAC	195–210
AFND37	AY291373	2R 15E	GATCGATACAATAAGTGTAGAAATAAT	TCACGATGTGCAACCTATAA	161–189
AFUB30	AY029737		GCCAGTTTGCAGAACCAAT	CTGCTGCTGATGTTGCTGAT	154–163
AFUB7	AY029714		ATGGGACGATGGATTACCAA	GCCAGTTTGCAGAACCAAT	220–223
AFUB16	AY029723		CGTGGATGGCAATGTTACTG	TGCGACTTATCAGTGCTCCT	179–209
Chromosome 3					
AFND21	AF171051		CCGCACACCAACTTACACTC	TGGCGTGGGATTAAATAGG	96–104
AFUB13			GACTTCCGCCACAGAACATC	CTCAGGCTCGCAGTAGGAGT	207–210
AFND19	AF171049	3R 34A	CAGAACCACTTCGATTCAAC	CCTGCACTCAGAAACACAC	172–205
FUND	AY6008	3R 35A	GCTAACTACTCCGAAGCGCT	GATCGCAAACTTCCGGTT	145–177
FUNI	AY6013		GCAACTAAGCTGGGACAGGA ^a	GCATCTAACCCCTGCTGCTT	181–197
AFND3	AF171033		ACGACTGTAACCACAACACC	TAGTAGCGAAGGCCGAAAGAT	171–195
FUNF	AY6010	3L 43A	CCTTCAGTTTTCGATTGGCG	AATAAGATGCGACCGTGGC	104–118
AFND10	AF171040		TTTTTTCTTCCCGTGTTC	TACCATTTGATTACAGCGCC	114–146
AFUB17	AY029724		GAAAACCGTACGAACGATGG	TGCGACAGTAGCACAGGGTA	187–196
AFUB1	AY029708		CAGCAGCAGCAGCAACAG	GACGTTAGCATCTCCACCAG	266–269
AFUB12	AY029719	3L 46C	TGGGGAACCTGGTCGTTAGAG	CTGGTGATGGGATTGAGGAT	152–158
FUNK	AY6015	3L 41AB	GCGCTTCCGCAAACATAC	ACTCACACCCCATTTCTGTG	184–202
263B12	NA		AGTGCGTCAGAGTTTGAA	TCGATTGATGGCGATGATAA	230–242
261H03	NA		CGCTCAAACGTGAAGCGATA	GGATGCGGAGATGATGTTGT	208–220
263A06	NA		CGTTCGGTTTCGCTAACTGT	CGTTCATTTCCGGGTGTGT	210–220
Unlinked markers					
AFUB21	AY029728		AACGCAGCAGTGGAGAGAAT ^a	AACACCAACCCTTGTGTGTC	224–230
AFND30	AY291369		GCCAGTTTGCAGAACCAAT	CTGCTGCTGATGTTGCTGAT	81–107

NA, not available.

^a Redesigned primer.

and AFUB5). Another 7 markers (FUNH, FUNI, FUNQ, AFUB19, AFUB21, AFND24 and AFND30) required a redesign of the original primers before reliable amplification was achieved. Thirty-four microsatellite markers were noninformative for all three families while 33 were informative for one or more family (21 for family 4, 12 for family 6, and 16 for family 11).

Thirteen of the loci deviated from the expected Mendelian ratio of 1:2:1 in one or more families (AFND6, AFND17, AFUB16, AFUB25, AFUB15, AFUB4, AFND10, AFND19, AFUB1, FUNI, AFUB12, AFUB21, and AFND30). These deviations were observed mainly at the $P < 0.05$ level. When the number of tests done in all the three

families was considered, the expected ratios were obtained in 25 cases from a total of 41 (Table 5). Several loci with deviation from the Mendelian ratio were genotyped a second time to confirm that the deviations were not a result of mis-scoring.

