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ARTICLE

Microsatellite Polymorphism in Tsetse Flies (Diptera: Glossinidae)

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ABSTRACT In sub-Saharan Africa, tsetse flies are the vectors of trypanosomes, the causative agents of sleeping sickness in humans and nagana in animals. Certain wild populations of the *palpalis* group exhibit intraspecific variation and are suspect of manifest differences in vectorial capacity. The current study reports the identification of 13 polymorphic microsatellite loci from *Glossina palpalis palpalis* Robinean-Desvoidy. The majority of these markers amplify corresponding loci from the related species *G. p. gambiensis* Vanderplank, *G. f. fuscipes* Newstead, and *G. tachinoides* Westwood. Only seven of 13 loci were amplified from *G. austeni* Newstead. Genetic variability was estimated in one field population of *G. p. gambiensis.* These results confirmed that microsatellite markers may be used to examine the subpopulation structure of tsetse flies.

KEY WORDS *Glossina,* microsatellite, tsetse fly, sleeping sickness, population genetics

IN AFRICA, TSETSE flies transmit *Trypanosoma* spp. that cause sleeping sickness in humans and nagana in livestock. Political factors including war and migration recently have caused a dramatic increase in the incidence of sleeping sickness in Zaire, Uganda, Sudan, Angola, and Central African Republic, with more than 1 million cases estimated by the World Health Organization (WHO 1999). Because the prevalence of this disease is increasing concurrently in many areas, eradication or management of tsetse from 38 countries covering >10 million square kilometers seems impractical (Leak 1998).

Based on isozyme analysis, morphological and ecological criteria and nuclear DNA sequence analysis, the 31 taxa of *Glossina* have been placed into four groups: *Austenina* (=*fusca* group), *Nemorhina* (=*palpalis* group), *Glossina* sensu stricto (=*morsitans* group), and *Machadomyia* (=*austeni* group) (Chen et al. 1999). Relationships among different species using isozyme markers, microsatellite DNA, and 16S rDNA of endosymbiont bacteria indicated that phylogenetically the *fusca* group is the most primitive, followed by the *austeni, morsitans,* and then *palpalis* groups (Gooding and Moloo 1994, Leak 1998, Chen et al. 1999). Although the genetic basis of vector competence in tsetse is largely unknown, flies within the *morsitans* group are highly competent vectors, whereas those within both *austeni* and *palpalis* groups are somewhat refractory to infection by trypanosomes (Moloo and Kutuza 1988a, 1988b).

Despite their importance, limited information is available on the genetics of tsetse flies because their reproductive biology makes study difficult. Tsetses are viviparous, retaining one fertilized egg at a time within the uterus. A single larva hatches, matures into a fully developed third instar and is expelled from the female fly. Each female can deposit five to seven offspring during its 3- to 4-mo life span. Genetic linkage studies have been based on isozyme polymorphism (Gooding et al. 1993). Crosses among several subspecies resulted in various degrees of sterility (Gooding 1987, 1988). Population genetics using isozyme markers revealed little or no variation and a low frequency of polymorphic loci (Gooding 1981, 1982; Gooding et al. 1993). Recently, three microsatellite markers from *G. p. gambiensis* Vanderplank were found to be polymorphic in several species of tsetse flies (Solano et al. 1997). Development of a large set of microsatellite markers will provide a powerful tool to examine gene flow among subpopulations and subspecies. Polymorphic microsatellite markers may be used to examine population structure that could explain intraspecific variation and related differential vectorial capacity suspected to occur among wild populations (Reifenberg et al. 1996, 1997). These markers also may be used to develop a genetic map that could be used to locate other traits of interest.

