

Population Genetics as a Tool to Select Tsetse Control Strategies: Suppression or Eradication of *Glossina palpalis gambiensis* in the Niayes of Senegal

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Abstract

Background: The Government of Senegal has initiated the “Projet de lutte contre les glossines dans les Niayes” to remove the trypanosomosis problem from this area in a sustainable way. Due to past failures to sustainably eradicate *Glossina palpalis gambiensis* from the Niayes area, controversies remain as to the best strategy implement, i.e. “eradication” versus “suppression.” To inform this debate, we used population genetics to measure genetic differentiation between *G. palpalis gambiensis* from the Niayes and those from the southern tsetse belt (Missira).

Methodology/Principal Findings: Three different markers (microsatellite DNA, mitochondrial *CO1* DNA, and geometric morphometrics of the wings) were used on 153 individuals and revealed that the *G. p. gambiensis* populations of the Niayes were genetically isolated from the nearest proximate known population of Missira. The genetic differentiation measured between these two areas ($\theta=0.12$ using microsatellites) was equivalent to a between-taxa differentiation. We also demonstrated that within the Niayes, the population from Dakar – Hann was isolated from the others and had probably experienced a bottleneck.

Conclusion/Significance: The information presented in this paper leads to the recommendation that an eradication strategy for the Niayes populations is advisable. This kind of study may be repeated in other habitats and for other tsetse species to (i) help decision on appropriate tsetse control strategies and (ii) find other possible discontinuities in tsetse distribution.

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Introduction

The Niayes of Senegal harbours the most northern and western population of *Glossina palpalis gambiensis* Vanderplank, which is a major vector of the debilitating diseases Human African Trypanosomosis (HAT) or sleeping sickness, and African Animal Trypanosomosis (AAT) or nagana (reviewed in [1]). Particular meteorological and ecological characteristics of this area provide great potential for agricultural development in general and animal production (cattle, donkeys, horses, small ruminants, pigs and poultry) in particular. Most of these animals are however susceptible to AAT which is seriously limiting the development

of efficient and productive, sustainable livestock systems. The socio-economic impact of the disease is therefore dramatic and very often underestimated [2]. In the 1970s and 1980s, it was attempted to eliminate the *G. p. gambiensis* population from the Niayes mainly using ground spraying of residual insecticides [3]. The tsetse and trypanosomosis problem seemed to have disappeared until flies were detected again in 1998 (unpublished report of the Direction de l'Élevage - DIREL). In 2005, the DIREL initiated a control campaign called «Projet de lutte contre les glossines dans les Niayes» with the objective of developing a sustainable solution to the tsetse and trypanosomosis problem in the Niayes. The programme is funded by the Government of

Author Summary

Tsetse flies transmit trypanosomes to humans (sleeping sickness) and animals (nagana). Controlling these vectors is a very efficient way to contain these diseases. There are several strategies and methods that can be used for control, each being more or less efficient depending on several factors. The Government of Senegal wants to sustainably eliminate trypanosomiasis from the Niayes region by controlling the tsetse vector, *Glossina palpalis gambiensis*. To reach this objective, two different strategies may be used: suppression (decrease in tsetse densities) or eradication (remove all the tsetse in the region until last one). For eradication, the approach has to be area-wide, i.e. the control effort targets an entire pest population within a circumscribed area, to avoid any possible reinvasion. Three different tools (microsatellite DNA, mitochondrial DNA and morphometrics) were used, and all showed an absence of gene flow between *G. p. gambiensis* from the Niayes and from the nearest known population in the south east of the country (Missira). This genetic isolation of the target population leads to the recommendation that an eradication strategy for the Niayes populations is advisable. This kind of study may be extended to other areas on other tsetse species.

Senegal and technically and financially supported by the Food and Agriculture Organization of the United Nations (FAO) and the International Atomic Energy Agency (IAEA). The project is implemented in the context of the African Union - Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC), a political initiative of the African heads of state that calls for increased efforts to manage the tsetse and trypanosomiasis problem.

