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Cytogenetic and isozymic comparisons of two laboratory lines of *Glossina palpalis gambiensis*

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The genetics of two laboratory colonies of *Glossina palpalis gambiensis* were characterized by C-banding and isoenzyme studies. The colonies, derived from flies collected in the same locality, had different histories in the laboratory and different susceptibilities to trypanosome infection. Although the two lines were also found to differ in the frequencies of chromosome and isozyme variants, the variation was not enough to put their specific status in doubt; it was probably the result of genetic drift since the foundation of the colonies.

There are indications that intraspecific genotypic variability in tsetse flies might play a part in their susceptibility to infection and transmission of trypanosomes (see Maudlin, 1991). Genetic characterization of the laboratory colonies of tsetse used in studies on vectorial capacity may help answer two important questions. Firstly, is the insect used in the laboratory comparable with the wild-caught insect, so that the results of laboratory studies can be usefully applied to the field? Secondly, do intraspecific variations in genotype affect a fly's ability to transmit trypanosomes?

Genetic differences are already known between geographical populations and between wild populations and laboratory-bred colonies of *Glossina* spp. (Jordan *et al.*, 1977; Van Der Geest *et al.*, 1978; Southern, 1980, 1981; Southern and Pell, 1981; Van Etten, 1982; Langley *et al.*, 1984; Agatsuma and Otieno, 1988; Kence *et al.*, 1990; Tarimo Nesbitt *et al.*, 1990; Gooding and Jordan, 1986; Gooding *et al.*, 1991). Any association between these differences and vectorial capacity remains to be established, although such an association may explain why experimental cyclical transmission of trypanosomes often gives very variable results (Maudlin, 1980; Le Ray, 1989). In the present study, the genotypes of two colonies of *G. palpalis gambiensis*, which appear to have very different susceptibilities to infection, were compared.

MATERIALS AND METHODS

Tsetse Flies

The two lines of *Glossina palpalis gambiensis* Vanderplank 1949 used, BO and MA, are maintained on guinea pigs in the Division for Sleeping Sickness, Centre for Medical Entomology, Prince Leopold Institute of Tropical Medicine, Antwerp. Although both lines developed from flies collected in the

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same locality in Burkina Faso, they have very different histories (Elsen *et al.*, 1993).

The MA line, initiated in 1990 using 547 pupae from a colony established at Maisons-Alfort, Paris, in 1972, now has 1500 productive females.

The BO line was initiated in 1986–1987 using 300 pupae, 200 from the membrane-fed colony of the CRTA, Bobo-Dioulasso, Burkina Faso (derived from wild-caught flies and the Maisons-Alfort colony) and 100 from the rabbit-fed colony at ILRAD, Nairobi, Kenya (derived from the CRTA colony). All adults alive 2 years after the initiation of the line were killed because of insecticide contamination but the colony was restored using the available pupae. The colony had 1000–2000 productive females between September 1989 and April 1991 but has since been reduced in size to about 500 productive females. The BO line has had a much more varied and unstable history than the MA line.

Cyclical Transmission

Teneral females and males of both lines, kept at 25°C and 80% relative humidity, were allowed to feed on rabbits infected with the Sb020588 stabilate of *Trypanosoma brucei gambiense* strain 'MBA' and then maintained on these infected hosts up to day 29. They were then fed on non-infected rabbits to days 39 or 40 and dissected on day 42.

Genetic Markers

Two types of genetic markers, isoenzymes and chromosome C-banding, were used.

ISOENZYME ELECTROPHORESIS

Thirteen enzymes were studied using cellulose acetate plates: esterase (EST; E.C. 3.1.1.1), phosphoglucomutase (PGM; E.C. 2.7.5.1.), glucose-phosphate isomerase (GPI; E.C. 5.3.1.9), hexokinase (HK; E.C. 2.7.1.1), aconitate hydratase or aconitase (ACON; E.C. 4.2.1.3), peptidase leu-leu-leu (PEP1; E.C. 3.4.11), peptidase leu-leu-ala (PEP2; E.C. 3.4.13), α-glycerol phosphate dehydrogenase (GPD; E.C. 1.1.1.8), isocitrate dehydrogenase (IDH; E.C. 1.1.1.42), malic enzyme (ME; E.C. 1.1.1.40), malate dehydrogenase (MDH; E.C. 1.1.1.37), 6-phosphogluconate dehydrogenase (6PGD; E.C. 1.1.1.44) and leucine amino peptidase (LAP; E.C. 3.4.11).

