

## Genetic and Morphometric Evidence for Population Isolation of *Glossina palpalis gambiensis* (Diptera: Glossinidae) on the Loos Islands, Guinea

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**ABSTRACT** Allele frequencies at four microsatellite loci, and morphometric features based on 11 wing landmarks, were compared among three populations of *Glossina palpalis gambiensis* (Diptera: Glossinidae) in Guinea. One population originated from the Loos islands separated from the capital Conakry by 5 km of sea, and the two others originated from the continental mangrove area close to Dubreka, these two groups being separated by  $\approx 30$  km. Microsatellites and wing geometry data both converged to the idea of a separation of the Loos island population from those of the mangrove area. Although occasional contacts cannot be excluded, our results support the hypothesis of the Loos population of tsetse flies being a completely isolated population. This situation will favor a sequenced intervention against human African trypanosomosis and the possibility of an elimination of tsetse from this island.

**KEY WORDS** *Glossina palpalis*, microsatellite DNA, geometric morphometrics, wings, Guinea

Human African trypanosomosis (HAT, or sleeping sickness) is showing some signs of declining due to recent efforts on case detection and treatment, notably in Central Africa (Jannin 2005). However the situation in West Africa is much less clear, and Guinea and Côte d'Ivoire are thought to be the two countries most affected by this disease (Camara et al. 2005, Kaba et al. 2006). Guinea has a long history of sleeping sickness, which was particularly prevalent in the years 1930–1940 (Bregues et al. 1964). Current data show prevalences up to 2–5% in villages of the coastal mangrove area (Dubreka focus) (Camara et al. 2005). The Loos islands are completely separated from this mangrove area of the mainland. The situation of HAT in these islands is not currently known, but there are historical reports of the disease: in 1942, a medical survey conducted by Med. Cap. Héricord detected 30 patients. In 1944, out of 1,924 inhabitants who were registered from the islands, 1,627 were visited, and 16 cases were detected (15 on Kassa island and one on Fotoba island). After that, little information has been

available except for seven cases originating from these islands between 1971 and 1987; these cases were passively detected and treated in Dubreka. It is not known whether the disease was autochthonous or was imported from other localities.

In West Africa, HAT is mainly transmitted by the tsetse fly species *Glossina palpalis* Van der Planck (Diptera: Glossinidae). Control of tsetse can be achieved through a variety of techniques, including traps, insecticide impregnated targets, live-baits, sequential aerial spraying, and sterile male release (Cuisance et al. 1980). Generally, however, the tsetse populations then tend to recover, due to either flies surviving the initial interventions, migrant flies coming from untreated regions, or both. To achieve and sustain local elimination of a target fly population, it is therefore preferable to define the area of intervention to include an entire panmictic fly population, such that natural immigration from neighboring localities is of low likelihood. This end is most readily achieved for isolated island populations, as shown by the elimination of *Glossina pallidipes* Austin from the Island of Principe in 1914 (Da Costa et al. 1916), and the elimination of *Glossina austeni* Newstead from Unguja Island of Zanzibar in 1997 (Vreysen et al. 2000). But for most mainland populations of tsetse, the geographical limits of target tsetse populations are less easily definable. Application of population genetics techniques can reveal the existing level of population differentiation in tsetse, providing guidance on the distribution of genetically defined subpopulations. In essence, the

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population genetics models are used to estimate rates of gene flow between populations, which are taken as a surrogate for the rate of migration of individuals (Patterson and Schofield 2005). Initial studies already showed evidence of strong structuring of *G. palpalis* populations in fragmented landscapes (Solano et al. 2000). With more detailed study, it should therefore be possible to determine key areas where tsetse control interventions can proceed with relatively low risk of reinvasion from neighboring areas.

To examine the population structure of *G. palpalis*, we used two approaches in the current study, one approach based on genetic variation at microsatellite DNA loci and the other approach based on phenetic variation as described by the geometry of the wings. The main objective was to assess whether the tsetse population from Loos islands was isolated from two other populations of the mangrove area of the HAT focus of Dubreka.