Tsetse populations may be reduced using a variety of techniques, including insecticide impregnated traps and targets, live-baits, sequential aerial spraying, and sterile male releases [4–9]. In the past, most control efforts were not implemented according to area-wide principles [10,11], and as a consequence, when the control effort was reduced or stopped, the tsetse populations tended to recover – due to either flies surviving the initial interventions, or migrant flies coming from untreated regions, or both [12]. This has fuelled a debate as to whether in some instances “eradication”, defined by FAO [13,14] as the creation of a tsetse free zone, may be more cost effective than “suppression” where tsetse densities are reduced to a level minimizing the risk of disease transmission. A sound decision whether to select an eradication or suppression strategy will be facilitated when the population structure within the target region, in particular the degree of genetic isolation of the target population from its adjacent populations is clearly understood. For isolated populations, eradication may be the most cost-effective strategy, as reported for *Glossina austeni* Newstead in Unguja Island, Zanzibar [5]. But for most mainland populations of tsetse, the geographical limits of target tsetse populations are less easily defined. Application of population genetics techniques can quantify rates of gene flow between sub-populations [15–19], and guide decisions on the choice of control strategies [20].

The level of isolation of the targeted tsetse populations will be an important parameter to guide the Government of Senegal to select the most optimal control strategy. Here we report population genetic analyses of microsatellite and mtDNA markers combined with morphometrics of *G. p. gambiensis* populations sampled from the Niayes area and from the nearest population in the south-

eastern part of the country (fig. 1) to assess their degree of isolation by measuring gene flow among the different populations. The genetic differentiation of the various *G. p. gambiensis* populations within the Niayes was also assessed to determine if the different populations of the Niayes can be targeted at the same time (if it is a single panmictic unit), or if a sequential control strategy can be contemplated (if substantial genetic differentiation between populations is found), which will also depend on their respective history, including effective population sizes and possible bottlenecks.

Methods

Study sites and sampling

In the Niayes region, four tsetse populations were sampled using Vavoua traps: Dakar Hann which is a swamp forest harbouring an animal park within the city of Dakar, Diacsaw Peul, an area of riparian thicket where tsetse and cattle are in intense contact, Sebikotan and Pout, which are mango and citrus-tree plantations where tsetse and people are in close contact. The tsetse flies collected in the Niaye area and analyzed in the present study are the sole property of the Senegalese authorities. They were collected by the national veterinary services through official mission orders, in one wildlife park (Parc de Hann) and three private sites, with the oral consent of the owners. No written consent is mandatory for tsetse fly collection in Senegal. In the south-eastern part of the country, the area of Missira was sampled: it is the nearest known infested area from the Niayes, according to a detailed tsetse survey implemented as part of the baseline data collection of the Niayes tsetse Control project (see Fig. 1). Areas between the Niayes and Missira are not favourable for tsetse, which was confirmed by zero tsetse catches (JB, BS unpublished data) despite intensive trapping efforts.

In total 153 tsetse individuals were analysed originating from Diacsaw Peul (22 females (F), 8 males (M)), Dakar Hann (23F, 6M), Sebikotan (21F, 11M), Missira (23F, 12M), and Pout (13F, 14M).

Microsatellite DNA markers

Markers used and PCR conditions. A total of 10 microsatellite markers were used (preceded by “X” for X-linked loci): X55.3 [21], XPgp11, Pgp1, XPgp13, Pgp24 [22], A10, XB104, XB110, C102 (A. Robinson, FAO/IAEA, pers. com.) and GPCAG [23]. The samples were then processed for Polymerase Chain Reaction (PCR) and genotyping on a 4300 DNA Analysis System from LI-COR (Lincoln, NE) exactly as described in [19].

Population structure analyses. Wright’s F -statistics [24], the parameters most widely used to describe population structure [25], were initially defined for a three-levels hierarchical population structure (individuals, sub-populations and total). In such a structure, three fixation indices or F -statistics can be defined. F_{IS} is a measure of the inbreeding of individuals (hence I) resulting from non random union of gametes within each sub-population (hence S). F_{ST} is a measure of the relatedness between individuals resulting from non-random distribution of individuals among sub-populations, relative to the total population; F_{ST} quantifies the differentiation between sub-populations in the total population (hence S and T). F_{IT} is a measure of the inbreeding of individuals resulting both from non-random union of gametes within sub-populations and from population structure (deviation from panmixia of all individuals of the total population, hence I and T). These F -statistics are classically estimated by Weir and Cockerham’s unbiased estimators f (for F_{IS}), θ (for F_{ST}) and F (for F_{IT}) [26]. When appropriate, these statistics were estimated with

