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Structure of some East African *Glossina fuscipes fuscipes* populations

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Abstract

Glossina fuscipes fuscipes Newstead 1910 (Diptera: Glossinidae) is the primary vector of human sleeping sickness in Kenya and Uganda. This is the first report on its population structure. A total of 688 nucleotides of mitochondrial ribosomal 16S2 and cytochrome oxidase I genes were sequenced. Twenty-one variants were scored in 79 flies from three geographically diverse natural populations. Four haplotypes were shared among populations, eight were private and nine were singletons. The mean haplotype and nucleotide diversities were 0.84 and 0.009, respectively. All populations were genetically differentiated and were at demographic equilibrium. In addition, a longstanding laboratory culture originating from the Central African Republic (CAR-lab) in 1986 (or before) was examined. Haplotype and nucleotide diversities in this culture were 0.95 and 0.012, respectively. None of its 27 haplotypes were shared with the East African populations. A first approximation of relative effective population sizes was Uganda > CAR-lab > Kenya. It was concluded that the structure of *G. f. fuscipes* populations in East Africa is localized.

Keywords

Glossina; breeding structure; genetic differentiation; tsetse

Introduction

The tsetse fly *Glossina fuscipes fuscipes* is classified with the *palpalis* group, the subgenus *Nemorhina* Robineau-Desvoidy (Gooding & Krafsur, 2005). Flies of the *palpalis* group generally occupy riverine and lacustrine habitats and are opportunistic feeders (Leak, 1998). *Glossina f. fuscipes* may be found within a large area of central Africa, from Cameroon, Gabon, Congo, Zaire and the Central African Republic eastwards to Uganda and western Kenya. Highly localized patches exist on the margins of Lake Victoria in Tanzania and small, isolated patches of *G. f. fuscipes* are also found in southwestern Ethiopia and southern Sudan (Rogers & Robinson, 2004). *Glossina f. fuscipes* are vectors of the trypanosomes (Kinetoplastida: Trypanosomatidae; *Trypanosoma brucei gambiense* and *Trypanosoma bruce rhodesiense*) that cause human African trypanosomiasis (HAT) or sleeping sickness, particularly in Uganda and western Kenya (Waiswa *et al.*, 2006). Uganda is an active focus of HAT (Welburn *et al.*, 2006), although *G. f. fuscipes* is principally zoophagic (Wamwiri *et al.*, 2007). Trypanosome reservoirs there include cattle and other domestic livestock, as well as numerous wild mammals (Waiswa *et al.*, 2003). Here, we examine patterns of mitochondrial diversity in *G. f. fuscipes*, with the objectives of estimating its population structure and the degree of population

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subdivision in East Africa. We also compare the East African population samples with the principal laboratory strain from which any others are derived. Because mitochondrial variation is single copy and inherited matrilineally, it shows divergence among lineages about four times as rapidly as autosomal loci such as microsatellites.

Materials and methods

Tsetse flies were sampled in 2003 in Nambale, Busia District (34.24 °E, 0.46 °S) and, Chakol Obekai, in Teso District (34.21 °E, 0.51 °S), both in western Kenya, and in 2000 in Lunyo in eastern Uganda (33.91 °E, 0.33 °S), about 55 km from the Kenyan sampling sites (Fig. 1). The Kenyan districts are adjacent and the sampling sites *c.* 32 km apart. Since 1986, the joint laboratory of the Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) has maintained a culture of *G. f. fuscipes* that originated from the Central African Republic (CAR-lab). The conditions and year of its original establishment are unknown. Its numbers have varied from as few as 375 to as many as 11 000 females. Population size was approximately 11 000 females at the time of genetic sampling. All extant cultures of *G. f. fuscipes* are derived from the FAO/IAEA stock (A. S. Robinson and A. G. Parker, FAO/IAEA Laboratories, year?, personal communication to E.S.K.).