Only the head and thorax of each fly were used for most enzymes (see Table 1), since preliminary experiments showed that results for the abdomen may be different from those for the thorax. Histochemical reactions (Table 1) and running conditions (Table 2) were adapted from those of Dujardin and Tibayrenc (1985) and Richardson *et al.* (1986).

C-banding

The C-banding in the cervical ganglions and testes (when present) from 7-day-old pupae was prepared and stained with Giemsa by a modification of Sumner's method [described in MacGregor and Varley (1983)]. Pupae were extracted from puparia and the ganglions and testes dissected out in physiological saline. The organs were transferred to freshly prepared 50% glacial acetic acid for 5–10 min, then placed on a slide with a drop of the same solution, triturated and squashed under a coverslip. The slide was then immediately frozen with solid CO_2 or liquid nitrogen and the coverslip removed with a scalpel blade. The preparation was allowed to air dry for 30–45 min, then submerged in 1 M HCl at room temperature for 15 min, distilled water for 2–3 min and then freshly prepared 5% (w/v) barium hydroxide at room temperature for 12 min. The slide was shaken gently from time to time in the barium hydroxide before being thoroughly rinsed, first in 5% glacial acetic acid, then twice in 2·5% glacial acetic acid and finally with abundant distilled water. Each slide was then transferred to a bath of 2 × SSC (8·8 g trisodium citrate 2-hydrate and 17·5 g NaCl in 1 litre distilled water, adjusted at pH 6·8–7·0 with crystallized citric acid), prewarmed to 60°C, for 30 min. After two or three rinses in distilled water, each for 3 min, the slides were stained with Giemsa (4% Merck or 2% Gurr) for 90 min. Finally, the slides were air dried and mounted in Neutral or Euparal.

Numerical Analysis

The general formula $h=2N(1-\Sigma x^2)/(2N-1)$ was used for estimating the genetic

TABLE 1
Isoenzyme electrophoresis conditions

Enzyme	*Migration buffer**	*Run time (min)*	*Application†*
IDH	Tris/citrate	18	Cathode
MDH	Electra (Helena)	24	Central
ME	Tris/versene/borate	18	Cathode
αGPD	Tris/citrate	18	Cathode
6PGD	Tris/citrate	18	Cathode
PGM	Tris/maleic	18	Cathode
GPI	Tris/citrate	18	Cathode
HK	Tris/versene/borate	18	Cathode
ACON	Tris/versene/borate	18	Cathode
Esterase	Tris/maleic	18	Cathode
LAP	Tris/versene/borate	24	Cathode
PEP1	Electra (Helena)	18	Cathode
PEP2	Electra (Helena)	24	Central

*The Tris/citrate and Tris/versene/borate buffers were as described by Shaw-Prassad (1970) and the Tris/maleic as described by Kreutzer *et al.* (1977).

†An extract of the abdomen (LAP, PEP1, PEP2) or of the head and thorax of a fly (all other enzymes) was applied in the position indicated.

and chromosomal variability (h); this is a measure of the 'expected heterozygosity' or 'genetic diversity' (Selander and Levin, 1980; Nei, 1987) when computed from electrophoretic data and of the 'C-banding diversity' when estimated from chromosomal markers. According to the marker used, it is the probability of sampling, from the same population, two individuals of different genotype or C-banding type.

An additional estimation of diversity that was used, based on the variety of different genotypes found respective to the size of the sample scored (Ben Abderrazak, 1993), is given by the equation $G=\Sigma g/n$, where n is the number of individuals examined and g the number of categories of genotypes (G_e and g_e referring to the different enzymatic multilocus genotypes and G_c and g_c to the different categories of C-banding cytotypes).