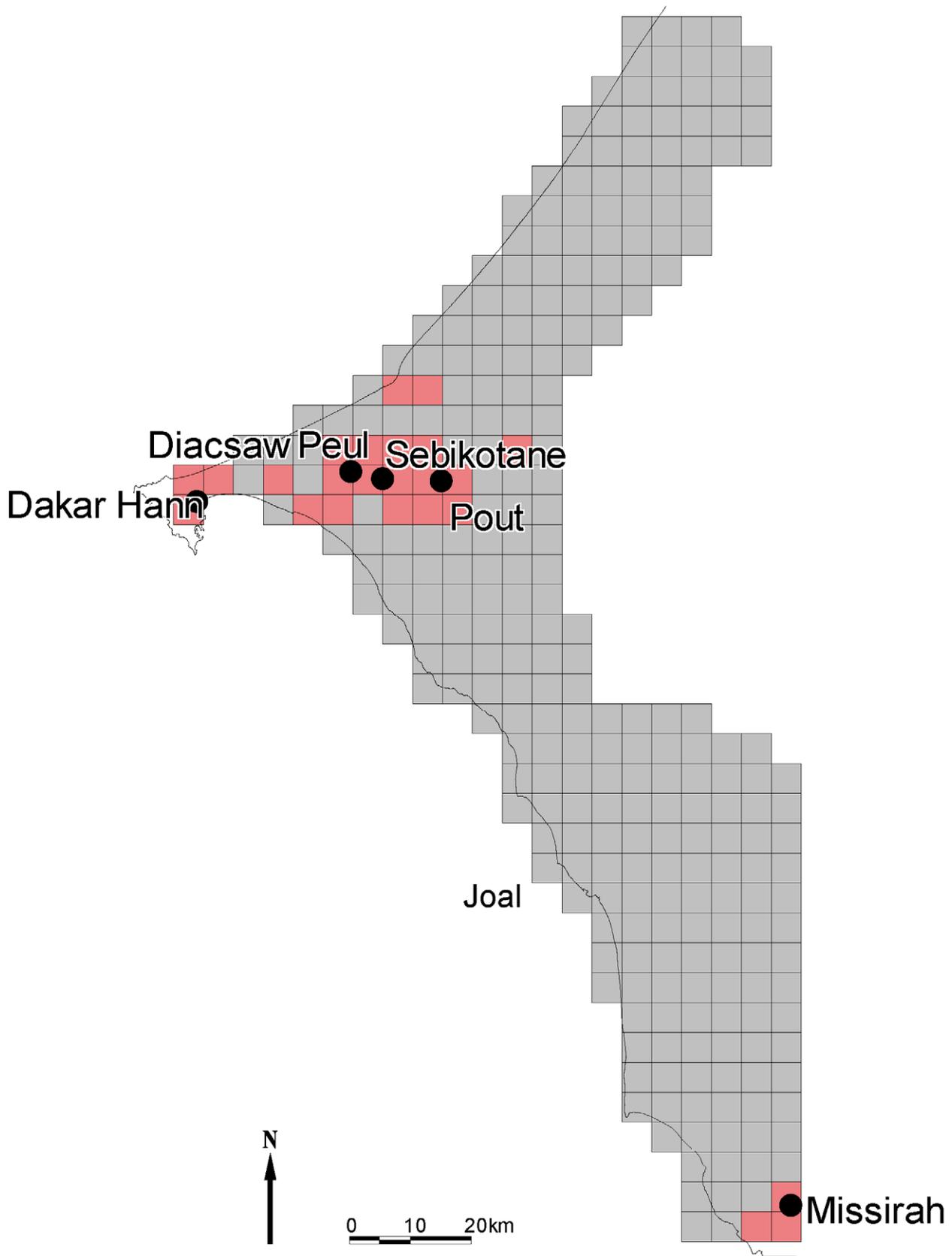


Figure 1. Geographic location of the study sites in Senegal. The study sites are located on the map, together with putative tsetse-infested area (red cells), according to the baseline entomological data collection of the tsetse eradication campaign in the Niayes of Senegal.
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Fstat 2.9.3.2 (updated from [27]). However, more than two levels (i.e., individuals, sub-populations and total) may exist. This is the case for the different subsamples belonging to the Niayes region when to be compared to tsetse from Missira. HIERFSTAT version 0.03–2 [28] is a package for the statistical software R [29]. This package computes hierarchical F -statistics from any number of hierarchical levels [28]. A user-friendly description of the software is presented elsewhere [30].