### Materials and Methods

**Study Area.** The HAT focus of Dubreka is located  $\approx 45$  km from Conakry, the capital of Guinea. HAT has shown incidences up to 5% in some of the visited villages from 1997 to 2005. The area is situated among the coastal mangrove, with anthropic Guinean savannah, and permanent or temporary inundated areas. Near the town of Dubreka (25,000 inhabitants), people live in villages of between 300 and 2,000 inhabitants, fragmented in many smaller localities. Main activities include fishing, salt extraction, and agriculture ("vergers" of *Elaeis guineensis*, mangoes, rice, and food crops). Loos islands are separated from the mainland, by 5 km of sea at the shortest distance. But the first mangrove area where tsetse occur are at  $\approx 20$  km from these islands (Fig. 1).

**Entomological Surveys.** In May 2005, six Vavoua traps (Laveissière and Grébaud 1990) were placed on Fotoba island (Loos islands), and 23 traps were placed in two mainland localities of the littoral (Magnokhoun) and mangrove (Touguissoury) areas. These two mainland localities are separated by  $\approx 15$  km, and the shortest distance between them and Loos islands is 30 km. Cages were changed daily during 2 to 4 days, and tsetse were counted and separated by sex. From each dissected tsetse, the wings were removed and put in individual, labeled, dry Eppendorf tubes, and three legs were removed and put in individual, labeled, dry Eppendorf tubes.

**Microsatellite Loci.** In total, 71 individuals were used for the genetic analyses at microsatellite loci: 23 in Loos islands (14 males [M], nine females [F]), 28 in Magnokhoun (14 M, 14 F), and 21 in Touguissoury (10 M, 10 F).

Four microsatellite loci were analyzed: Gpg55,3 (Solano et al. 1997); pgp11 and pgp1 (Luna et al. 2001), and B104 (kindly provided by A. S. Robinson, IAEA, Vienna, Austria). Locus Gpg55,3 has been reported to be located on the X chromosome (Solano et al. 1997, Gooding et al. 2004), and given an absence of heterozygotes on a subsample of males (data not shown),

B104 and pgp11 also were interpreted to be located on the X chromosome.

To each tube containing the legs of the tsetse, 200  $\mu$ l of 5% Chelex chelating resin was added (Walsh et al. 1991, Solano et al. 2000). After incubation at 56°C for 1 h, DNA was denatured at 95°C for 30 min. The tubes were then centrifuged at  $12,000 \times g$  for 2 min and frozen for later analysis.

The polymerase chain reaction (PCR) reactions were carried out in a thermocycler (MJ Research, Cambridge, United Kingdom) in 10- $\mu$ l final volume, by using 1  $\mu$ l of the supernatant from the extraction step. After PCR amplification, allele bands were routinely resolved on a 4,300 DNA Analysis System from LI-COR (Lincoln, NE) after migration in 96-lane reloadable (3 $\times$ ) 6.5% denaturing polyacrylamide gels. This method allows a multiplex by the use of two infrared dyes (IRDye), separated by 100 nm (700 and 800 nm), and read by a two-channel detection system that uses two separate lasers and detectors to eliminate errors due to fluorescence overlap. To determine the different allele sizes, a large panel of  $\approx 30$  size markers was used. These size markers had been previously generated by cloning alleles from individual tsetse flies into pGEM-T Easy Vector (Promega, Madison, WI). Three clones of each allele were sequenced using the T7 primer and the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). Sequences were analyzed on an Applied Biosystems 310 automatic DNA sequencer, and the exact size of each cloned allele was determined. PCR products from these cloned alleles were run in the same acrylamide gel as the samples, allowing the allele size of the samples to be determined accurately.