DNA was extracted using the Qiagen DNeasy Extraction Kit (Qiagen, Xxx, XX, Xxx). Subsequently, 300 bp of the ribosomal 16S (*r16S2*) and 440 bp of the cytochrome oxidase I (*COI*) mitochondrial genes were amplified in 50 μL consisting of 48 μL of 1.1x ReddyMix PCR Master Mix (ABGene, New York, NY, U.S.A.), 2 μL of DNA extract (approximately 80 ng), and 0.5 μm each of oligonucleotide primers N1-J-12585 (5′GGT CCC TTA CGA ATT TGA ATA TAT CCT3′) and LR-N-12866 (5′ACA TGA TCT GAG TTC AAA CCG G3′) for *r16S2*, and C1-J-1751 (5′GGA TCA CCT GAT ATA GCA TTC CC3′) and C1-N-2191 (5′ CCC GGT AAA ATT AAA ATA TAA ACT TC3′) for *COI* (Simon *et al.*, 1994). Thermocycling was performed in a PTC-100 (MJ Research, Woburn, MA, U.S.A.) thermocycler and consisted of 30 cycles at 93°C for 45 s, 52°C for 1 min and 72°C for 1 min. DNA sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility using an ABI Model 3730 automated sequence analyser. BioEdit (Hall, 1999) was used to edit the nucleotide sequences. Sequence alignment was accomplished using ClustalX (Thompson *et al.*, 1997).

Genetic statistics

Haplotype and nucleotide diversities were calculated according to Nei (1986) and Nei & Kumar (2000). Haplotype diversity refers to the probability that two randomly chosen flies will have different haplotypes. Nucleotide diversity π_S is the average proportion of nucleotide differences between all pairs of sequences and is estimated over all possible pairwise comparisons. Genetic differentiation of populations was estimated in several ways. Student's *t*-tests were used to test hypotheses that haplotype and nucleotide diversities were homogeneous. Raymond & Rousset's (1995) exact test was also used; it is analogous to Fisher's exact test. Departures from homogenous dispersion of haplotypes were estimated by $F_{ST} = 1-(H_w/H_b)$, where H_w and H_b are the average pairwise nucleotide differences within and between samples, respectively (Hudson *et al.*, 1992). As used here, F_{ST} measures the probability that two randomly chosen sequences from any two samples differ by one or more nucleotide substitutions. Analysis of molecular variance (AMOVA) on haplotype frequencies was carried out using ARLEQUIN. AMOVA also yields estimates of *F*. F_{ST} can be used to estimate gene flow in terms of the mean number of reproducing females per generation according to Wright's island model.

Theta (θ) is the product of the effective population size $2N_e$ and the mutation rate μ. It was estimated from the average number of pairwise nucleotide differences among sequences (Tajima, 1989). The main underlying assumption is that of mutation-drift equilibrium. Its

biological meaning here is a scaling factor relating the relative effective sizes N_e of the sampled populations. Effective size is the harmonic mean reproductive population size averaged over many generations.

Pairwise nucleotide differences (mismatch distributions) and frequencies were also used as the basis for tests of selective neutrality and demographic equilibrium. Tests used F_S (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) statistics. If neutrality can be assumed and there is no genetic 'hitchhiking', these tests are the most powerful available to detect historical demographic expansions (Rogers & Harpending, 1992;Ramos-Onsins & Rozas, 2002). It should be noted, however, that the power to reject a null hypothesis when it is false varies with the interval between a population expansion and sampling and the magnitude of the expansion. The genetic statistics were calculated by using DnaSP Version 4.10.9 (Rozas *et al.*, 2003) and ARLEQUIN Version 3.11 (Excoffier *et al.*, 2005).

Results

The concatenated fragments of *r16S2* and *COI* genes numbered 668 nucleotides, of which 59 were variable (8.8%) and 54 were shared between two or three populations. There were no alignment gaps or missing data. The haplotype distributions for each sample are set out in Table 1. A total of 48 mitochondrial haplotypes were recorded, 29 of which were singletons (60.4%). It can be seen at a glance that the East African *G. f. fuscipes* populations shared no haplotype with the laboratory culture (CAR-lab).

Natural populations

A total of 21 haplotypes were recorded in the field samples, of which four (19%) were shared, eight (38%) occurred in only one population and nine (43%) were singletons. Only one Kenyan haplotype was shared with the Ugandan *G. f. fuscipes*.