The significance of the F -statistics was tested by randomization (10000 permutations in each case). The significance of F_{IS} was tested by randomizing alleles between individuals within subsamples. The significance of F_{ST} was tested by randomizing individuals among sub-samples. These tests were performed with Fstat. The significance of F_{SR} , i.e., the homozygosity due to subdivision into subsamples within the Niayes region, was tested by randomising individuals among subsamples of the Niayes. The significance of F_{RT} , which measures the relative homozygosity due to the geographical separation between the Niayes region and Missira was tested by randomizing subsamples (with all individuals contained) between the two regions. These two last tests were performed with HIERFSTAT. For F_{IS} the statistic used was directly the f (unbiased estimator of F_{IS}). For other differentiation measures (F_{ST} , F_{SR} , F_{RT}), the statistic used was the maximum likelihood ratio G [28,31].

Non random association of alleles within individuals ($F_{IS} > 0$) may be due to the presence of null alleles. We used Micro-Checker 2.2.3 [32] to detect null alleles and estimate their frequency p_n at each locus according to Brookfield's second method [33]. For X-linked loci, males were not included in such analyses and null frequency was also directly estimated by the proportion of missing genotypes. The global expected frequency of blanks (null homozygotes) was then compared to the observed one with an exact unilateral (H1: there are less blanks than expected) binomial test with R.

Linkage disequilibrium between pairs of loci was tested using Fstat by randomising loci combinations (free recombination) across loci with a G -based statistics permitting a global test across sub-samples for each pair of loci. Because there are as many tests as locus pairs tested, we used the exact binomial test to check if the proportion of tests found significant at the 5% level was significantly above 0.05 with the "Test alpha' and look for k" option of Multitest V.1.2 [34]. To optimise power we disregarded loci displaying one allele at a frequency above 0.9, which was the case of locus XB110.

Some loci are X-linked. For F_{IS} based analyses males were coded as missing data at these loci, otherwise these loci were coded as homozygous (differentiation and LD based analyses).

In order to visualize the partition in genetic differentiation among all samples, a NJTree dendrogram was constructed with the software MEGA 3.1 [35] from a chord distance matrix (noted $D_{C-S@E}$) [36], as recommended by [37], and computed using Genetix V4 [38].

Effective population sizes. Effective population sizes were computed with three methods. Estim 1.2 [39] uses the connection between migration and effective population size with heterozygosity and linkage disequilibrium between loci. Linkage disequilibrium is indeed connected to effective population size, a property exploited by the method from Bartley et al. [40], which is in fact coming from Hill [41] and modified by Waples [42]. From equation 2 of [40], modified as in NeEstimator Help file [43], allelic correlations (r) can be estimated as $r = D / \sqrt{p(1-p)q(1-q)}$, where p and q are frequencies of allele A at locus 1 and allele B at locus 2, respectively and D is the so called (e.g. [40,44]) Burrow's composite measure of disequilibrium [45]. The r^2 -values across all pairs of alleles

are averaged to yield a single r^2 for each pair of loci. Finally, an arithmetic mean of the r^2 values for all pairs of loci is used to obtain a single correlation coefficient and to obtain an N_e estimate using the equation $N_e = 1 / [3(r^2 - 1/S)]$ where S is the harmonic mean of the sample sizes of each pairwise comparison between loci [40,46]. Males were coded homozygous for X-linked loci, which should not be a problem for a composite based linkage disequilibrium measure. The method was implemented with NeEstimator [43]. Heterozygote excess method from Pudovkin et al. [47] (see also [48]) corrected by Balloux [49], uses the fact that, in dioecious (or self incompatible) populations, alleles from females can only combine with alleles contained in males and a heterozygote excess is expected as compared to Hardy-Weinberg expectations, and this excess is proportional to the effective population size. This method was implemented using Weir and Cockerham estimator of F_{IS} in the equation $N_e = 1 / (-2F_{IS} - F_{IS} / (1 + F_{IS}))$ [49] and was only applicable in subsamples with heterozygote excess.

For intra locus based methods (Estim and Balloux's methods), loci showing evidence of stuttering and null alleles were removed.

Bottleneck detection. Signatures of bottleneck events were investigated by comparing the expected heterozygosity for a sample (H_E) with the heterozygosity that would be expected for a sample taken in a population at mutation/drift equilibrium with the same size and allele number (H_{EQ}). As allele number decreases faster than heterozygosity, a bottleneck is signed by $H_E > H_{EQ}$ in subsequent generations [50]. This analysis was performed with BOTTLENECK v1.2 software [51] assuming that mutations of microsatellite loci followed either an IAM (infinite allele model), a SMM (stepwise mutation model), or a TPM (two phase model), in the last case of which we assumed that 70% of mutations consist of one step and 30% consist of multistep change with a variance of 30 (default values). Tests were performed using unilateral Wilcoxon tests as recommended [50].