**Microsatellite Data Analysis.** For the total sample subdivided into the three localities, Wright's  $F_{is}$  (within sample heterozygote deficiency, a measure of deviation from panmixia) and  $F_{st}$  (measure of population differentiation) were estimated using Weir and Cockerham's unbiased estimators ( $f$  for  $F_{is}$ ,  $\theta$  for  $F_{st}$ ) (Weir and Cockerham 1984). For random mating (within samples) or random distribution of individuals (between samples),  $F$  values are expected to be zero. When  $F_{st}$  was measured, it was compared with  $F_{st\ max} = 1 - H_s$  (Hedrick 1999, 2005).

The significance of  $F_{is}$  (deviation from panmixia) at each locus, and over all loci, also was tested separately within each sample by using 10,000 permutations of alleles between individuals. Males were hemizygous at loci on the X chromosome. For these loci, measure of  $F_{is}$  and its significance were conducted only on females. The significance of  $F_{st}$  (population differentiation) was assessed using 10,000 permutations of genotypes among samples. To evaluate significance when multiple tests were performed, the sequential Bonferroni procedure was applied (Rice 1989).

An unweighted pair-group method with arithmetic average (unweighted pair group method with arithmetic mean) dendrogram was built based on Cavalli-Sforza and Edwards (1967) chord distance between the three populations. This distance is indeed the most

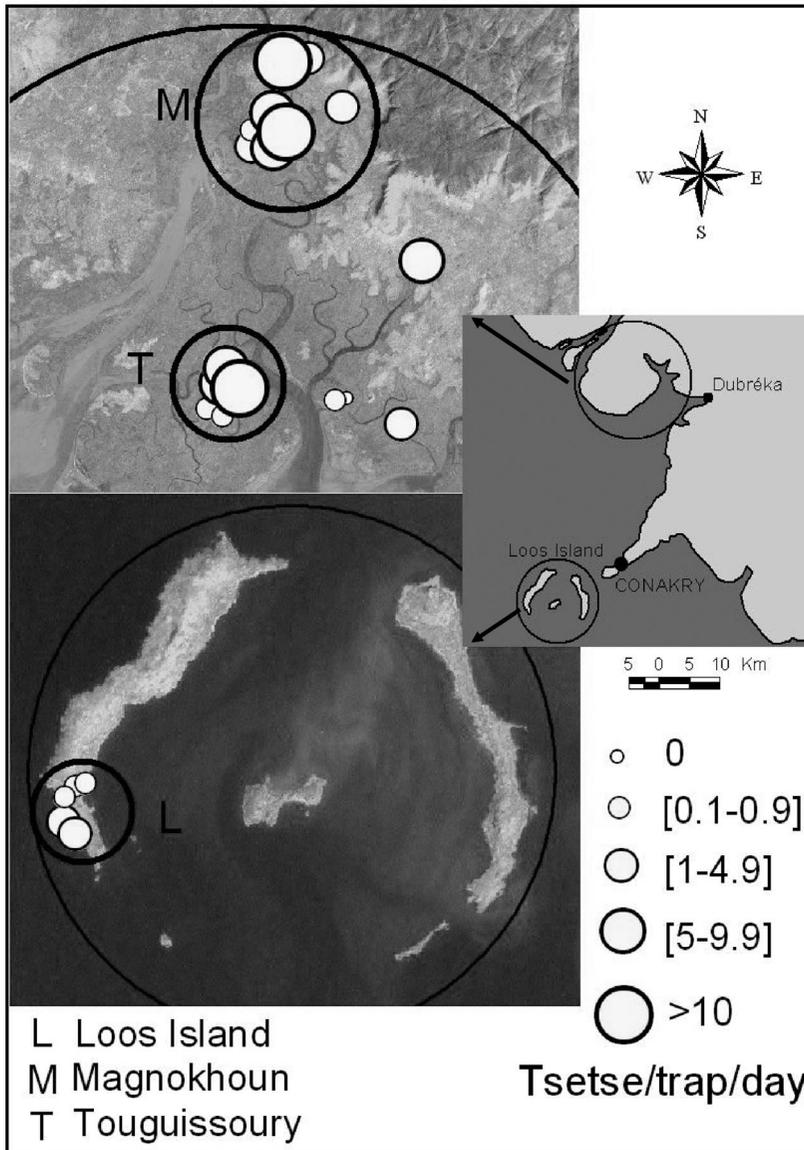


Fig. 1. Geographic location of study area. The circles correspond to the three localities of the tsetse samples. L, Loos islands; M, Magnokhoun; and T, Touguissoury.

appropriate for tree construction (Takezaki and Nei 1996).

