for a high incidence of null alleles. These characteristics make these markers invaluable for studies of mating patterns and genetic diversity in *C. ferox*.

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Isolation of polymorphic microsatellite loci from the malaria vector *Anopheles funestus*

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Anopheles funestus is one of the three most important insect vectors of human disease in terms of human morbidity and mortality caused, making a major contribution to the transmission of malaria in sub-Saharan Africa. Evidence exists for natural population heterogeneities in *A. funestus* from West Africa. In Senegal, allopatric populations are characterized by different frequencies of chromosomal inversions that are in Hardy–Weinberg equilibrium unless pooled between locations (Lochouarn *et al.* 1998). In Burkina Faso, significant departures from Hardy–Weinberg equilibrium have been recorded for inversions within samples from the same locale, suggesting that at least two cryptic species with different vectorial capacities may be present (Boccolini *et al.* 1994). There is a clear need for further population-level studies, but very little molecular information exists for this species. We therefore undertook the isolation of a set of microsatellite loci for use as markers.

A genomic library was prepared from a *Sau*3AI-digest of genomic DNA from four Kenyan specimens of *A. funestus*. The DNA, without size selection, was directly ligated to *Bam*HI-digested dephosphorylated pBluescript (Stratagene). From the resulting library, 5120 white colonies were transferred onto nylon membranes (Stratagene) and screened with an equal mixture of $(TC)_{13}$ and $(TG)_{15}$ oligonucleotide probes end-labelled with $\gamma^{32}P$. Hybridizing colonies were picked, suspended in 50 µL of TE and used for PCR amplification across the insert. The PCR products were electrophoresed through agarose gels, blotted to nylon membranes and re-screened with the same probes for verification. DNA from 31 positive PCR reactions was purified using Wizard PCR preps (Promega), and sequenced on PE-ABI 377 (Perkin-Elmer) apparatus with M13 Universal and Reverse primers. FASTA sequence searches showed that none of these clones were identical.

Primers were designed flanking each suitable microsatellite sequence (one per clone where multiple microsatellites occurred), using GCG (Genetics Computer Group) Prime software, to amplify short (70–250 bp) PCR products. Primer pairs were selected which had a similar melting temperature (Tm) and an annealing temperature as close as possible to 55 °C, to allow PCR multiplexing. Primers were tested in 15-µL PCR reactions containing 7.5 pmol primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, and 0.75 µL of 100-µL genomic template DNA from whole single mosquitoes extracted as described by Collins *et al.* (1987). Cycling conditions in a Perkin-Elmer GeneAmp cycler were: 94 °C denaturation for 5 min, followed by 35 cycles of 94 °C for 25 s, 55 °C for 28 s and 72 °C for 30 s, with a final 72 °C extension of 5 min.

For those primers which produced successful and consistent amplification (with products visualized on 2% agarose gels), one primer per pair was re-synthesized incorporating a fluorescent label, FAM, TET or HEX. Resulting PCR products

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Table 1 Primer sequences of 22 *Anopheles funestus* microsatellite loci. GenBank accession nos are listed below each locus. Sequence interruptions between repeats are indicated as '+'. Number of alleles are based on sample sizes of 10, 8 and 18 specimens collected from Kenya, Tanzania, and Burkina Faso, respectively. The three trinucleotide repeat loci included were isolated fortuitously