Twelve haplotypes were recorded in Nambale and Chakol Obekai, four of which were shared between them (Table 2). Ten haplotypes were recorded in the Ugandan samples, five (50%) of which were singletons and only one (10%) of which was also found in Nambale and Chakol Obekai. Average pairwise differences in mitochondrial gene sequence variation (Table 3) between the Kenyan samples did not differ significantly (P ~ 0.72, Student's *t*-test). The Kenyan data were then pooled for analysis. Exact tests of differentiation based on haplotype frequencies, however, indicted that even the two Kenyan populations differed from each other significantly ($P = 0.02 \pm 0.002$). By the same two tests, the Kenyan flies differed greatly from the Ugandan population sample ($P << 0.001$). The pairwise estimate of F_{ST} (Table 2) between Kenyan samples did not differ from zero, in keeping with a null hypothesis of much gene flow between them. F_{ST} between Kenyan and Ugandan samples was 0.28, equivalent to the exchange of 1.3 reproducing females per generation, according to the simplifying assumptions of the island model of population structure. Haplotype diversities (H_s) varied from 0.72 in Nambale, Kenya, to 0.94 in Lunyo, Uganda (Table 3). Here, H_s is the probability that two randomly chosen flies from a sample will have different haplotypes; these probabilities differed significantly among populations. Nucleotide diversities per nucleotide π_s were five times greater in eastern Uganda than in western Kenya. Mean pairwise nucleotide differences between populations θ were also very much greater in Uganda than in Kenya. The foregoing data suggest that the Ugandan population was very much larger than the Kenyan populations. Mean haplotype and nucleotide diversities over the three field samples were $H_s = 0.84$ (± 0.05) and $\pi_s = 0.009$ (± 0.001), respectively.

Tests for selective neutrality and demographic equilibrium in the East African populations included Fu's F_S and Ramos-Onsins and Rozas' statistic R_2 (Table 3). These tests can be used to detect recent demographic expansions or contractions because they cause 'waves' in

mismatch distributions. Two such waves are apparent in Fig. 2. All tests were consistent, however, with the null hypothesis thereby providing no statistical support for demographic disequilibria arising from earlier bottlenecks or selective sweeps. Estimates of θ support the suggestion that the Ugandan population was about four times as large as the Kenyan populations (Table 3).

Laboratory-cultured flies

In CAR-lab flies, 27 haplotypes (lineages) were recorded, 20 of which were detected in the sample of 48 only once (Table 1). The proportion of singletons in the laboratory culture was much greater than that in the field populations (74% vs. 43%, respectively; $\chi^2_{(2)} = 4.81$, $P < 0.03$). Diversities in the laboratory flies were similar to diversities in Ugandan populations, but the number of haplotypes was much greater. The number of lineages is consistent with a recent population expansion. The mismatch distribution shows clear departures from neutral expectations (Fig. 3) that are statistically significant according to the F_S and R_2 statistics (Table 3). The pattern of mitochondrial gene sequence variation is consistent with laboratory records that show the *G. f. fuscipes* culture was maintained for *c*. 115 generations at *c*. 325–1700 females until 2001, when it was increased to approximately 11 000 females (A. G. Parker, year?, personal communication to E.S.K.). Some eight generations later, genetic samples were taken.

Relative effective population numbers

The estimate of θ for the laboratory culture (8.4; Table 3) provides a measure of relative population numbers (sizes), assuming mutation-drift equilibria and equal mutation rates. This number, 8.4, can be related to the effective numbers in the tsetse stock culture. There were approximately 11 000 females at the time of sampling, but the culture's effective number is related to its harmonic mean over the 117 generations of its establishment. Thus, we estimate the laboratory culture N_e to be *c*. 1060. Kenya θ, at 3.2 (Table 3), was 0.38 that of CAR-lab. Uganda θ was 17.8, 2.13 times the laboratory culture estimate. We can then estimate N_e to a first approximation for the Kenyan and Ugandan *G. f. fuscipes* populations at 403 and 2258 female adults, respectively.