**Morphometrics.** Out of the 71 individuals submitted to microsatellite analyses, 64 showed wings in good state for morphometric studies. Wings were dry-mounted between two microscope slides and scanned at 3,200 dpi. On this image, 11 landmarks defined by vein intersections were recorded (Fig. 2). Their coordinates were subjected to generalized Procrustes analysis (GPA) (Rohlf 1990, 1996). Centroid size (Bookstein 1991) was used to describe size changes among sexes and localities (Fig. 3).

For geographic comparisons, 18 “partial warps” (PW), corresponding to 11 landmarks (Fig. 2) were computed from the right wings by using the total

sample, mixing males and females: 18 individuals from Loos islands (10 M, 8 F), 24 from Magnokhoun (13 M, 11 F), and 22 from Touguissoury (13 M, 9 F). To circumvent the problem of small sample sizes relative to the large number of variables (18 PW), the 11 first “relative warps” (principal components of the PW) were used instead, representing >95% of the total shape variation. The residual allometry was estimated by multivariate regression of PW on size, on the total sample and separately in each sex, and statistical significance estimated by 1,000-runs permutation tests (Good 2000). To estimate the contribution of size variation to the geographic distinction provided by the discriminant functions, each of these was regressed on size variation (Fig. 4). The Mahalanobis distances were

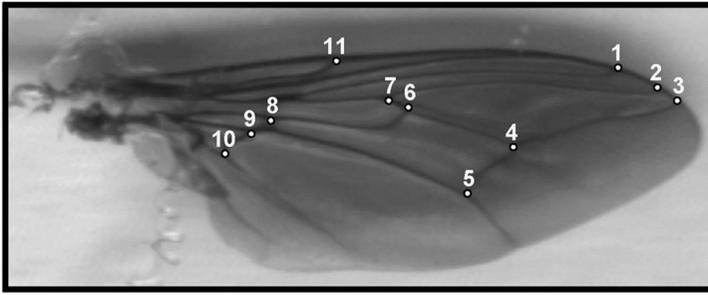


Fig. 2. Location of the 11 landmarks that were recorded for each tsetse wing.

examined for significance by permutation tests (1,000 runs) and used to construct an unweighted pair-group method with arithmetic average dendrogram. Based on these distances, the percentage of correctly assigned individuals was also computed for each locality.

For bilateral differences, only a subset of the total sample was used (9 M, 6 F from Loos, 8 M and 5 F from Magnokhoun, and 11 M and 7 F from Touguissoury). Five landmarks could be retained (Fig. 2, see landmarks 1, 3, 5, 10, and 11). To provide a better estimate of digitizing error, both wings of each individual were recorded three times (Møller and Swaddle 1997).

Size asymmetry was estimated on the basis of centroid size and followed the analysis of variance (ANOVA) procedure recommended by Palmer and Strobeck (1986). In the absence of significant directional asymmetry, the distribution of signed differences was examined for kurtosis to assess the existence of fluctuating asymmetry (or reject the existence of antisymmetry).

**Software.** The  $F_{is}$  and  $F_{st}$  estimators were calculated with FSTAT version 2.9.3 software (Goudet 1995). Cavalli-Sforza and Edwards (1967) chord distances were computed by the GENETIX version 4 software package (Laboratoire Génome et Populations, Centre National de la Recherche Scientifique Unité Propre de Recherche 9060, Université de Montpellier II, Montpellier, France).