The method of bottleneck detection described by Cornuet and Luikart [50] allows a rough estimate of the effective population size right after the bottleneck event (N_{e-pb}). From the Figure 3A of [50], with 10 loci and mean sample sizes of 10–30 individuals, a bottleneck can be detected if it occurred between $\tau_1 2N_{e-pb}$ and $\tau_2 2N_{e-pb}$ generations before sampling with $\tau_1 = 0.1$ and $\tau_2 = 2.5$.

The difference in genetic diversity, as measured by H_s (Nei's unbiased estimator [52]), between the Niayes and Missira was evaluated with a bilateral Wilcoxon signed rank test for paired data. The different microsatellite loci were used as the pairing factor. This test was undertaken with R.

The Bonferroni procedure [53] was used each time multiple testing was done and individual tests significance required.

User-friendly descriptions of most of the tests and procedures used in the genetic data analyses can be found in a recent review [54].

Mitochondrial DNA markers

A portion of the 5' end of the mitochondrial gene COI was amplified, purified and sequenced using the primers CI-J-2195 TTGATTTTTTGGTCATCCAGAAGT [55] and CULR TGAAGCTTAAATTCATTGCACCTAATC using the same conditions reported by [56]. The following statistics were calculated using DNAsp version 4.50.3 [57]: Hd Haplotype diversity, was calculated using equations 8.4 and 8.12 in [58], P_i the nucleotide diversity, which is the average number of nucleotide differences per site between two sequences, and its sampling variance was calculated using equations 10.5–10.7 in [58], K_i the average number of nucleotide differences and the total variance of K_i is (sampling plus stochastic), assuming no recombination were calculated using equations from [59]. F_{ST} was calculated according

to equation 3 in [60], H_{ST} was calculated according to equation 2–4, and K^*_{ST} according to equations 7–11 in [60]. H_{ST} is a haplotype frequency based genetic differentiation statistic that does not take into account the number of differences separating different haplotypes. $H_{ST} = 1 - (H_S/H_T)$, where H_S is the weighted average of subpopulation genetic diversities and H_T is the estimated haplotype diversity of the total population. $K^*_{ST} = 1 - (K_S/K_T)$, where K^*_S is a weighted average of the log corrected average number of sequence difference in the populations being compared, and K_T is the average number of difference between sequences. A permutation test, in which haplotypes or sequences were randomly assigned to the different localities 10000 times, was used to test the significance of H_{ST} and K^*_{ST} [60]. The average number of nucleotide difference (equation A3, [61]) and its variance (sampling plus stochastic) were also calculated using DNAsp version 4.50.3. Haplotype trees for 738 nucleotides of *COI* from *G. p. gambiensis* (data from a total of 148 individuals in this study) were generated using the algorithm of [62]. The TCS 1.21 programme was used to estimate the haplotype tree, with the connection limit (probability of parsimony) at 95% [63]. The maximum number of connection steps at 95% was 11.

Geometric morphometrics

In total, the number of analysed wings was 20, 21, 18, 34 and 18 for Diacsaw Peul, Dakar Hann, Sebikotan, Missira and Pout respectively. These analysed individuals were all also analysed by the molecular markers.

Wings were dry-mounted between two microscope slides and scanned with a scanner. From this picture, 10 landmarks defined by vein intersections were recorded as previously described [64]. Each landmark has X and Y coordinates, and the 10 LM defined per wing represent a polygon. After scaling, translating and rotating all these polygons so that they can be compared, data were subjected to generalized Procrustes analysis (GPA) [65,66] allowing to implement shape variables, here represented by 16 “partial warps” (PW) (including uniform component of shape). These PW were used to conduct a discriminant analysis to allow for individual reclassification based on Mahalanobis distances (noted D_M), which were calculated between populations. The statistical significance of Mahalanobis distances was estimated by 1,000-runs permutation tests [67]. A re-classification score was computed where individuals are assigned to each group and the percentage of good classification was then calculated.

Evaluating correlation between genetic distances and morphometric distance matrices

To evaluate the correlation between distance matrices we undertook three Mantel tests [68] with the “Mantelize it” option of Fstat 2.9.4 [27] between $D_{C-S\&E}$ and Kst^* , between $D_{C-S\&E}$ and D_M , and between Kst^* and D_M .