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	No. of alleles	H_O
AF1	F: CGGTTGAAAATTAAGGACCA	$(GA)_{11}$	210–234	13	0.44
AF171031	R: CAAAAGACACCAAACACCAG				
AF2	F: ATAAACCCGTCCATTCCCTT	$(GT)_9$	131–151	12	0.69
AF171032	R: CCTATGATTCGCTCCTGACA				
AF3	F: ACGACTGTAACCACAACACC	$(GGT)_{5+4}$	171–195	6	0.53
AF171033	R: TAGTAGCGAAGGCGAAAGAT				
AF4	F: CACACAAGCCAGAACATTCC	$(GT)_{4+2+3+4}$	65–83	5	0.36
AF171034	R: ACACTGAACAATCACGCCAA				
AF5	F: CCTCTCGTTGTGTTGCCTAC	$(GT)_{5+3}$	169–183	6	0.50
AF171035	R: GTTCATACGTTGCCCGATTT				
AF6	F: GCTTCTTCTCCCCTAATCTG	$(GT)_{7+3}$	184–212	19	0.78
AF171036	R: TCCTGCTTTTTAGTTTGTCG				
AF7	F: TGCATCATTCGACTCGGAAG	$(GT)_8$	70–84	9	0.83
AF171037	R: AACGGCACTACCGTTCACTG				
AF8	F: ACCATTTGCATCCTAGAACC	$(GT)_{7+2}$	117–136	13	0.78
AF171038	R: CATGAACTATTGTTAACCACCC				
AF9	F: GTGTTGAAAACCCCGTCTTG	$(ATT)_{4+2+3}$	165–168	2	0.03
AF171039	R: AATCGTTGATTGGTTCGCTG				
AF10	F: TTTTTTCTTCCCGTGTTGC	$(GT)_{6+4}$	114–146	15	0.44
AF171040	R: TACCATTTGATTACAGCGCC				
AF11	F: TTTCAACTATTTCCGTAACGAG	$(GCT)_5$	125–134	4	0.42
AF171041	R: GATCATCACCACTACCAGGC				
AF12	F: GTTCTCCATCGCTGTTCTACTC	$(GA)_6$	87–107	11	0.44
AF171042	R: TATAACGTTTCGTACACACGCC				
AF13	F: TTATGAACAGCACTGTAGCA	$(GT)_8$	181–196	12	0.44
AF171043	R: GTCTCATCTATTCACTTTCCG				
AF14	F: CGATGCCCACTAATTCAAG	$(GT)_{12}$	169–194	17	0.72
AF171044	R: GCCACCCAAAGTATAGTGAAG				
AF15	F: TGACATCCAGTGACTCACC	$(GA)_7$	203–223	12	0.50
AF171045	R: GAACAGATTTTCCGTTTAGC				
AF16	F: TGACCGTAAATTGCCAATCC	$(GT)_8$	99–101	2	0.75
AF171046	R: TCAGTCACATTCCATTCGTTTC				
AF17	F: AAAACGCCACAAAGAGCAC	$(GT)_{5+2}$	129–157	14	0.56
AF171047	R: CGGGTCAAATTCTACCGTAAG				
AF18	F: AAAGCGCACTTTATGAACG	$(GA)_{10}$	210–231	14	0.33
AF171048	R: AAAACAAACACAGGAAGGC				
AF19	F: CAGAACCACTTCGATTCAAC	$(GT)_{12}$	172–205	12	0.75
AF171049	R: CCTGCACTCAGAAACACAC				
AF20	F: AGAACCACATTAGGGAACAG	$(GT)_{7+2}$	123–139	9	0.69
AF171050	R: TTTACAACCAGCAGCACAC				
AF21	F: CCGCACACCAACTTACACTC	$(GA)_9$	96–104	4	0.39
AF171051	R: TGGCGTGGGATTAAATAGG				
AF22	F: CGAATTCATCTCGTAGAGACC	$(GT)_{2+3+6}$	162–182	9	0.36
AF171052	R: TAATACACACGCTTCGCTTC				

were resolved on 5% denaturing polyacrylamide gels using PE-ABI 377 apparatus running Genescan software and following PE/ABI protocols. Products from up to three microsatellite loci were run per lane, either from multiplexed PCR or combining products post-PCR. Gels were re-loaded for a second run where possible, which did not incur any significant loss of sizing accuracy. At some of the loci peak doublets separated by 1 bp were produced for each allele, thought to be caused by some untemplated addition of an Adenosine nucleotide to the PCR product. The size of the first peak of such doublets was recorded regardless of the relative peak heights, using PE-ABI Genotyper software.