Discussion

Averaged over all field samples, mitochondrial diversity in *G. f. fuscipes*, at 0.84, was similar in magnitude to that found in *Glossina morsitans morsitans* Westwood (0.81), *Glossina m. submorsitans* in Ethiopia (0.84), *Glossina pallidipes* Austin in Tanzania (0.82), but not *Glossina swynnertoni* Austin (0.59), all in the *morsitans* group (Krafsur *et al.*, 2000; Ouma *et al.*, 2005, 2006; Marquez *et al.*, 2006). The chief determinants of diversity are mutation rates and effective population numbers. The greater diversities in the Ugandan sample, only ~ 55 km from the Kenyan populations, suggested much larger effective population numbers. That inference is supported by the θ estimates that we use to estimate relative effective population sizes among the three populations. Why the great difference? Declining *G. f. fuscipes* numbers in Kenya must be considered, given high human populations on the Kenyan side of the border and ongoing loss of natural habitats. We obtained no statistical evidence for bottle-necks or recent population expansions because the neutrality tests were consistent with hypotheses of selective neutrality and demographic equilibrium.

Mitochondrial mutation rates are known to be substantial and inherited matrilineally, leading to rapid lineage sorting (Avise, 1994). Effective population numbers of tsetse flies are generally very much smaller than those of more common Diptera such as *Drosophila* spp., mosquitoes, house flies and other common, noxious species. Genetic drift is inversely proportional to effective population size. The chief demographic reason for comparatively smaller tsetse

populations is their low reproductive rates – a single fed female must live nearly a month before she can deposit two mature larvae. The low reproductive rates are compensated, however, by longevities that greatly exceed those of other Diptera.

According to the exact test of Raymond & Rousset (1995), mitochondrial variation in the two Kenyan populations differed by more than chance alone. The populations were only *c.* 32 km apart and each was highly differentiated from the Ugandan population sample. This level of differentiation results from the patchy distribution of *G. f. fuscipes* populations that seem more typical of savannah-inhabiting tsetse flies whose demes seem to form islands (Krafsur, 2003). It can be assumed that the Kenyan and Ugandan populations are sympatric by virtue of sharing the same river drainage formation (the rivers Malaba and Sio).

In the riverine and lacustrine tsetse taxa, wet season dispersion from dry season refugia is thought to establish demes in which genetic drift leads to differentiation. Later, the retreat of flies to dry season refugia downstream can lead to mixing with survivors of formerly differentiated demes. Such a view is supported by the work of Cuisance *et al.* (1985), who used mark-release-recapture methods to demonstrate dispersals of *Glossina palpalis gambiensis* up to 21 km along gallery forests in Burkina Faso and related studies showed dispersals up to 5.1 km across savannahs and watersheds. Genetic support for mixing of genetically differentiated demes (the Wahlund effect, indicative of non-random mating within demes) can be provided by detecting significant heterozygote deficiencies in samples. Indeed, heterozygote deficiencies were detected at microsatellite loci in *G. p. gambiensis* in Burkina Faso (Solano *et al.*, 1999) and *Glossina p. palpalis* in Cote d'Ivoire (Ravel *et al.*, 2007). A deficiency of heterozygotes will also occur if two or more reproductively isolated cryptic species are sampled. Spatial examination of mitochondrial variation in *G. p. gambiensis* in Senegal and Mali demonstrated significant genetic differentiation among demes in different watersheds (Marquez *et al.*, 2004). To these examples may be added *G. f. fuscipes* in East Africa, demes of which share little mitochondrial variation in common, even in the same watershed. Would a continuous cycle of annual mixing of autosomal genotypes and mitochondrial haplotypes, followed by dispersion and genetic drift, lead to the degree of spatial differentiation observed in *palpalis* group flies? It seems doubtful, but the question requires sophisticated simulation studies for proper evaluation. For now, it seems that *palpalis* group taxa show population structures that are not greatly different from those of *morsitans* group flies.