Collection of anatomical landmarks, GPA, multivariate analyses as well as asymmetry detection and measurement were performed using software freely available at <http://www.mpl.ird.fr/morphometrics> (developed by J.P.D.).

PHYLIP package (by J. Felsenstein, <http://evolution.genetics.washington.edu/phylip.html>) was used to construct the unweighted pair-group method with arithmetic average tree, and NJPLOT (<http://pbil.univ-lyon1.fr>) was used for tree edition (Perrière and Gouy 1996).

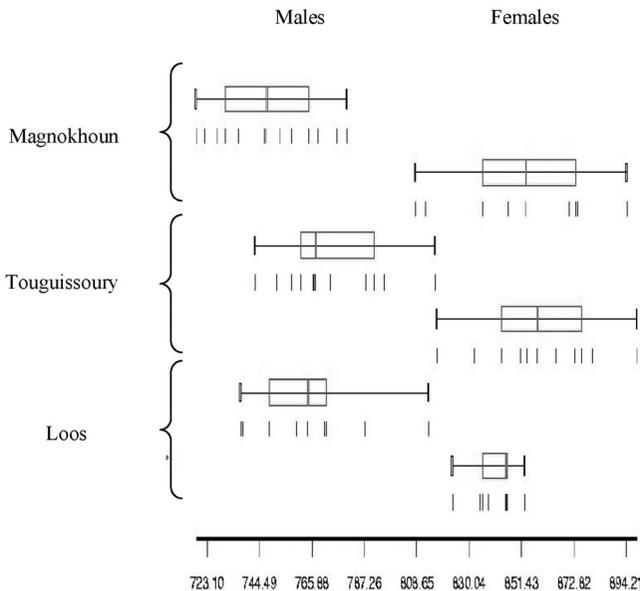


Fig. 3. Quantile plots showing in each sex the distribution of individuals along the isometric estimator of size (centroid size). Each box shows the group median separating the 25th and 75th quartiles, with the 10th and 90th quartiles shown as lines on the right and left sides of the box.

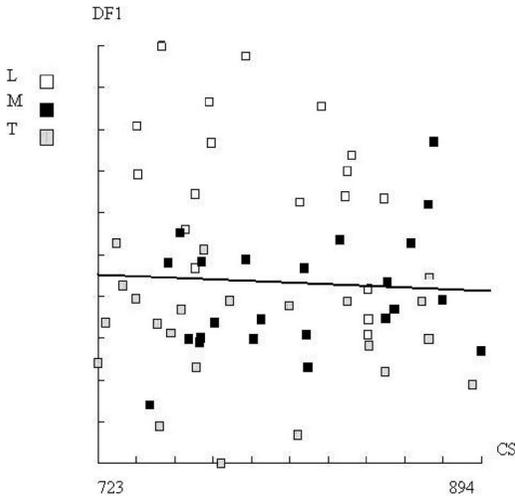


Fig. 4. Regression of first discriminant factor on centroid size. Vertical axis is the DF1, representing 84% of the total discrimination. Horizontal axis is the centroid size of the wing, in pixels. White squares, Loos (L); black squares, Magnokhoun (M); and gray squares, Touguissoury (T). Regression line is shown.

**Results**

**Entomological Surveys.** Of 421 tsetse (240 M, 181 F) that were trapped during the survey, all were *G. p. gambiensis* based on morphological characters and on geographical distribution. The apparent density (AD) averaged 4.53 tsetse per trap per day. On Loos islands, 26 tsetse in total were caught (AD of 1.44 tsetse per trap per d), 277 were caught at Magnokhoun (AD of 6.15 tsetse per trap per d), and 118 at Touguissoury (AD of 4.07 tsetse per trap per d).

**Genetics.** From the 71 individuals analyzed, the number of alleles at each locus was 18, 13, 16, and 14 for loci B104, Pgp11, Pgp1, and Gpg55,3 respectively (Table 1). The Loos sample had the lowest number of alleles. Weir and Cockerham (1984) estimators indicated an overall  $F_{is}$  of 0.15 ( $P < 0.001$ ) and an averaged  $F_{is}$  per population of 0.18, 0.16, and 0.13, respectively, for Loos, Magnokhoun, and Touguissoury, none of these values being significant (Table 2).