SNP markers: SNP markers were identified by analyzing sequence traces of 11 physically mapped DNA fragments or cytochrome P450 of *A. funestus* amplified by PCR from genomic DNA. Fifteen informative SNPs were identified from these sequences with most of them informative in just one family. Ten SNPs were informative in family 6, 4 in family 4, and 3 in family 11. Eight of these SNPs involve transition mutations and seven

TABLE 2
Sequence origin for the informative SNPs used in this study

Genes	Putative function	Accession no.	Chromosomal location	Left primer 5'–3'	Right primer 5'–3'	Size of fragment sequence (bp)
BU21	Phosphoribosylamino-imidazole carboxylase	BU038921	X: 3A	TTTCAAGGTGAACGGTGTGA	CCATCAAGATGACGACCAGA	475
BU021	Tubulin β -3 chain	BU039021	3L: 39B	GAGTTGGTTGATGCCGTGTT	CGTCCGGAAACAAATATCGT	400
BU92	Microtubule binding	BU038892	3L: 39A	CATGCGACCGAAGAGAAAGTT	ATCTGATTTCTGGCTCATGG	550
9J12	Cytochrome P450	AY729663	NK	TACGGGTGTGACGCTTGA	CTTTGGGCGAAGGTAAA	195
9K1	Cytochrome P450	AY987362	NK	GTACGAGCTGGCCGTTAATC	CCTTTCTGTAGCTGCACCTTG	240
9J14	Cytochrome P450	AY729665	NK	CGGACAACTGATGATCGAATT	TTTGGCTTGCAATAAAAGGTG	215
BU66	Lysozyme	BU038966	3R: 30A	TAGCTCATAGTGGCGGTTAT	ACTACAACATGCTGTGCAAA	830
BU19	Chitinase	BU038919	2R: 12B	CTGTTGCTGCTGTACATAC	CGGTCACGTACAAATAGTC	770
BU40	Glutathione peroxidase	BU038940	2R: 14B	AGGCAAAATCAATTTTGAA	CGTAACAATTTCTCGACCAT	1150
6P4	Cytochrome P450	AY987359	NK	GTACGAGACTGGCAAGAAT	AAGCAAGACGTATGGATGG	480
BU82	Unknown	BU038882	2R: 14D	AGGGCGGTACAACAAATCT	GCATCGGAGCGTTTCTCTA	860

NK, not known.

involve transversions. Ten of the SNPs are derived from physically mapped loci (Table 2). The majority of the loci were scored using the HOLA method but, for five of the loci, the polymorphisms in the sequence flanking the SNP interfered with the design of the reporter and/or the detector primers and, for these loci, the primer extension method was used.

Genetic map: A total of 174 F₂ progeny from three families were genotyped at 49 informative loci (33 microsatellites, 15 SNPs, and 1 RFLP).

In the first instance, males and females were treated separately because the frequency of recombination can differ between sexes in Diptera (CLEMENTS 1992). In general, recombination frequencies were comparable between the sexes and similar genetic distances were observed between the loci (for example, the overall genetic distance was 79.6 cM for females and 80.3 cM for males for the 3L chromosomal arm). The exception was chromosome 2, where analysis of solely male progeny resulted in two linkage groups whereas the female progeny formed a single linkage group for this chromosome. For subsequent analysis, both sexes were used (with the exception of the X chromosome; see below).

Similarly, linkage groups initially were resolved for each individual family before pooling the data from all progeny. Four linkage groups were unambiguously defined in families 4 and 11 and were anchored to chromosome X, chromosome 2, and chromosomal arms 3R and 3L using the known physical map location of the markers in each linkage group (SHARAKHOV *et al.* 2004). However, for family 6, six linkage groups were defined, with loci on chromosomes 2 and 3 split into three different groups (there were no markers informative for the X chromosome in this family). The genetic order was generally consistent among the loci across all families and all markers were consistently assigned to the same linkage groups. A good concordance was observed between the genetic order from single family genetic map and the physical map of SHARAKHOV *et al.* (2002, 2004).

Given the general concordance between the sexes and among the different families, we constructed a composite map combining the genotypes from all progeny from the three families. Linkage groups corresponding to the three chromosomes were unambiguously defined but, as with the single-family analysis, chromosome 3 formed two distinct linkage groups (Figure 1).