Results

Microsatellite loci

Within subsamples analyses. The per-locus number of alleles was 3.4, 3.7, 3.9 and 4 for Diacsaw Peul, Dakar Hann, Sebikotan and Pout respectively (all coming from the Niayes), but it was 8.7 for Missira. The Wilcoxon test confirmed that mean genetic diversity in the Niayes ($H_s = 0.5$) was lower than the one found in Missira ($H_s = 0.7$) (P -value = 0.0059).

There was a global and significant homozygote excess over all loci ($F_{IS} = 0.17$, P -value = 0.0001). Figure 2 shows that this is mainly due to certain loci (i.e. pGp1, pGp24, A10 and XB110). This strongly suggests locus specific technical problems. Micro-

checker analysis confirmed this view as only A10 and XB110 did not display enough blanks (not enough male blanks for XB110) as compared to expected ones if F_{IS} were only due to null alleles. However, both loci seemed to suffer from stuttering. Without these four loci, the six remaining loci did not result in rejection of the hypothesis of panmixia ($F_{IS} = 0.035$, P -value = 0.0983).

Among the 36 possible tests, four pairs of loci displayed a significant linkage at the 5% level of significance. This proportion (4/36) is not different from the proportion expected under the null hypothesis (exact binomial test, P -value = 0.1037).

Hierarchical population structure. The overall mean F_{ST} value was estimated at $\theta = 0.11$ (CI_{95} : 0.088 < θ < 0.143). The mean θ value between Missira and all the Niayes populations was high and significant, $\theta = 0.12$ (CI_{95} : 0.08 < θ < 0.14, $p < 0.001$). Within the Niayes, the mean θ value between Dakar Hann and the three others was also high and significant $\theta = 0.12$ (CI_{95} : 0.05 < θ < 0.20, $p < 0.001$), which is of the same order of magnitude as the one between Missira and the Niayes. This is illustrated in Fig. 3, and this explains why HierFstat analysis reveals a substantial and highly significant contribution of sites ($F_{SR} = 0.069$, P -value = 0.001) and an absence of significant contribution of region (P -value = 0.209). This latter point is due to the fact that Dakar-Hann is as different from the other Niayes subsamples, than Niayes are from the remote Missira subsample.

Effective population sizes. A constant seemed to be that Diacsaw Peul, Dakar-Hann and Sebikotan displayed very small effective population sizes (3 to 28), and Pout and Missira appeared much bigger (51, 83 respectively). However, some of the computations led to infinite population sizes and sometimes the different methods did not converge to a reliable estimator (see Table S1), which suggests bigger effective population sizes than those found.

Bottleneck. Only two subsamples provided some evidence for a bottleneck: Dakar-Hann with P -values of 0.001, 0.001 and 0.116 for IAM, TPM and SMM mutation models respectively; and Pout with P -values 0.012, 0.348 and 0.935 for IAM, TPM and SMM (see Table S2). These values were still significant after Bonferroni procedure for multiple testing in Dakar Hann only: $P = 0.015$ and 0.022 for IAM and TPM respectively. It is thus probable that a bottleneck affected the population from Dakar-Hann, between $0.2N_{e-pb}$ and $5N_{e-pb}$ generations ago. Using 16 as a proxy for the effective population size in this site (obtained with Bartley’s method) ranges the bottleneck between 3.2 and 80 generations ago. With the 95% confidence interval upper limits (i.e. 23) the ranges increases to [5–115]. This bottleneck provides a good explanation for the genetic isolation noticed for this site during the population differentiation analysis.

Mitochondrial DNA

The statistics of genetic differentiation between the Niayes population samples from Sebikotan, Pout, Dakar Hann and Diasa Peuhl and the south-eastern Senegal site Missira based on 738 nucleotides of mitochondrial *cytochrome oxidase 1 (COI)* are shown in table 1. Statistics of genetic differentiation based on nucleotide sequence and haplotype frequency both indicate a very high level of genetic differentiation between Niayes populations and Missira, since there were no common haplotypes between the two regions (see Fig 4). Genetic differentiation between Diacsaw Peul and Pout was also significant ($0.05 > P > 0.01$). Otherwise, there was no significant evidence for differentiation between populations within the Niayes region. The haplotype and nucleotide diversity of the Niayes populations was very low in all cases (see table 2).