Overall  $F_{st}$  averaged +0.032 ( $P < 0.0001$ ), indicating genetic differentiation between the three populations. When measured among the three populations taken by pairs, pairwise  $F_{st}$  were 0.014 between Magnokhoun

and Touguissoury, 0.032 between Loos and Magnokhoun, and 0.062 between Loos and Touguissoury.

Because  $F_{st}$  between Magnokhoun and Touguissoury was low and nonsignificant, these two samples were mixed into one population and compared with Loos. The resulting  $F_{st}$  was 0.057 (highly significant,  $P < 0.0001$ ).

Because  $H_s$  was 0.832,  $F_{st\ max} = 1 - H_s$  was 0.168, and our maximum  $F_{st}$  value was 0.057. A standardized estimate of  $F_{st}$  would thus give  $F_{st'} = F_{st}/F_{st\ max} = 0.34$ . Should this value have been close to 1, a complete lack of migrant would have been supported. The lower value (0.34) suggests that either there are migrants, or there have been migrants in the past and the separated populations did not yet reach equilibrium. If equilibrium is assumed in a two island model, then the corresponding number of migrants would be  $Nm = (1 - F_{ST})/8F_{ST}$ , which would give, with our "standardized" measure of  $F_{ST}$ ,  $Nm = 0.24$  migrant per generation or one migrant every four generations.

**Morphometrics.** A strong sexual dimorphism for size was disclosed in each locality, in favor of females (Fig. 3). Size variation was not significant between localities.

The multivariate regression of PW on size was significant on the total sample, mixing males and females (1,000 runs,  $P = 0.0040$ ), but it was not significant within each sex (1,000 runs,  $P = 0.508$  in males and  $P = 0.083$  in females). This suggests that allometric effects were mainly due to sexual size dimorphism.

The first discriminant function (DF1, 84% of the total variation) clearly separated the Loos population from the other two populations (Fig. 4), whereas these latter populations were slightly separated by the second discriminant function (DF2, 16% of the total variation). Comparing flies from Loos islands with the group formed by flies from Magnokhoun and Touguissoury, the reclassification scores were 77% for Loos and 91% for mainland group. Size contribution to the geographic separation provided by DF1 was not significant (Fig. 4;  $r^2 = 0.001$ ,  $P > 0.05$ ).

Bilateral differences of centroid size showed distinct patterns in males and females, varying with geography. In females, directional and nondirectional asymmetries were found to be significant for the Loos population only. This suggests that nondirectional asymmetry on the island was antisymmetry, although kurtosis could not be detected. In females of the continental area as well as in males from the three localities, nondirectional (but no directional) asymmetry was detected, at a slightly higher amount in males (Table 3), and no kurtosis was detected in the distri-

Table 1. Gene diversity and number of alleles sampled by locus per population

Locus	Gene diversity/locus/pop			No. alleles sampled		
	L	M	T	L	M	T
B104	0.917	0.937	0.939	8	12	12
Pgp11	0.764	0.846	0.811	6	9	7
Pgp1	0.918	0.926	0.833	10	12	7
Gpg55,3	0.830	0.824	0.800	5	11	10

L, Loos islands; M, Magnokhoun; and T, Touguissoury.