In total, 22 loci were characterized for their degree of polymorphism (Table 1), using 36 unkaryotyped specimens from East and West Africa. Specimens from Burkina Faso were collected in September 1998 by indoor spray-sheet or resting

catch in Kuiti and Goden villages, near Ouagadougou; 16 F1 male offspring were also assayed. Kenyan specimens were collected in March 1998 at Rota village near Kisumu, and Tanzanian specimens were collected in indoor light traps (December 1997) at Kibanda village near Muheza (all specimens were preserved by desiccation). The levels of heterozygosity found by genotyping 36 individuals at all of the loci were high (Table 1), and in this respect almost all of these loci appear to be good candidates for further population work.

NCBI BLAST searches showed no significant homologies to GenBank sequences for 20 of the 22 clones. However, clones AF4 and AF16 showed homology to rDNA 18S and ITS2 regions, respectively. AF16 showed only two variants ('alleles') present in the three populations, and 16 F1 male offspring of wild-caught females from Burkina Faso all showed two variants. The most parsimonious explanation for this pattern is that more than one variant per X chromosome is present within the multicopy rDNA array. The low variation observed is in agreement with low ITS2 sequence variation observed by Mukabayire *et al.* (1999). Any barriers to gene flow which have arisen recently might not be detectable by studying this region.

The distinction between X-linked and autosomal loci has been made by genotyping 16 F1 male offspring from wild-caught female parents. At locus AF12 none of the males were heterozygous, while 12 of 16 F1 females were heterozygous, which is strong evidence that AF12 is X-linked (males being hemizygous at X-linked single copy loci). AF16 and AF4 are X-linked based on homology to rDNA sequences, while AF9 showed insufficient polymorphism to draw any conclusions. Male heterozygosity observed at all of the other loci indicates that they are autosomal.

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Characterization of highly polymorphic microsatellite markers in the marsupial honey possum (*Tarsipes rostratus*)

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The honey possum *Tarsipes rostratus* is a tiny (males 7–9 g; females 10–12 g) diprotodont marsupial, endemic to the heathlands of southwestern Australia (Wooller *et al.* 1981). They are the sole species in a monotypic family (Tarsipedidae; Kirsch *et al.* 1997). Honey possums are one of the few mammals which feed solely on nectar and pollen, and their adaptations to this specialized diet include an elongated snout, a long brush tongue and highly reduced dentition (Richardson *et al.* 1986). Reproductively, *Tarsipes* has a number of extraordinary attributes. Similar to the macropod marsupials, they show embryonic diapause (Renfree *et al.* 1984). Females carry 3–4 pouch young, which at birth are the smallest mammalian young and weigh less than 5 mg. The testes represent 4.2% of total body weight, proportionately the largest among mammals. They also have the longest sperm of any mammal (up to 360 μm). These male reproductive traits suggest a promiscuous mating system with gamete selection (Renfree *et al.* 1984), but this has yet to be confirmed.

Clearly, highly polymorphic markers would be an invaluable tool for investigating the mating system and mechanisms of sperm competition. Consequently we describe the characterization of a series of extremely polymorphic microsatellite markers from the honey possum, *T. rostratus*.

A partial genome library was constructed for *T. rostratus* by digesting genomic DNA (5 μg) to completion with *Sau*3AI. Size-selected DNA fragments (200–700 bp) were ligated into *Hin*dIII-cut pGem (Promega Corp.), transformed into competent ElectroMAX DH 10B cells (Life Technologies) and spread onto agar plates containing ampicillin, IPTG and X-gal. A total of 800 recombinant clones were individually picked into 96-well microtitre plates containing 200 μL of LB-Ampicillin. These were grown overnight and transferred onto nylon membranes (Hybond-N; Amersham) by vacuum dot blotting using a multichannel pipette (which facilitated sample handling). The filters were probed with two oligonucleotides (CA_{12} and GA_{12}) which had been end-labelled with [γ^{33}P]-dATP.

Nucleotide sequences for 36 positive clones were determined on an ABI 377 automatic sequencer. Of the clones sequenced, many (55%) were found to contain microsatellites with greater than 20 repeat units; several additional clones contained imperfect or lower numbers of repeat units, and another contained a complex locus containing a tetranucleotide repeat unit.

Oligonucleotide primers were designed to enable PCR amplification at 20 of these loci, five of which were highly