Nei's standard genetic distance (Ds) (Nei, 1972) and the rate of polymorphism (Pm, the proportion of variable loci; Nei, 1987) were only calculated for isoenzymes.

Hedrick's similarity coefficient (Hedrick, 1971), based on a probabilistic approach, was applied to isoenzymes (S) and to C-banding (S_c), following Panzera *et al.* (1992) for Triatomine bugs.

Hardy–Weinberg equilibrium was tested by a χ^2 goodness of fit, which was corrected according to Yates.

The association between isoenzyme genotype and C-banding type was examined using the f-test developed by Tibayrenc *et al.* (1990). This test, for linkage disequilibrium, is based on a Monte Carlo simulation and permits the significance of a result to be tested even when sample sizes are small.

RESULTS

Transmission

The susceptibilities of the MA and BO lines for *T. b. gambiense* are summarized in Table 3. Flies of the MA line were five times more likely to carry procyclics when dissected than BO flies and metacyclics were only found in MA flies. Similar differences exist in the susceptibilities of the two lines to infection with *T. b. brucei* EATRO 1125, although the proportion of MA flies supporting mature

TABLE 2

Staining conditions for the isoenzymes, showing the staining solutions mixed with molten 1·2% agar (10 ml/10 ml)

	Staining buffer							
Enzyme	*Buffer*	*pH*	*Volume (ml)*	*Substrate*	*Linking enzyme*	*Volume of activator (1 M $MgCl_2$)*	*Coenzyme**	*Stain†*
IDH	0·1 M Tris–HCl	8	10	10 mg DL-isocitric acid	—	250	NADP	NBT and PHS
MDH	1 M Tris–HCl	8	1·2	900 µl 1 M malate, pH 7	—	0	NAD	NBT and PHS
ME	1 M Tris–HCl	7	2·5	600 µl 1 M malate, pH 7	—	250	NADP	NBT and PHS
αGPD	0·1 M Tris–HCl	8	10	10 mg DL-glycero-3-phosphate	—	0	NAD	NBT and PHS
6PGD	1 M Tris–HCl	8	2·5	10 mg 6-phosphogluconic acid	—	250	NADP	NBT and PHS
PGM	1 M Tris-HCl	8	2·5	20 mg glucose-1-phosphate	8 I.U. G6PDH	250	NADP	NBT and PHS
GPI	1 M Tris–HCl	7	2·5	10 mg fructose-6-phosphate	4 I.U. G6PDH	250	NADP	NBT and PHS
ACON	1 M Tris–HCl	8	1·7	14 mg cis-aconitic acid	1 I.U. IDH	250	NADP	NBT and PHS
HK	1 M Tris–HCl	8	2·5	20 mg glucose	4 I.U. G6PDH	250	10 mg ATP	NBT and PHS
Esterase	0·1 M phosphate	6·3	10	60 mg α-naphtylacetate and 12 mg β-naphtylacetate.	—	0	—	10 mg Fast Blue BB
LAP	0·2 M Tris/maleic	6	10	10 mg L-leu-naphtylamide	—	0	—	10 mg Fast Black K-salt
PEP1	0·1 M Tris-HCl	8	10	10 mg L-leu-leu-leucine	1 mg L-amino-acid-oxidase and 1 mg peroxidase	150	—	10 mg 3-amino-ethyl-carbazol
PEP2	0·1 M Tris-HCl	8	10	5 mg L-leucyl-L-alanine	1 mg L-amino-acid-oxidase and 1 mg peroxidae	150	—	10 mg 3-amino-ethyl-carbazol

*NADP, 100 µl of a 50 mg/ml solution; NAD, 200 µl of a 50 mg/ml solution.
†NBT, 500 µl of a 10 mg/ml solution; PMS, 300 µl of a 10 mg/ml solution.