Table 2.  $F_{is}$  per locus per population

Locus	$F_{is}$ /locus/pop			P value (0.05 level)		
	L	M	T	L	M	T
B104	0.377	0.161	-0.065	0.02	0.05	1.00
Pgp11	0.127	0.071	0.137	0.40	0.39	0.32
Pgp1	0.109	0.064	0.333	0.20	0.36	0.04
Gpg55,3	0.097	0.353	0.125	0.42	0.0042	0.28
Total	0.182	0.159	0.126	0.017	0.013	0.013

**Table 3.** Left-right comparisons between the three tsetse populations

	Directional asymmetry	Nondirectional asymmetry
Females		
Loos	34**	20**
Magnokhoun	ns	8*
Touguissoury	ns	8*
Males		
Loos	ns	16**
Magnokhoun	ns	16**
Touguissoury	ns	29**

Values are mean squares (MS) of an ANOVA output with individuals, side and their interaction as effects, and centroid size as dependent variable. ns, not significant (value not shown).\*\*,  $P < 0.00001$ ; \*,  $P < 0.0010$ .

bution of signed differences. These results were compatible with fluctuating asymmetry in males and in females, although the nondirectional asymmetry in the island females suggest antisymmetry as the cause of their nondirectional asymmetry.

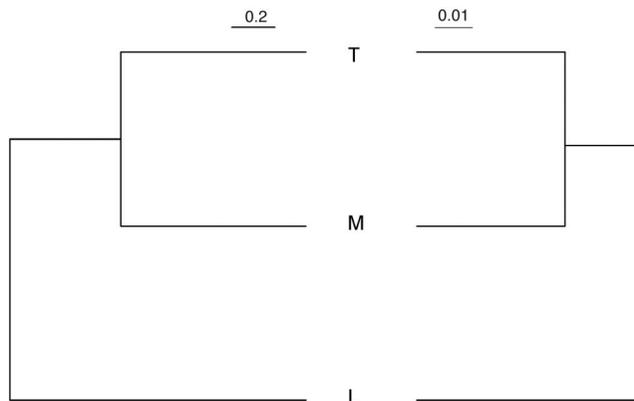
**Classification Trees.** The Mahalanobis distances ( $D_m$ ) derived from shape variation of the wings of the three populations were significant only when comparing the Loos population with Magnokhoun ( $D_m = 1.82$ ,  $P = 0.014$ ) or Touguissoury ( $D_m = 2.30$ ,  $P < 0.001$ ), so that the resulting unweighted pair-group method with arithmetic average tree produced a pattern isolating the tsetse from the island and grouping the tsetse from the mangrove localities ( $D_m = 1.09$ ,  $P = 0.450$ ) (Fig. 5, left). The unweighted pair-group method with arithmetic average tree based on the Cavalli-Sforza and Edwards chord distances ( $D_{cve}$ ) at microsatellite loci between the three populations gave similar branching (Fig. 5, right), and significant values were again found only when comparing the Loos population with populations of the mainland (1,000 permutations,  $D_{cve} = 0.13$ ,  $P = 0.001$  between Loos and Magnokhoun;  $D_{cve} = 0.140$ ,  $P = 0.002$  between Loos and Touguissoury; and  $D_{cve} = 0.085$ ,  $P = 0.141$  between Magnokhoun and Touguissoury).

## Discussion

This study was undertaken to explore the population structure of *G. p. gambiensis*, the main tsetse species found in the very active HAT focus of Dubreka, Republic of Guinea. We feel that knowing the genetic structure of a vector population is useful for understanding an epidemic and will contribute to a rational control operation. Adapted tools are genetic markers like microsatellite DNA markers (Jarne and Lagoda 1996), which have proven successful for population studies of many arthropod species, including insect vectors (Lanzaro et al. 1995, de Meeis et al. 2002) and tsetse (Solano et al. 1999, Gooding and Krafur 2005). In the current study, the use of morphometrics was explored as a complementary, low-cost tool, to get information on population structure (Dujardin and Slice 2006). Thus, allele frequencies at four microsatellite loci, and morphometric features based on 11 wing landmarks, were compared among three populations of *G. p. gambiensis*, one originating from an island located 5 km from the capital Conakry, and the two others from the continent in the HAT focus of Dubreka, these two groups being separated by  $\approx 30$  km.