Experimental studies in Uganda have indicated high dispersal rates in male *G. f. fuscipes*, random movements of 338 m/day; for *G. fuscipes* and *morsitans* group tsetse; analysis suggested a mean displacement rate of 252 m/day (Rogers, 1977). Estimates of tsetse dispersal distances vary and are contentious because the underlying assumptions of random diffusion models do not always apply in the field (e.g. Hargrove, 1981). It is clear, however, that tsetse have the capacity to disperse far and wide. Such dispersal rates among the *G. f. fuscipes* populations sampled here would predict a rapid fusion of Kenyan and Ugandan populations. It seems, however, that forces of genetic drift in East African *G. f. fuscipes* are very much stronger than gene flow. Its dispersal tendencies seem to be either overestimated or thwarted by environmental circumstances, unapparent to us, in the habitats lying between the three sampled populations. It will be interesting to learn the population structure of this species in the central part of its range.

None of 49 composite haplotypes were shared between the IAEA CAR-lab sample and East African *G. f. fuscipes*. The CAR-lab flies had been in culture for more than 117 generations since the population's establishment, but there was no detectable evidence of a genetic bottleneck that greatly reduced its original mitochondrial diversity. The pattern of variation in the *G. f. fuscipes* laboratory culture, at least 16 years after its establishment, indicates rapid population growth, already known from insectary records, and numerous (27) lineages, also

indicative of rapid growth. The magnitude of mitochondrial diversity suggests that the colonization procedure was efficient in that existing genetic diversity was maintained. The much greater number of singular haplotypes in CAR-lab flies (20) than in Ugandan flies (9) testifies to highly effective insectary husbandry practices without which haplotypes in low frequency would be lost rapidly through drift. Careful husbandry is absolutely necessary in culturing tsetse flies because of their slow reproduction rates.

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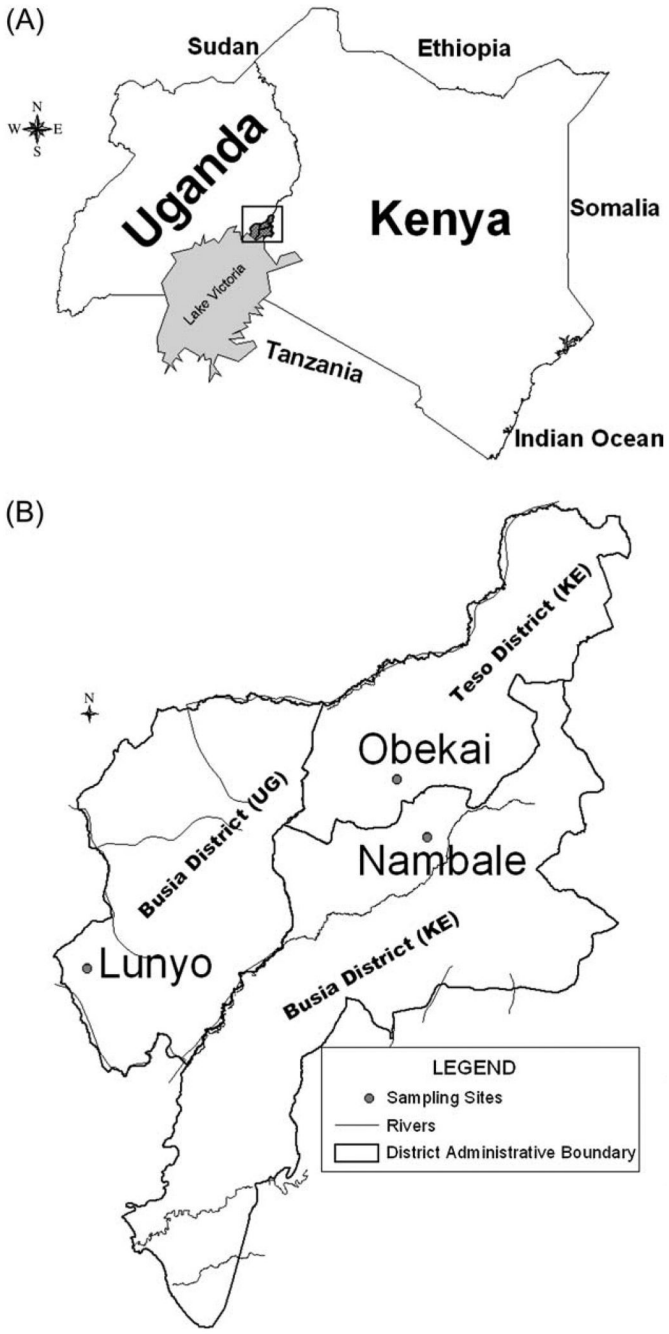

Fig. 1.
(A) Sampling region north of Lake Victoria. (B) Detail of sampling sites near the Uganda-Kenya border.