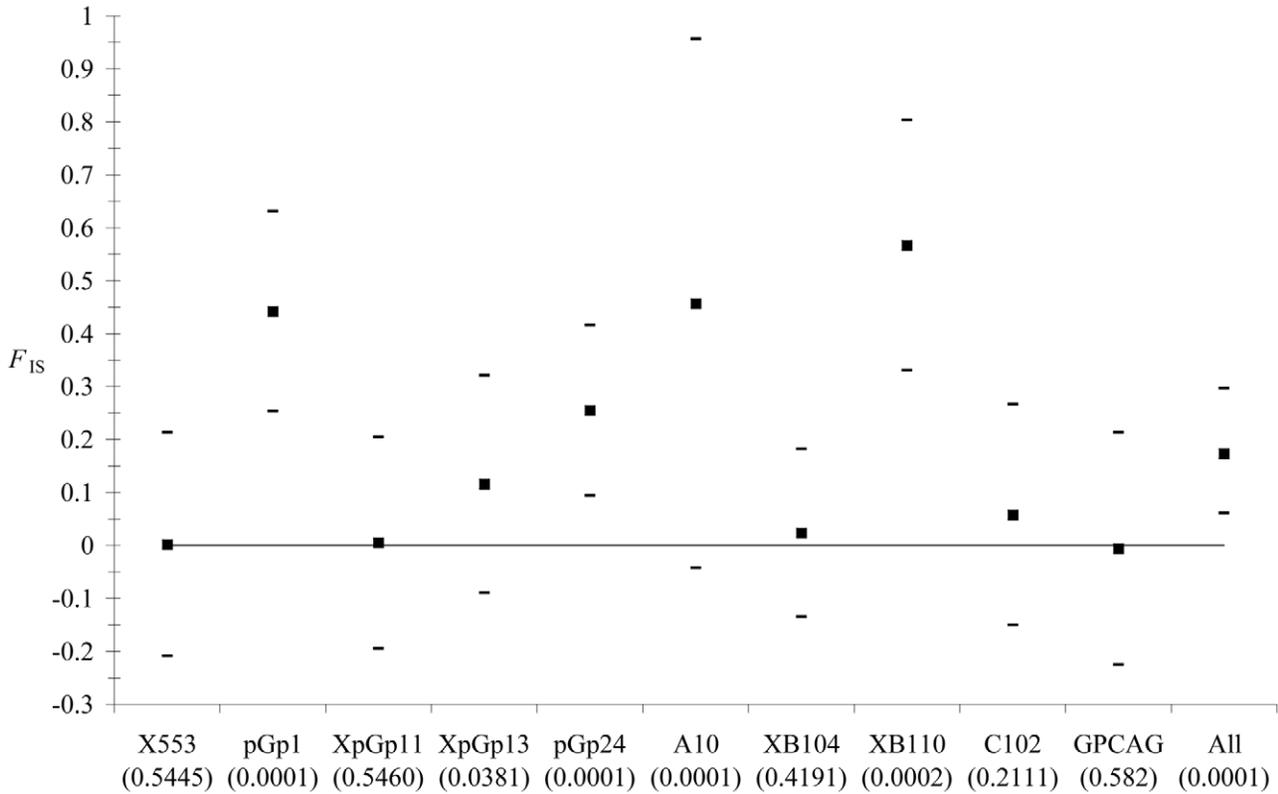


Figure 2. F_{IS} across loci and overall loci. For each locus 95% confidence intervals (CI) were obtained by jackknives over populations. Overall loci (All), CI were obtained by bootstrap over loci. Deviations from panmixia are given as corresponding P -values between brackets.
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Morphometrics

An analysis of the shape of the wings was conducted by using Procrustes analysis to compare wing shape among the five different populations. All Mahalanobis distances between populations were significant except between Pout and Sebikotan. This is illustrated by reclassification scores which were over 70% indicating differentiation between populations (see Table 3). Missira sample

showed the best one (85%) confirming this sample was easily distinguished from the others.

Correlation between distance matrices

The correlation between the genetic distances and morphometric distances were all strong and significant (see Figure 5). The strong correlation between microsatellites and COI