In the current article we describe 13 microsatellite markers from *G. p. palpalis* that constitute an important tool to examine the population genetics of tsetse fly species. These microsatellites were highly polymorphic in laboratory colonies of *G. p. palpalis* and in field-collected specimens of *G. p. gambiensis, G. f. fuscipes* Newstead, *G. tachinoides* Westwood, and *G. austeni* Newstead. Population genetic analysis re-

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Table 1. Sources of tsetse flies

Species and strain	No. of individuals	Location and date	Source
G. p. palpalis	≈30	Laboratory colony	A. Robinson, IAEA
G. p. palpalis Tan	5	Laboratory colony	R. Goodings, University of Alberta
G. p. palpalis Brick	5	Laboratory colony	R. Goodings, University of Alberta
G. p. gambiensis	34	Burkina Faso, December, 1995	CIRDES
G. tachinoides	10	Burkina Faso, December, 1995	CIRDES
G. g. fuscipes	10	Central African Republic, May 1998	Medical Emergency Relief International
G. austeni	10	Shimba Hills region in Kenya, June, 1999	S. O'Neill, Yale University

IAEA, International Atomic Energy Agency, Seibersdorf, Austria. CIRDES, Centre International de Recherche-Development sur l'Elevage en zone Subhumide.

vealed a phylogenetic tree that is consistent with that derived from DNA sequence analysis.

Materials and Methods

Fly Samples. The numbers of individuals of each species and strain and the source are summarized in Table 1.

Isolation of Microsatellite Loci from a Partial Genomic Library. Standard molecular biology (Sambrook et al. 1989) procedures were followed. Genomic DNA of *G. p. palpalis* was prepared from 30 individuals from a colony established at International Atomic Energy Agency (IAEA), Seibersdorf, Switzerland, as described by (Cheng and Aksoy 1999) and digested with *Sau3*AI. Fragments with an average size of 500 bp (bp) purified from agarose gel were cloned into *Bam*HI site of pBluescript (Stratagene, La Jolla, CA) and transformed into *Escherichia coli* DH5α competent cells. Approximately 6,000 recombinant colonies were lifted onto nitrocellulose membranes (Millipore, Bedford, MA) and screened using radioactively (^{32}P) end-labeled oligonucleotides $(GA)_{15}$ and $(GT)_{15}$ as described by Zheng (Zheng 1997). Forty putative clones were rescreened by Southern hybridization, resulting in 29 positive clones. Double stranded DNA was prepared from positive colonies and sequenced by the dideoxynucleotide termination method (Sanger et al. 1977). Eighteen microsatellite clones confirmed by sequencing are listed in Table 2. The primer pair for marker 55.3 developed for *G. p. gambiensis* by Solano et al. (1997), 5′-GTACTCAACGTGGTGCTTAAAGTTG-3′ and 5′-GTCTGAGATAGGACCATTTATCG-3′, was used to examine *G. p. palpalis* and was found to yield variable levels of amplification by polymerase chain reaction (PCR). PCR products with this primer pair using genomic DNA from one *G. p. palpalis* fly as a template were cloned in pGEM-T (Promega, Madison, WI), sequenced and yielded *Pgp1.* Nucleotide sequences of these clones were deposited in GenBank (accession numbers AY008353-AY008370).

PCR Reactions and Genotype Scoring. Pairs of oligonucleotides were designed for 16 clones using the computer software "Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Microsatellite amplification was performed using PCR with one of the primer pair radioactively end-labeled with γ-^{32}P ATP. PCR products were resolved on 6% denaturing polyacrylamide gels, and the bands were visualized by autoradiography on X-ray films (Zheng 1997).

Data Analysis. The number of alleles per locus, the observed and expected heterozygosity, deviations from Hardy–Weinberg equilibrium expectations and linkage disequilibrium were computed using the GENEPOP version 3.1 software (Raymond and Rousset 1995). The test of Hardy–Weinberg for <5 alleles is an exact test. For ≥ 5 alleles, a Markov chain is used to obtain an unbiased estimate of exact probability. Genotypic linkage disequilibrium between pairs of loci was estimated using the common correlation coefficient.

The frequency of null alleles (N*o*) was estimated for a natural population of *G. p. gambiensis* (Table 1) presumed to be in Hardy–Weinberg equilibrium according to Brookfield (1996): $He\text{-}Ho/he+1$, where H*e* and H*o* are the expected and observed heterozygote frequencies, respectively. The inbreeding coefficient, F*is*, and the F*st* index were calculated using GENEPOP. A heterozygote deficiency is indicated by $Fis > 0$. For a subpopulation in Hardy–Weinberg equilibrium, $Fis = 0$. Differentiation between populations was measured using Wright's *F*-statistics (Weir and Cockerham 1984). F*st* values range from 0 to 1, the greater the value, the greater the differences among populations.