TABLE 3

The susceptibilities of the two lines to T. b. gambiense, *as measured in two separate experiments*

Line	*No. dissected*	*No. with procyclics*	*No. with metacyclics*	*Vectorial competence**
BO				
Experiment 1				
Males	23	2	0	0
Females	86	1	0	0
Overall	109	3	0	0
Experiment 2				
Males	11	1	0	0
Females	82	5	0	0
Overall	93	6	0	0
MA				
Experiment 1				
Males	59	18	3	0·05
Females	82	26	0	0
Overall	141	44	3	0·02
Experiment 2				
Males	28	9	0	0
Females	173	57	1	0·006
Overall	201	66	1	0·005

*The proportion of the flies dissected that had metacyclic forms.

infections is considerably higher with *T. b. brucei* than *T. b. gambiense* (P. Kageruka and J.-M. Kasadi, unpubl. obs.).

Isoenzymes

Only five of the 13 enzymes studied (EST, GPI, HK, PEP1 and PGM) gave useful variation (Table 4). ACON was monomorphic except for a single MA fly. GPD (with three bands), ME (one band) and IDH (one band) were always monomorphic. Although two variable loci were present with EST, only the slowest (EST2) has been retained as the allelic interpretation of the EST1 results was difficult. PEP2, 6PGD, MDH and LAP also exhibited polymorphism but allelic interpretation was again not possible.

The relative mobilities (R_f) for the interpretable enzymes were 0·60 and 0·51 for ACON, 0·22, 0·18 and 0·13 for EST2, 1·0, 0·94 and 0·87 for GPI, 0·80 and 0·77 for HK, 0·78 and 0·74 for PEP1 and 0·60 and 0·55 for PGM.