The smallest linkage group consisted of five loci (two SNPs, one RFLP, and two microsatellites). Two of the markers were derived from the phosphoribosylamino-imidazole carboxylase gene, which has been physically mapped to the X chromosome (SHARAKHOV *et al.* 2002), allowing us to anchor this group to the X chromosome. As *Anopheles* mosquitoes have heteromorphic sex chromosomes, with females being XX and males XY, only females are informative for X chromosome markers. The five markers spanned a genetic distance of 44.7 cM

TABLE 3
SNP loci scored by HOLA method

Locus	Sequence immediately upstream from SNP	Chromosomal location	Detector primers 5'-3'	Reporter primer 5'-3'	Alleles
7BU21	GATTCGGA	X: 3A	7BU21C-dtc biotin-GGACGGCCCAAAGGGCGCCACGAG	7BU21-rpt	C/T
8BU21	GTGACCGC	X: 3A	7BU21T-dtc biotin-GGACGGCCCAAAGGGCGCCACGAA	PO4-TCCGAATCGATCACATCCCGCTA-Fluo	A/T
6P9K1	GAGGATA	NK	8BU21A-dtc biotin-TTACCGGTGTCACAGATCGGCT	8BU21-rpt	C/T
6BU40	CTAGGGCG	2R: 12B	8BU21T-dtc biotin-TTACCGGTGTCACAGATCGGCA	PO4-GCGGTTCACATTAGCCAGATTG-Fluo	C/T
2BU19	CTGCCACA	2R: 14B	6P9K1T-dtc biotin-TGTTGCTGCACCTTGTGCCCCGTTA	6P9K1-rpt	C/T
2BU021	CACTCGCT	3L: 39A	6P9K1C-dtc biotin-TGTTGCTGCACCTTGTGCCCCGTTG	PO4-TAATCCCTCCAGAACGTACGGTT-Fluo	C/T
5P9J12	TTAAAATG	NK	2BU19C-dtc biotin-AAATATTTACGCGCCATTACG	2BU19-rpt	G/T
9BU66	CTAGCCAA	3R: 30A	2BU19T-dtc biotin-AAATATTTACGCGCCATTACG	PO4-TGTGGCAGCCATCTTTTGGGA-Fluo	G/T
12BU66	AAAGTTTG	3R: 30A	6BU40T-dtc biotin-TTACCTATAAGTGTGTAGCAA	6BU40-rpt	A/C
2P9J14	TGGACCTG	NK	6BU40G-dtc biotin-TTACCTATAAGTGTGTAGCAC	PO4-CGCCCTAGGAAGGTGCTGACAA-Fluo	A/G
4P9J14	CGCAGAAA	NK	2BU021T-dtc biotin-ATACCGGAACCCAGTACCACTCCA	2BU021-rpt	G/T
			2BU021G-dtc biotin-ATACCGGAACCCAGTACCACTCCC	PO4 AGCGAGTGGTCAATTGGA-Fluo	A/C
			5P9J12A-dtc biotin-TTGGCGGGAAGGTAAAGCCTAT	5P9J12-rpt	A/G
			5P9J12C-dtc biotin-TTGGCGGGAAGGTAAAGCCTAG	PO4 CATTTTAAGCACCTTTTACAA-Fluo	A/G
			9BU66A-dtc biotin-GATGCCATTATTTGCCAAAGCTT	9BU66-rpt	A/G
			9BU66G-dtc biotin-GATGCCATTATTTGCCAAAGCTC	PO4-TTGGCTAGCTCGCAITTTGGA-Fluo	A/G
			12BU66A-dtc biotin-AATCCAAGAGTAGTTAAATCTT	12BU66-rpt	G/T
			12BU66G-dtc biotin-AATCCAAGAGTAGTTAAATCTC	PO4-CAAAACTTTTCCCCAGATCAAGA-Fluo	A/T
			2P9J14G-dtc biotin-AACAAATTAGCTCCCGATCGAC	2P9J14-rpt	
			2P9J14T-dtc biotin-AACAAATTAGCTCCCGATCGAA	PO4-CAGGTCCACTTCAAGCTTTTCC-Fluo	
			4P9J14A-dtc biotin-ATACCTTGCACTTCTCGCGT	4P9J14-rpt	
			4P9J14T-dtc biotin-ATACCTTGCACTTCTCGCGA	PO4-TTCTCCTCGTCTCGAAGACGAT-Fluo	

NK, not known.

TABLE 4
SNP loci scored with the Beckman SNP analysis method

Locus	Sequence immediately upstream from SNP	Chromosomal location	Primer used for the extension reaction	Alleles
11BU92	GCGGCCGA	3L: 39A	GCT GAT AAG CAG GCG GCC GA	(A/G)
3P6P4	AGCATTTA	NK	GAA AAGTAATGT GAA AGT	(G/T)
1BU19	ACCCGGAG	2R: 12B	CTG AAG AAA GAG CAC CCG GAG	(C/T)
3BU82	ATCGTTTA	2R: 14D	GA ACT GGA AAT GAT CGT TTA	(A/G)
4P9K1	TACGAAAC	NK	ATG TAG CGC ATC GAT TGG AG	(C/T)

NK, not known.

with the greatest distance of 26.7 cM between the RFLP loci G17 and the SNP locus 4P9K1. The smallest distance, 1.0 cM, as expected was between the two SNPs located within the same gene (separated by just 95 bp).