Results

Identification and Characterization of Microsatellite Loci. A total of 18 microsatellites were isolated from the genomic library of *G. p. palpalis* and one (*Pgp1*) was redesigned based on the corresponding locus in *G. p. gambiensis* described in Solano et al. (1997). Seventeen of 18 microsatellites contained $(CA)_n/(GT)_n$ repeat arrays (Table 1). The only exception was one TA repeat in a clone originally positive by GA/GT screening. Three repeat sequences were not used because the repeat arrays were too close to the end (*Pgp40*) or too complex and too long (*Pgp15*) for the design of the reverse primer. Clone *Pgp14* contained too few repeat arrays and most likely was not polymorphic.

Eight of 18 loci were classified as perfect repeats (*Pgp1, 8, 11, 17, 20, 22, 35, 40*), eight as imperfect repeats (*Pgp13, 14, 16, 28, 29, 33, 34, 38*), and three as compound repeats (*Pgp15, 24, 37*) (Weber 1990).

Table 2. Molecular structure of 18 microsatellite loci from *G. p. palpalis*

Locus	Repeats	Type of repeat	Primers (5′-3′)	Allele Size (bp)	No. of alleles
Pgp1[a]	$(GT)_9$	P	TCTCACGCAATTAAAATCGG TGTTGAGTACACTTTTAAGCCTATGA	124	14
Pgp8[a]	$(GT)_{10}$	P	GTACGTGTGAGGGCCAGAAT TTGAAAATCCATCCCGCTAT	192	8
Pgp11[a]	$(GT)_{10}$	P	GTGCGTGGTTGTCTCTGTGT GCGTTTCGAAAGGAGTAACG	178	10
Pgp13[a]	$(GT)_{17}TT(GT)_3$	I	AAAATAACGAACGGGTGCAG GACGCACGCACATAGTTACG	201	6
Pgp17[a]	$(GT)_{21}$	P	TGGCAAACTCTTCCATGTTT AAGGCGGCTCTCTTCTAAGC	191	6
Pgp20[a]	$(GT)_{11}$	P	TTCTCGCTCTCTCCATTCCT TGTACAGTTGACCCGTCGTC	194	8
Pgp22[a]	$(GT)_9$	P	GCTACTGCGACTGCTACTGC CAAGGAATTGGTTCTGCCAT	279	9
Pgp24[a]	$(TA)_6CA(TA)_3T(GT)_{16}$	C	AATTCCCGCCGGTAAAGTT TGTTTGCGTATGCATCTGTG	215	11
Pgp28[a]	$(GT)_4GC(GT)_7$	I	TCAAATTGTTCCCATCAAGGA ATCGTTTTTAAAGGGTTTTAAGTTT	103	11
Pgp29[a]	$(GT)_3TT(GT)_6TT(GT)_3$	I	CGCAATTAAGTCGTGCAATG GCGTGATAGTTTGCGACGTA	237	12
Pgp33[a]	$(GT)_8AT(GT)_3$	I	TGTTTGCACCCATTCTACCA CACAAAACCCAGGCAAGTTT	208	4
Pgp34[a]	$(GT)_6TG(GT)_7$	I	GGCAACTTAATGCCCATAA CAGCAAAAGGCCAATTTTGT	364	6
Pgp35[a]	$(GT)_{14}$	P	AATACGGGCATGGATAAGCA TCGGAGGAGAGCTTTTTCAG	202	13
Pgp38[a]	$(TA)_2TC(TA)_4TC(TA)_2CA(TA)$	I	GGCCCTCATCAAAACAAAA CACATAAAAAGGGCGAGCAA	225	1
Pgp37	$(GT)_3TT(GT)_{11}G(CACG)_7$	C	TGTCATGTGTTGCGGGTACT TTTAACGATGGGCGTGGTAT	217	
Pgp14	$(GT)_4ATGG(GT)_3$	I			
Pgp16	$(GT)_3A(GT)_8$	I			
Pgp40	$(GT)_{10}$	P			

P, perfect arrays; I, imperfect arrays, and C, complex arrays. The fifth column indicates the size of the allele sequenced. Numbers in the sixth column represent the total allele number obtained within all the individuals.
[a] Markers that were genotyped.