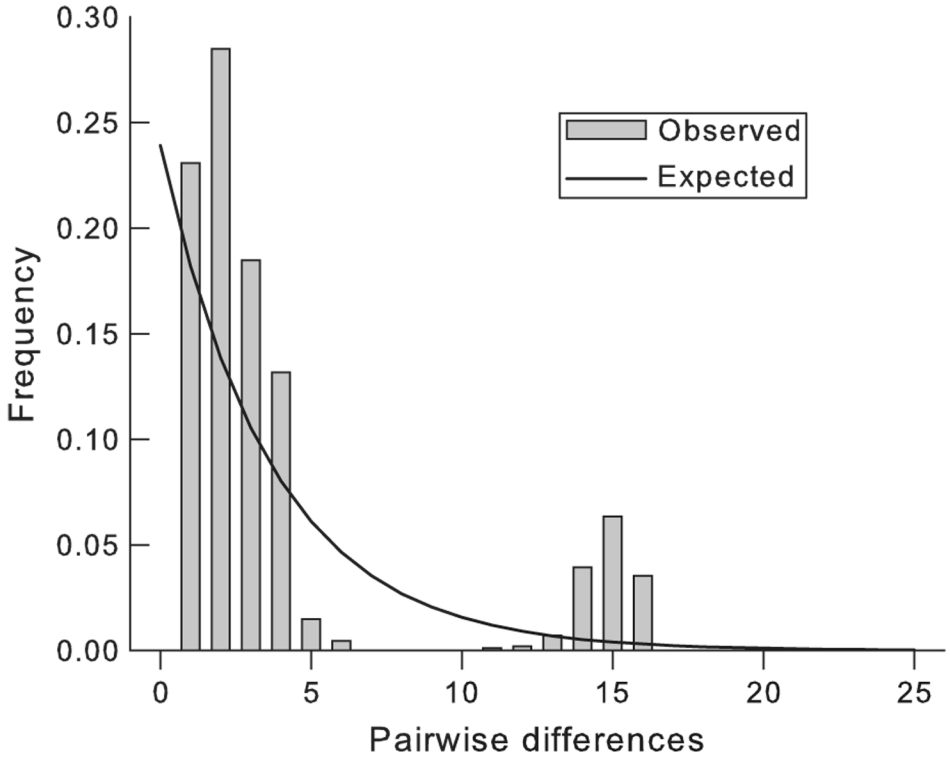

Fig. 2.
Mismatch distribution at mitochondrial loci in Kenyan *Glossina fuscipes fuscipes*.