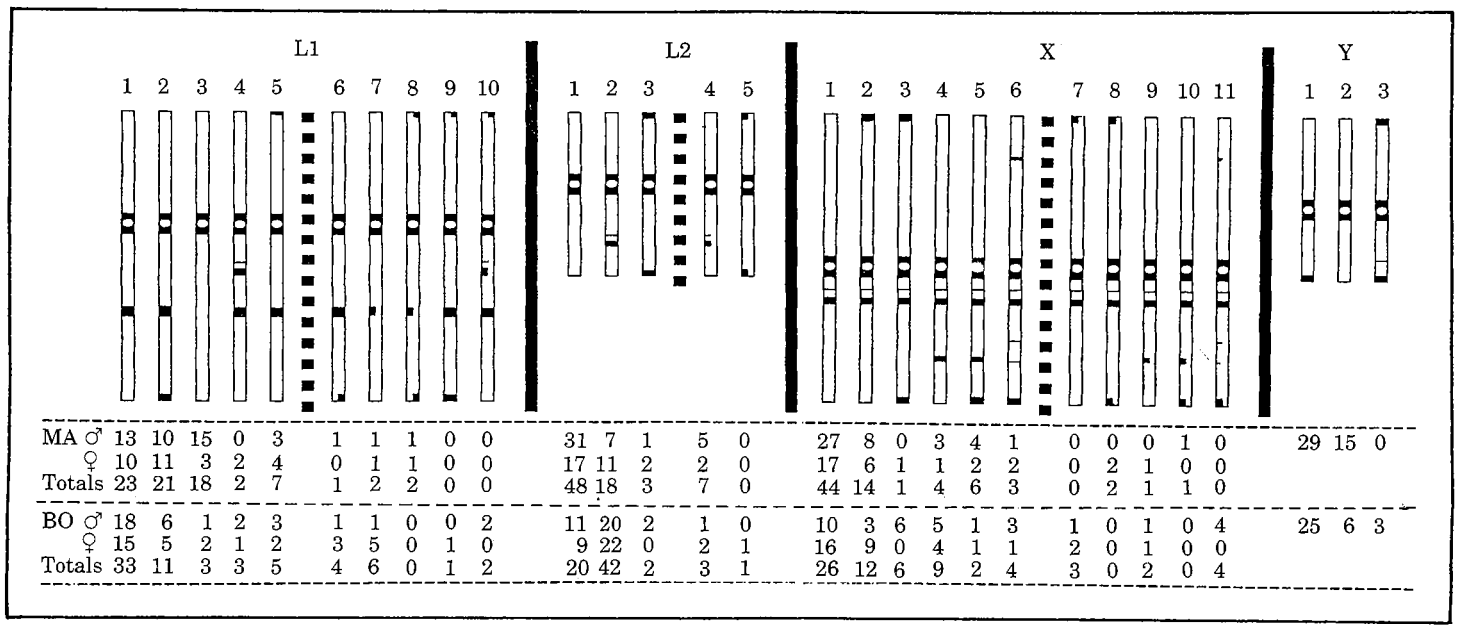
C-banding

Well marked C-banding polymorphism was found for all chromosomes, but were most marked for the autosome L1 and the X-chromosome; these had 10 and 11 different forms, respectively (Fig.). The most apparent difference between the two lines was the larger proportion of odd C-banding types in BO males than in MA males, especially in the X-chromosome. However, differences between the lines could also be clearly observed in the frequency of the two main forms of autosome L2.

Numerical analysis

The results of tests for Hardy-Weinberg equilibrium in the allozyme data are given in Table 4; evidence for sex-linked genes was revealed by the lack of heterozygotes in males at three loci: HK, PGM and EST2.

Tests for differences in gene frequency between the two populations gave significant values for various loci (Table 5). Nei's



MA ♂	13	10	15	0	3	1	1	1	0	0	31	7	1	5	0	27	8	0	3	4	1	0	0	0	1	0	29	15	0
♀	10	11	3	2	4	0	1	1	0	0	17	11	2	2	0	17	6	1	1	2	2	0	2	1	0	0			
Totals	23	21	18	2	7	1	2	2	0	0	48	18	3	7	0	44	14	1	4	6	3	0	2	1	1	0			
BO ♂	18	6	1	2	3	1	1	0	0	2	11	20	2	1	0	10	3	6	5	1	3	1	0	1	0	4	25	6	3
♀	15	5	2	1	2	3	5	0	1	0	9	22	0	2	1	16	9	0	4	1	1	2	0	1	0	0			
Totals	33	11	3	3	5	4	6	0	1	2	20	42	2	3	1	26	12	6	9	2	4	3	0	2	0	4			

Fig. A diagrammatic representation of the different types of C-banding for the autosome (L1 and L2) and the sex chromosomes (X and Y) and their frequency in the MA and BO lines of *Glossina palpalis gambiensis*.

TABLE 4
Observed numbers of flies for each polymorphic enzyme

Line and enzyme	*No. of males with allele:*				*No. of females with allele:*				*No. of flies overall with allele:*			
	11	*12*	*22*	χ^2*	*11*	*12*	*22*	χ^2	*11*	*12*	*22*	χ^2
BO												
HK	16	1	13	26·1	9	16	11	0·42	25	17	24	15·51
PEP1	5	7	15	4·3	2	9	17	0·27	7	16	32	3·91
ACON	45	0	0	0	44	0	0	0	89	0	0	0
PGM	63	1	18	76·38	45	13	1	0·003	108	14	19	63·28
GPI†	14	25	8	0·31	13	11	12	5·43	27	36	20	1·32
EST2†	13	2	10	17·54	7	10	5	0·15	20	12	15	10·99
MA												
HK	7	0	27	34·0	5	13	20	1·36	12	13	47	20·01
PEP1	6	8	10	2·37	5	10	19	2·89	11	18	29	5·69
ACON	43	1	0	0·006	44	0	0	0	87	1	0	0·003
PGM	69	0	15	84	38	18	4	0·81	107	18	19	52·02
GPI†	20	15	8	2·55	20	8	7	7·72	40	24	15	8·33
EST2†	13	4	8	11·11	9	11	6	0·53	22	15	14	8·04

*All χ^2 values are for one degree of freedom.
†11 represents the most frequent allele, 22 the other alleles and their heterozygotes and 12 the heterozygotes between 11 and the other homozygotes.

standard genetic distances for the allozyme frequencies were between 0·018 and 0·036 (Table 6). Hedrick's similarity coefficient varied between 0·970 and 0·980 for the electrophoretic data and between 0·823 and 0·904 for the C-banding (Table 6).

Unexpectedly for laboratory lines, autosomal loci such as PEP1 and GPI were not in equilibrium (Table 4). Linkage disequilibrium was not detected between different genotypes among the five enzymatic loci (data not shown), but a significant linkage disequilibrium between C-banding types was found in the BO line, in males only (the probability of a disequilibrium equal to or higher than that observed was 0·009).