With the exception of *Pgp37* that harbored a tetranucleotide array immediately adjacent to a dinucleotide repeats, all the sequences contained dinucleotide repeats (Table 2). The average length of the perfect array was 12.2 units and the maximum uninterrupted dinucleotide was 21 (*Pgp17*). Primer pairs were designed for 15 microsatellite sequences.

Conservation of Flanking Primer Sequences. Microsatellite polymorphism was examined in individual tsetse from two laboratory strains (five adults each) of *G. p. palpalis* and from field-collected adults of *G. p. gambiensis* (34), and 10 each of *G. f. fuscipes*, *G. tachinoides*, and *G. austeni*. *Pgp16* and *37* failed to amplify in most populations, even when new primers were designed (data not shown). *Pgp38* was monomorphic in all six populations. The other 13 microsatellites showed polymorphism in at least one population. Only five loci (*Pgp24*, *28*, *29*, *34*, and *35*) were amplified in all of the species examined.

All 13 loci were amplified in closely related *G. p. gambiensis*, *G. p. palpalis*, and *G. f. fuscipes*. A majority (85%) could be amplified in *G. tachinoides*, whereas only 54% could be amplified in *G. austeni* (Tables 3 and 4).

Although all 13 loci were amplified in *G. f. fuscipes*, between 20 and 80% of individuals did not amplify with most of the primers. This also was found with other species but at a lower value. However, for loci *Pgp8* and *Pgp13* in *G. p. gambiensis*, DNA from ≈30% of individuals could not be amplified (Table 3).

Number of Alleles. The average number of alleles per locus ranged from 2.0 in *G. tachinoides* to 5.5 in *G. p. gambiensis*. The highest number of alleles (12) at locus *Pgp1* was observed in *G. p. gambiensis*. The average number of alleles for all loci per individual per species decreased in order from *G. p. palpalis*, *G. f. fuscipes*, *G. austeni*, *G. tachinoides* to *G. p. gambiensis* (Table 4).

Population Structure. Allele frequencies were calculated from genotypes for each of the 34 *G. p. gambiensis* to estimate genetic variability in a natural population. The inbreeding coefficient values (F_{is}) were positive at 11 of 12 loci in *G. p. gambiensis*, indicating extensive heterozygote deficiencies. Three loci (*Pgp1*, *13* and *22*) exhibited significant departures from Hardy–Weinberg expectations, primarily due to heterozygote deficiencies (Table 3). These deficiencies may be due to null alleles present at locus *Pgp13* (see below).

The frequency of null alleles for loci *Pgp13* and *Pgp34* were estimated at 37 and 38%, respectively. Other loci (*Pgp8*, *20*, *22*, and *28*) had null allele frequencies between 10 and 20%. Although the inbreeding coefficient and Hardy–Weinberg departure indi-

Table 3. Genetic variability at 13 loci in *G. p. gambiensis*

Loci	No. alleles	H	Het(ob)	Het(ex)	F*is*	H-W(P)	*n*	No
Pgp1	12	0.87	27	27.44	0.016	0.022	31	0.015
Pgp8	5	0.43	10	15.23	0.206	0.156	23	0.0187
Pgp11	6	0.72	23	25.44	0.097	0.252	32	0.092
Pgp13	4	0.37	9	14.77	0.395	0.006	24	0.366
Pgp17	4	0.53	17	18.97	0.105	0.131	32	0.098
Pgp20	6	0.48	16	20.25	0.212	0.122	33	0.2
Pgp22	8	0.59	19	23.2	0.184	0.001	32	0.174
Pgp24	6	0.57	19	19.91	0.046	0.068	33	0.043
Pgp28	3	0.12	4	4.76	0.162	0.146	34	0.132
Pgp29	6	0.62	21	22.09	0.05	0.307	34	0.047
Pgp33	1	—	—	—	—	—	32	—
Pgp34	4	0.28	9	15.25	0.414	0.047	32	0.385
Pgp35	7	0.72	24	22.44	0	0.713	33	—
Mean1	5.5							
Mean2	2.1							