The number of traps (six in Loos islands, a total of 23 in the two other localities) and the time during which they were left (between 2 and 4 d) do not allow comparison of tsetse densities between localities. It may explain the limited number of individuals of sample L available for genetic and morphometric comparisons. Adapted statistics were used based on non-parametric tests.

Overall, and within each of the three populations,  $F_{is}$  values were positive, indicating within population heterozygote deficiency. We attributed this apparent heterozygote deficiency mainly to the occurrence of null alleles, as suspected by the high variance of  $F_{is}$  values among loci for each population, and as it has previously been reported in tsetse (S.R., unpublished data). Thus, we assume that there was random mating within each population.



**Fig. 5.** Unweighted pair-group method with arithmetic average tree on genetic distances based on wing morphometry (on the left, Mahalanobis distance) and on microsatellite DNA loci (on the right, Cavalli-Sforza and Edwards distance) of the three tsetse populations.

The  $F_{st}$  value measured among the three populations was positive and significant, indicating genetic differentiation among the three populations. Pairwise Cavalli-Sforza and Edwards chord distances always distinguished the sample from Loos islands from the two other samples, whereas this same distance was the lowest and was not significant when measured between the two samples from the mangrove. When the two mainland populations were grouped and compared with the those from the island,  $F_{st}$  value was nearly 2 times higher (0.057) than the value comparing the three populations (0.032). Taking into account the high degree of polymorphism of our microsatellite loci, and using the suggestion of Hedrick (1999, 2005) for a standardized estimate ( $F_{st} = F_{st}/F_{st\ max}$  with  $F_{st\ max} = F_{st}/(1 - H_s)$ ) provided a corrected estimate of 0.34. Despite being relatively high, this upper bound seems far from 1, suggesting that either there are some migrants (e.g., one effective migrant each four generations), or there have been migrants in the past, and the separated populations did not yet reach equilibrium. According to mark-recapture experiments on *G. palpalis* (Cuisance et al. 1985, Bouyer et al. 2007), flies should be able to reach a distance of 30 km if the populations had been separated by homogeneous riverine forest. But given the geographic location of our study, it seems unlikely for them to actively disperse from the islands to the mangrove or vice versa. However, passive transport by the numerous boats in the area cannot be ruled out. Our working hypothesis is that tsetse from the mangrove colonized the Loos islands probably by passive transport, and probably at the time when the islands harbored important economic activities such as the bauxite exploitation, and the Conakry peninsula harbored natural mangrove vegetation. The frequency of passive tsetse exchanges probably dropped with the regression of economic activities and the degradation of this vegetation. According to that hypothesis, both low migration rates and small population sizes would have contributed to the observed genetic differentiation.

Metric properties are under the influence of both environmental and genetic factors, and as continuous traits they are among the earliest characters to change between physically separated populations (Falconer 1981). Environment typically acts primarily on size (Glasgow 1961) and then on shape, frequently as an allometric effect of size change (Dujardin and Le Pont 2004). The three populations were not distinct on the base of size, but they could be discriminated by shape variation. In our data, although some residual allometry was found due to sexual dimorphism typical of tsetse species (Sané et al. 2000, de La Rocque et al. 2002), no allometric effect was detected to explain the shape discrimination between island and mainland populations. Pure shape differences among conspecific populations are more likely to result from adaptive or genetic causes than from environmental effects (Dujardin and Slice 2006). This interpretation of metric variation was supported by the detection of directional asymmetry (and possibly antisymmetry) only in the island population, although limited to the females.

Directional asymmetry is a heritable trait very common in Diptera (Klingenberg et al. 1998), and the finding of intraspecific variability in this trait also suggests genetic differences.

Data from microsatellites and from wing geometry both converged to the idea of a separation of the Loos island population from the mainland. The level of separation in terms of number of migrants per generation seems high, which conforms to the known dispersing behavior of the insect. Although occasional contacts cannot be excluded, our working hypothesis is that the Loos population of tsetse flies is a completely isolated population. If true, this situation will favor control interventions, with the possibility of eliminating tsetse from this island.

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