The map obtained for chromosome 2 encompasses 16 loci and spans 158 cM, giving an average resolution of 9.8 cM (Figure 1). The greatest distance is 22.2 cM between loci AFUB15 and AFND2. For this chromosome, the genetic order showed some discordance with the physical map. Marker 2BU19 was physically mapped to division 2R:12B but the genetic map puts it closer to the centromere (Figure 1). The microsatellite marker AFND37 physically maps to the 2R chromosome arm according to SHARAKHOV *et al.* (2004) but our study found this marker genetically linked to the left arm of chromosome 2, close to the telomere.

Two linkage groups were consistently resolved for chromosome 3. These were anchored to the 3R or 3L arms of this chromosome according to the physical map. The genetic map corresponding to the 3R arm is formed of 10 loci spanning a distance of 90.9 cM (average resolution, 9.1 cM). There is a complete agreement between the genetic order of the loci and their physical location as described by SHARAKHOV *et al.* (2002, 2004). The map obtained for the 3L arm comprises 11 loci and spans 89.1 cM (average resolution of 8.1 cM). Again, the genetic order of these 3L loci is in agreement with their physical location (Figure 1).

Seven markers were not linked to any of the four defined linkage groups (AFUB21, AFND30, FUNQ, 6P9K1, 2P9J14, 4P9J14, and 3P6P4). Six of these markers show deviation from the Hardy-Weinberg ratio.

DISCUSSION

We present here the first genetic map of *A. funestus* consisting of microsatellite, RFLP, and SNP markers. The linkage groups defined have been assigned to corresponding chromosomes using the physical map described by SHARAKHOV *et al.* (2002, 2004). The overall average distance between markers in this study is 9.1 cM for 42 markers successfully mapped. This study has in-

creased the total number of genetic markers that have been physically and/or genetically mapped in this species from 32 to 64 markers.

Recombination was observed in both males and females as previously noted in *A. gambiae* (ZHENG *et al.* 1996). The frequencies observed in both sexes are generally comparable with the exception of chromosome 2 where in males markers AFUB30 and AFUB7 formed a separate linkage group not genetically linked to the remainder of the chromosome 2 markers. This observed difference may reflect a locus-specific effect rather than a genome-wide one since, apart from these two loci, recombination frequencies between both sexes are comparable. This is reinforced by the fact that in other anopheline mosquitoes studied so far, there have always been comparable recombination frequencies between males and females (MITCHELL *et al.* 1993; SEAWRIGHT and NARANG 1993; ZHENG *et al.* 1996).

In general, there is a good agreement between the genetic order of loci and their physical location as described by SHARAKHOV *et al.* (2002, 2004). This agreement was complete for loci on chromosomes X and 3 while the genetic order of two loci on chromosome 2 (2BU19 and AFND37) was in disagreement with their cytological location. The male parents of the crosses were obtained from the FUM0Z strain, which is polymorphic for inversion 2Ra; however, this inversion, which spans from division 13C to 14B on chromosome 2R, does not include either of these markers. The FANG strain has not been karyotyped and it was not possible to examine the parents of each of the individual crosses for the presence of inversions and so the effect, if any, on the genetic map is unknown. Locus 2BU19 is located within inversions *au* and *t*, two of the multiple chromosomal inversions found on the 2R chromosomal arm of *A. funestus* (GREEN and HUNT 1980). A polymorphism in one of these two inversions may explain the difference observed in the location of this locus between the genetic and the physical map. The discrepancy is more important because locus AFND37 mapped genetically to the end of chromosome 2 but located physically at division 15E of the 2R arm. There is no obvious explanation for this disagreement. The recombination