Column 2 and 3 show the number of alleles and heterozygote frequency, respectively. The observed [Het(ob)] and the expected heterozygotes [Het(ex)] are shown in the fifth and sixth columns, respectively. Confirmation to Hardy–Weinberg equilibrium is shown in the seventh column. The eighth column indicates the number (N) of amplified individual at each locus. Estimated null allele frequency (No) are shown in the ninth column. The average numbers of alleles per locus (mean1) and per individual fly (mean2) are shown. —, sign indicates calculation cannot be performed.

cated extensive heterozygote deficiency, most loci exhibited a high level of polymorphism with H ranging from 12 to 87% (Table 3).

Linkage disequilibria were detected between the loci *Pgp20/Pgp22, Pgp22/Pgp24, Pgp20/Pgp34, Pgp22/Pgp34, Pgp17/Pgp24,* and *Pgp28/Pgp35* (data not shown). These results indicated departures from random mating (Wahlunds effect). Alternatively, selection of genes linked to these microsatellite markers also could have contributed to the observed linkage disequilibria.

The analysis of the inbreeding coefficient in the other species showed two loci with a complete absence of heterozygotes (F*is* = 1), *Pgp20* and *13* in *G. tachinoides*, and *Pgp13* in *G. f. fuscipes* (Table 4). The *G. tachinoides* individuals analyzed were all males, indicating that both *Pgp13* and *20* could be located on the *X* chromosome. Locus *Pgp13* failed to amplify in 70% of the *G. f. fuscipes* individuals probably due to null alleles. Because of the low number of individuals of *G. f. fuscipes* and *G. tachinoides* ($n = 10$) analyzed, we could not eliminate sampling artifacts as a possible explanation for these observations.

Discussion

We have identified 18 clones with microsatellite sequences from a small genomic library of 6,000 clones with an average size of 500 bp. The frequency of microsatellite markers in the genome of *G. p. palpalis* was estimated to be one every 160 kilobases, similar to that estimated for *Anopheles gambiae* Giles, the principal vector for malaria (Zheng et al. 1993). A recent survey of end sequences obtained from a library of

Table 4. Genetic variability in four *Glossina* taxa

	G. fuscipes ($n = 10$)			*G. p. palpalis* (Tan) ($n = 5$)			*G. p. palpalis* (Brick) ($n = 5$)			*G. tachinoides* ($n = 10$)			*G. austeni* ($n = 10$)		
locus	No. alleles	F*is*	H-W (P)	No. alleles	F*is*	H-W (P)	No. alleles	F*is*	H-W (P)	No. alleles	F*is*	H-W (P)	No. alleles	F*is*	H-W (P)
Pgp1	2	0	1	3	0	0.771	2	0	1	1			NA		
Pgp8	1			1			1			NA			2	0	1
Pgp11	2	0	1	2	0.273	0.619	1			NA			5	0.311	0.692
Pgp13	2	1	0.333	1			2	0	1	2	1	0.077	NA		
Pgp17	4	0	0.931	2	0	1	3	0.733	0.048	1			NA		
Pgp20	4	0.636	0.011	2	0	1	2	0	1	3	1	0.001	NA		
Pgp22	1			2	0	1	2	0	1	2	0	1	NA		
Pgp24	2	0	0	2	0		3	0.448	0.142	1			3	0.079	0.189
Pgp28	6	0.13	0.387	2	0	0.771	2	0.667	0.238	3	0	0.482	3	0.794	0.007
Pgp29	3	0.647	0.077	3	0		3	0	1	2	0	1	5	0.167	0.305
Pgp35	7	0.385	0.047	2	0		2	0	1	1			2	0	1
Pgp33	1			1			2	0.667	0.238	3	0	0.776	NA		
Pgp34	1			5	0	1	3	0	1	3	0.105	0.471	4	0	1
Mean1	2.8			2.2			2.2			2.0			3.4		
Mean2	3.6			5.6			5.6			2.2			2.4		