As the rate of polymorphism (*Pm*) does not need a rigorous allelic interpretation at each

TABLE 5
P-values for the differences between the gene frequencies for particular isoenzymes in the MA and BO lines (not adjusted for multiple comparisons)

Enzyme	*Male flies*	*Female flies*	*Overall*
HK	<0·001	<0·02	<0·02
PEP1	NS	NS	NS
ACON	NS	NS	NS
PGM	NS	NS	<0·02
GPI	NS	<0·02	<0·02
EST2	NS	NS	NS

NS, Not significant (P>0·05).

TABLE 6

Nei's standard genetic distances and identities at 10 enzymatic loci and Hedrick's similitude index at these loci and based on C-banding-type frequencies, measured between the MA and BO lines

	Males flies	*Female flies*	*Overall*
Nei's genetic distance (*Ds*)	0·036	0·018	0·031
Nei's genetic identity (*I*)	0·964	0·982	0·979
HEDRICK'S SIMILITUDE INDEX			
Based on enzymes	0·970	0·980	0·977
Based on C-banding	0·823	0·904	0·843

locus, all loci may be considered together: 10 of 15 were found variable ($Pm=0·66$). This high value was the same for each sex and each line. Nei's gene diversity index (Table 7) was also high, although estimated from 10 loci: the five loci with interpretable variation (HK, PGM, EST2, PEP1 and GPI) and the five monomorphic loci mentioned above. Both this index and the chromosomal diversity index (Table 7) based on the same equation indicated more genetic variability in the BO line than in the MA line, especially in the males.

The G_e diversity index, when used with the five polymorphic loci, was almost 100% for both lines, with very minor differences between them. However, when used with any four of the five polymorphic loci, G_e revealed significant differences between the two lines, the BO line being more variable than the MA line ($P=0·005$; Table 7). Similarly, another marked difference was found between the lines using G_c with the four chromosomes ($P=0·02$; Table 7), particularly in the males ($P=0·01$).

DISCUSSION

The clear differences in the susceptibilities of the two lines of *G. p. gambiensis* led to the present study of their genetics. Three loci were sex-linked, of which one (HK) is described here for the first time. HK, monomorphic in the *morsitans* group (Agatsuma and Otieno, 1988; Tarimo Nesbitt *et al.*, 1990; Gooding *et al.*, 1991; Tarimo Nesbitt, 1991), exhibited a polymorphism typical of a sex-linked locus. Similar patterns were found for PGM and EST2. PGM has already been described as sex-linked in *G. pallidipes* and *G. longipennis* (Agatsuma and Otieno, 1988; Tarimo Nesbitt *et al.*, 1990; Gooding *et al.*, 1991). The only esterase described as sex-linked in the *G. palpalis* group, including *G. p. gambiensis*, is the one named EST-T by Gooding (1982). We have investigated the testes and thoraxes of several flies for esterase; although the thorax shows two loci the testes shows only one and the latter has exactly the same mobility as the second, sex-linked locus in the present study. It seems likely that the EST-T of Gooding (1982) and EST2 are the same.

The disequilibrium observed at the PEP-1 and GPI loci was due to heterozygote deficiency and was more pronounced at the GPI locus in females than in males. This non-random association of alleles within loci was not verified between loci: no linkage disequilibrium could be detected using a Monte Carlo stimulation (Tibayrenc *et al.*, 1990), even taking into account the sex-linked loci. A selection force reducing the number of heterozygotes at two unlinked loci is unlikely, and we believe some deviation from random mating within laboratory strains could be the cause of this disequilibrium. This unexpected situation could have resulted from the procedure used to produce each new generation in the MA and BO colonies, i.e. the grouping of males and females for reproduction into separate cages, each of 60 specimens, could produce an artificial structuring, leading to transient Wahlund effects.