TABLE 5
Observed Mendelian ratios for microsatellite and SNP markers

Locus	Chromosome	χ^2 values		
		Family 4	Family 6	Family 11
Chromosome X				
7BU21 ^a	X: 3A		0.333	
8BU21 ^a	X: 3A	0.037		
FUNE ^a		2.13		
FUNQ ^a	X: 5D	2.13		
4G21 ^a		2.19	4.94	0.297
6P9K1 ^a		17.2**		
4P6K1 ^a			2.4	
Chromosome 2				
2BU19	2R: 12B		7.75*	
AFND6	2R: 12E	8.86*		16.6**
AFND17		4.33	2.65	9.88*
3BU82	2R: 14D		5.6	
AFUB14		3.74		
AFUB16		11.7*		
AFND32		2.2		
AFUB25		12.2*	2.13	4.91
AFUB30			2.13	
AFUB7			2.09	
AFUB15			16.2**	12.7*
AFUB4				8.3*
6BU40	2R: 14B		12.1*	
AFND37	2R: 15E			1.64
AFUB10	2L: 26CD			3.12
Chromosome 3				
9BU66	3R: 30A	1.43		1.03
12BU66	3R: 30A		9.87*	
AFND3		0.378		
AFND10		6.65*	2.61	3.42
AFND19		2.37	6.74*	
AFUB1		4.87		8.3*
FUND	3R 35A	2.32		
263B12		1.57		
261H03		5.98		
2BU021	3L: 39A		2.35	
11BU92	3L: 39B			1.19
FUNF	3L 43A	2.73		7.33
FUNI		1.65	45.0**	3.46
FUNK	3L 41AB	5.0		3.45
263A06		1.65		
AFUB17			4.16	
5P9J12	3L: 44–46		11.9*	
AFUB12	3L 46C			13.5**
AFUB21		9.27*		
ND30		28.1**	8.29*	
AFND21				3.7
2P9J14	3L		5.95	
4P9J14	3L		14.5**	
Unlinked locus				
3P6P4				7.21*

The second column shows (if known) the chromosome and the cytological division of the locus. * $P < 0.05$, ** $P < 0.01$.

^a χ^2 values were calculated with the expected ratio of 1A:1H.

frequency does not always closely correlate with physical distance on the polytene chromosome. For example, on chromosome 2, markers from 4 (12–15) of 22 divisions span nearly half of the genetic map (Figure 1).

An independent assortment of markers on chromosomal arms 3L and 3R was observed during the construction of this map. Each time that these two chromosomal arms were joined together, a genetic distance of >60 cM was observed between the two markers making the link and the overall distance of this combined chromosome 3 was very high. For this reason we decided to split this chromosome into two separate linkage groups. This situation was not observed in the genetic map of *A. gambiae* (ZHENG *et al.* 1996). This independent assortment may be due to a lack of markers in the vicinity of the centromere linking the two arms. In fact, we did not find any informative marker in the three chromosomal divisions around the centromere of this chromosome.

The overall consistency observed between the recombinational and the cytogenetic order of the markers in this study is an indication of the robustness of this genetic map. The map presented here is statistically the best estimate from the data available. Nevertheless, the map is limited by the size of the data sets that have been used to construct it (both progeny numbers and informative markers) and by possible polymorphisms in chromosome arrangement. Certainly, with more informative markers, this map would have gained much more resolution and robustness. Efforts to identify more SNP markers should be increased with the aim of building a high-resolution map or a fine-scale map for this species. To improve the quality of the map, it would be desirable to select *A. funestus* strains that are chromosomally homogeneous, but sequence divergent and robust in the laboratory. Given the difficulty of colonizing *A. funestus* with only two strains in stable colonies worldwide, this is a major task.

The genetic map of *A. funestus* presented in this study is similar to that described for *A. gambiae*, another malaria vector using mainly microsatellite markers (ZHENG *et al.* 1996). However, there was no independent assortment of the markers on the 3L and 3R chromosomal arms as observed here for *A. funestus*. The map described for *A. gambiae* presents a higher resolution than the one constructed here for *A. funestus* especially because of the greater availability of an important number of microsatellite markers in *A. gambiae* than in *A. funestus*.

The construction of this first genetic map of *A. funestus* is a significant advance in the genetics of this important malaria vector in Africa. This integrated genetic and physical map for *A. funestus* provides a useful platform for the effort to use positional cloning to isolate genes of interest in this species. In addition, the new markers identified during this study could be used in population genetics studies in this species.

FIGURE 1.—Integration of the genetic linkage map and the physical map of *A. funestus*. The genetic map was constructed from pooled data from three F_2 intercross families. A scale of genetic distance in centimorgans is shown. Thick lines link the genetic loci to their appropriate location on the physical map.

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