Number of alleles, inbreeding coefficient (F*is*) and Hardy–Weinberg equilibrium probability are shown in the three columns for each species/populations, respectively. The average numbers of alleles per locus or per individual fly are listed as mean1 and mean2, respectively. N, sample size of each population analyzed. Empty entry indicates calculation not applicable. NA, not amplified.

bacterial artificial chromosome clones revealed that microsatellite markers gave a similar estimate of the frequency of microsatellite markers as the *An. gambiae* genome (unpublished data).

Thirteen microsatellite markers were characterized in detail for *G. p. palpalis* and four other tsetse species. The degree of conservation of primer sequences defined by the microsatellites of *G. p. palpalis* is probably a reflection of their close phylogenetic relationship. Increasing phylogenetic distance may lead to the accumulation of mutations in the flanking region, yielding "null alleles" or unusable markers (Harr et al. 1998). All of the microsatellite loci could be amplified from the three closely related species of the *palpalis* group: *G. p. palpalis, G. p. gambiensis,*and *G. f. fuscipes.* These species also exhibited the greatest similarity at isozyme loci (Gooding 1981). Overall, 85% of the loci could be amplified from *G. tachinoides,* which also belongs to the *palpalis* group. The most distantly related species, *G. austeni,* had only 54% of the loci amplified. Previous phylogenetic studies based on isozymes (Gooding 1981) and on genomic DNA analysis using the 408 bp of the ITS2 region (Chen et al. 1999) also indicated that *G. austeni* was the most distantly related among the tsetse species examined here.

Previous studies comparing homologous loci in different species demonstrated that higher polymorphism and a greater number of alleles were found in the focal species, in which the primers were characterized first (Jarne and Lagoda 1996, Hutter et al. 1998). In this study the focal species, *G. p. palpalis,* had a greater number of alleles when the number of individuals were taken into consideration. The extent of polymorphism of field samples of *G. p. palpalis* could be even higher. The high degree of polymorphism could be somewhat masked by mutations in the flanking sequences that also could lead to null alleles or homoplasy. Complex mutations at corresponding loci from distantly related species, given sufficient time, could lead to alleles of apparent identical sizes, but with different sequences or the same sequence with a different history (Estoup et al. 1995, Garza and Freimer 1996, Viard et al. 1998).

A natural population of *G. p. gambiensis* showed heterozygote deficiency that could be due to (1) extensive inbreeding, (2) null alleles, or (3) Wahlund effect, when sympatric populations are sampled as a single population. Heterozygote deficiency also had been detected at two microsatellite markers in one population of *G. p. gambiensis* from Burkina Faso (Solano et al. 1999). Excessive consanguineous matings could contribute to positive inbreeding coefficients for 11 of 12 loci examined here. Null alleles are frequent in population studies using microsatellites in other organisms (Dumas et al. 1998, Kamau et al. 1998). In *G. p. gambiensis* six of 12 loci had estimated null allele frequencies from 10 to 38%. However, this heterozygote deficiency also could be an indication of a population structure in *G. p. gambiensis,* because this population also had extensive linkage disequilibria. Linkage disequilibrium could be due to the lack of recombination, in this case five out of 12 loci should be on the same chromosome, or could be explained if populations that differ in allelic frequencies across several loci are pooled. Examination of larger natural populations will be necessary to address heterozygote deficiencies.

Glossina p. gambiensis showed great genetic plasticity of behavior in populations from Burkina Faso and great variability in their ability to transmit *Trypanosoma* (Solano et al. 1996) that could reflect its subpopulation structure. Solano et al. (1999) also detected significant genetic differentiation among three populations of *G. p. gambiensis* from Burkina Faso and Senegal. The high level of allelic diversity detected using microsatellite in this group of species, with an average of 9.08 alleles over 13 loci, confirmed the value of microsatellites as tool for population studies. Future studies with larger field samples of different tsetse fly species would be helpful in elucidating the population structure of tsetse flies.

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