TABLE 7

Parameters of genetic diversity in flies of the MA and BO lines

	Males		*Females*		*Overall*	
	MA	*BO*	*MA*	*BO*	*MA*	*BO*
ENZYMES						
Heterozygosity (h)						
HK	0·332	0·503	0·428	0·505	0·385	0·504
PEP1	0·496	0·440	0·421	0·363	0·456	0·400
PGM	0·295	0·352	0·342	0·224	0·314	0·302
GPI	0·468	0·497	0·437	0·506	0·452	0·499
EST2	0·490	0·503	0·503	0·507	0·493	0·500
Mean heterozygosity (H) ± S.E.*	0·208 ± 0·072	0·229 ± 0·078	0·213 ± 0·072	0·211 ± 0·075	0·210 ± 0·072	0·221 ± 0·076
Genotype diversity (G_e)†	0·786	0·882	0·750	0·941	0·605	0·824
					α=0·05	
CHROMOSOMES						
Diversity index (h_c)						
L1	0·738	0·674	0·582	0·290	0·660	0·480
L2	0·464	0·577	0·546	0·525	0·490	0·554
X	0·576	0·828	0·615	0·643	0·596	0·735
Y	0·449	0·489	—	—	0·449	0·489
Mean diversity (H_c) ± S.E.	0·557 ± 0·115	0·641 ± 0·125	0·571 ± 0·041	0·486 ± 0·146	0·549 ± 0·086	0·565 ± 0·136
Cytotype diversity (G_c)‡	0·523	0·824	0·625	0·697	0·566	0·761
	α=0·01				α=0·02	

*S.E. computed according to Nei (1987).

†For four polymorphic loci.

‡For all chromosomes.

Disequilibrium in the C-banding types of BO males may be the result of the mix of different populations used to initiate this line. This may have generated a spurious linkage disequilibrium by pooling samples with different C-banding-type frequencies.

Since gene frequency differences were statistically significant at various loci (Table 5), Nei's distance values may also be regarded as significant. The two lines, MA and BO, may be considered genetically distinct although there are no indications that they are not conspecific populations. Similar conclusions may be drawn from the Hedrick's similarity coefficients for both the isoenzymes and chromosomal types. Differences were slightly more pronounced between males (Table 6). The coefficients for the electrophoretic data appeared to be slightly higher than those based on the cytogenetic analysis but the former were based on 10 loci among which five identical monomorphic loci, whereas the latter were only based on four chromosomes.

The overall genetic variability revealed by the isozymes and C-banding was higher than expected for laboratory lines, particularly for the MA line which was founded in 1972 with 191 specimens and subsequently maintained in isolation (Elsen *et al.*, 1993). Since disequilibrium was frequently observed and found due to a deficit in heterozygotes rather than excess (Table 4), balanced polymorphism is unlikely to be the cause of this high genetic variability. A testable hypothesis would be that natural populations of *G. palpalis gambiensis* could be very highly variable for the enzymes examined in the present study. Indeed, Gooding (1981), using other enzymes, observed little genetic variation in *G. p. gambiensis*, but he states that 'the genetic variation in the [Bobo Dioulasso] laboratory population of this species is lower than in natural populations'.

Nei's gene diversity indices (Table 7) gave concordant values with both markers, the BO line, especially the males, being slightly more variable than the MA line. As the two populations are closely related it would be irrelevant to compare their respective genetic variability using the variance of H (Nei, 1987). However, a classical t-test for paired observations can be used on h for the different enzymatic loci (Nei, 1987) or the different chromosomes, and the standard deviate can be used in comparing the G indexes. While the standard deviate for G gave significant results between the two lines whichever marker was used, the t-test results for h were not significant. G, although interesting when indirectly comparing the genetic diversity between two populations, is quickly saturated. For instance, G_e reached about 100% in both lines when five loci were used. Any combination of four loci was therefore used to perform the comparisons. The results of the statistical analyses of both types of nuclear markers were in agreement with the hypothesis that the BO line is more variable than the MA line, which is consistent with the much more heterogeneous conditions under which BO has been maintained (Elsen *et al.*, 1993; present study).

To conclude, two lines of *G. p. gambiensis* exhibiting unequal susceptibilities to *T. b. gambiense* were found to also exhibit significant genetic differences, as shown by two distinct types of genetic markers. These differences probably reflect differences in the breeding history of each line and may be attributed to genetic drift since their foundation.

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