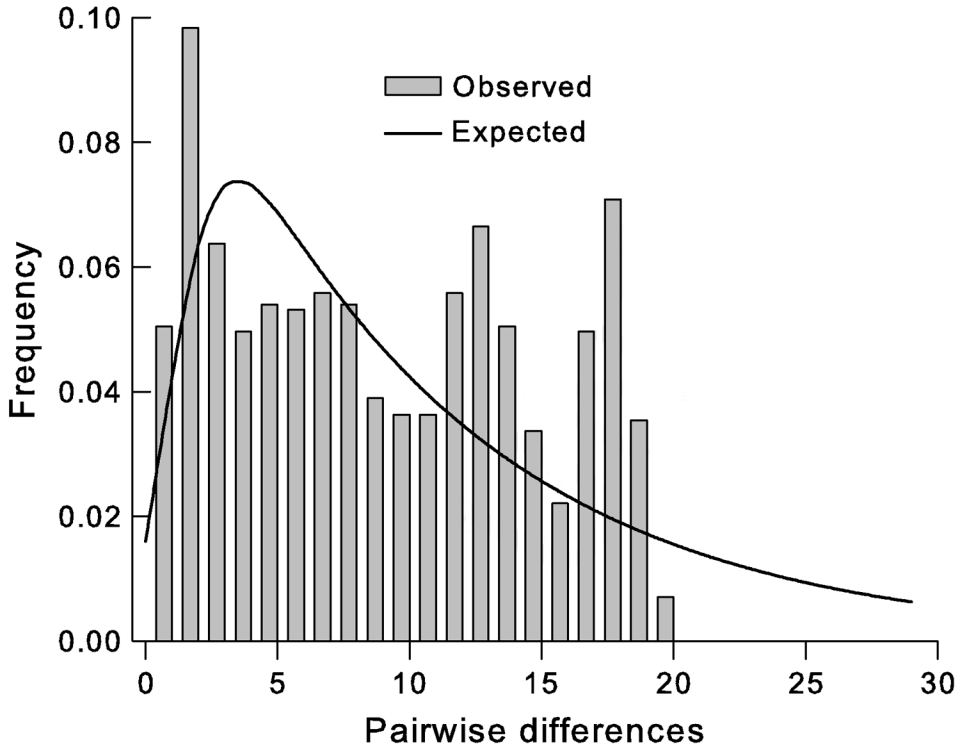

Fig. 3.
Mismatch distribution at mitochondrial loci in laboratory-reared *Glossina fuscipes fuscipes*.

Table 1

Glossina fuscipes fuscipes mitochondrial haplotype frequency distribution.

	Kenya		Uganda	Central African Republic	
Haplotype	**Nambale**	**Chakol Obekai**	**Lunyo**	**Laboratory**	**Totals**
1	1				1
2	13	5			18
3		1			1
4			1		1
5				4	4
6				1	1
7				1	1
8				1	1
9				1	1
10				2	2
11	1				1
12		2			2
13	21	3			24
14				3	3
15	1				1
16			2		2
17	1	2	3		6
18		2			2
19				1	1
20				1	1
21				1	1
22				2	2
23	2				2
24	2				2
25	5	1			5
26			2		2
27			2		2
28			2		2
29			1		1
30			1		1
31				1	1
32				1	1
33				1	1
34				3	3
35				1	1
36				1	1
37				1	1
38				1	1
39				1	1
40				1	1
41				6	6
42				1	1
43				1	1
44				1	1
45				8	8
46				1	1
47			1		1
48			1		1
n	47	16	16	48	127
Haplotypes, *n*	9	7	10	27	48
Variable Sites, *n*	17	17	53	22	—

Table 2

Number of haplotypes shared (upper diagonal) and genetic differentiation (F_{ST}) among samples (lowest diagonal).

	Chakol	Nambale	Uganda	CAR-lab
Chakol		4	1	0
Nambale	−0.026		1	0
Uganda	0.28	0.28		0
CAR-lab	0.58	0.46	0.58	

CAR-lab, Central African Republic laboratory strain.

Table 3

Indices of molecular variation in *Glossina fuscipes fuscipes* including haplotype (H_S) and nucleotide (π_S) diversities. Kenyan samples were pooled for tests for selective neutrality (R_2) and demographic equilibrium (F_s).

Sample	n	Mean pairwise differences (θ)	R_2 [*]	F_S [†]	H_S [‡]	π_S [§]
Chakol Obekai	16	3.18			0.87 ± 0.06	0.005 ± 0.001
Nambale	47	3.22	0.094	− 0.891	0.72 ± 0.05	0.005 ± 0.0006
Uganda	16	17.80	0.160	2.160	0.94 ± 0.04	0.027 ± 0.002
CAR-lab	48	8.36	0.189 [*]	−7.611 [¶]	0.95 ± 0.02	0.012 ± 0.0004

[*] Test according to Ramos-Onsins & Rozas (2002).

[†] Test according to Fu (1997).

[‡] Between field-collected samples: Student's $t = 13.12$, $P < 0.006$.

[§] Between field-collected samples: $t = 1.99$, $P = 0.19$.

[¶] $P < 0.001$.

CAR-lab, Central African Republic laboratory strain.