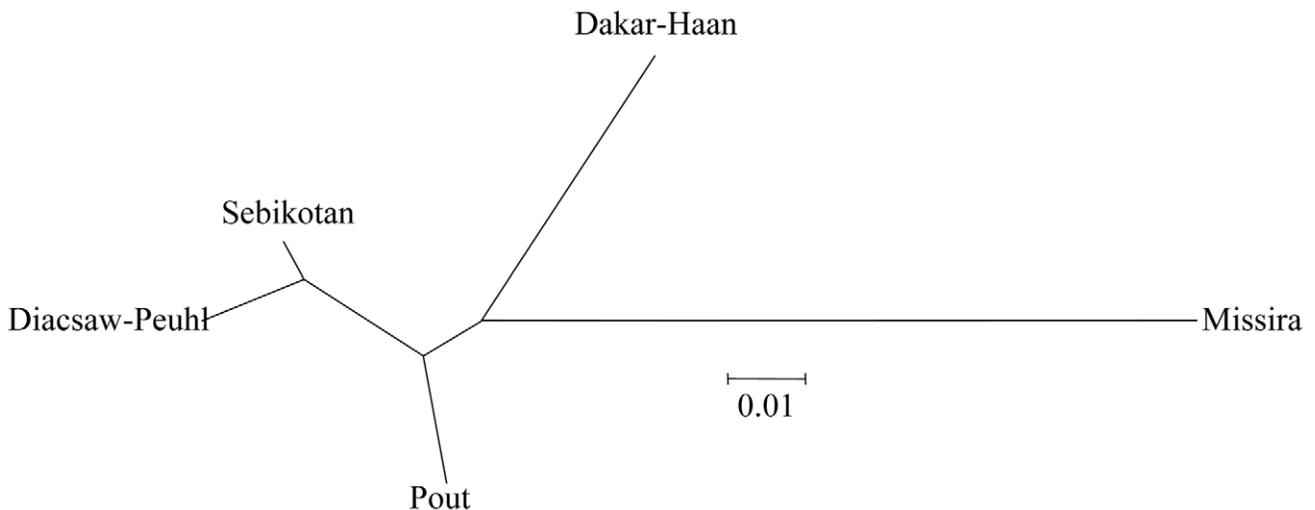


Figure 3. Unrooted Cavalli-Sforza and Edward's chord distance based Neighbour-Joining-Tree. This tree shows the respective genetic distribution of the five *G. palpalis gambiensis* tsetse samples from Senegal.
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Table 1. Population differentiation statistics for mitochondrial *cytochrome oxidase 1 (COI)* sequences (738 sites in alignment, no gaps).

Population	Population	Hs	Fst	Kst*	Hst
Missira	Sebikotan	0.431	0.88	0.642	0.405
Missira	Diacsaw Peul	0.415	0.883	0.646	0.426
Missira	Dakar Hann	0.499	0.876	0.607	0.350
Missira	Pout	0.611	0.865	0.561	0.253
Sebikotan	Diacsaw Peul	0.032	0.000	-0.002 ns	-0.002 ns
Sebikotan	Dakar Hann	0.099	-0.02	-0.007 ns	-0.007 ns
Sebikotan	Pout	0.200	0.137	0.087 ns	0.087 ns
Diacsaw Peul	Dakar Hann	0.067	0.038	0.022 ns	0.025 ns
Diacsaw Peul	Pout	0.171	0.208	0.134 (0.05>P>0.01)	0.134 (0.05>P>0.01)
Dakar Hann	Pout	0.256	0.062	0.035 ns	0.035 ns

Bold type indicates significant tests ($P < 0.001$) after sequential Bonferroni correction for multiple testing.

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distance matrices was mainly due to the fact that there are two sets of points: those from the Niayes sites and those differentiating the Niayes from Missira (very strong distances for both markers). For the correlation between genetic and morphometric data, Figure 5 shows that around 50% of the variance in Mahalanobis distance can be explained by genetics, meaning that the other 50% are probably explained by environmental parameters.

Discussion

The results of the genetic and morphometric analyses indicated limited gene flow between the *G. p. gambiensis* population of the Niayes and those of the main tsetse belt in the south-eastern part of

Senegal. Using three different kinds of markers, i.e. microsatellite DNA, mitochondrial DNA and geometric morphometrics, the data led to the conclusion that the *G. p. gambiensis* population from the Niayes can be considered isolated with very little risk of re-invasion should the population eventually be eradicated. These data corroborate the results of the entomological baseline data collection and observations in the field indicating the absence of *G. p. gambiensis* in the 120 km long area between Missira and the Niayes (JB, BS unpublished results.). On this basis, eradication of *G. p. gambiensis* from the Niayes can therefore be recommended as an appropriate control strategy. The data from this study, which was part of a comprehensive baseline data collection effort, confirmed that eradication can be recommended as an appropriate control strategy, and as such the study greatly assisted in the decision making on which strategy to select. Although the isolation of the target population is not an absolute prerequisite for AW-IPM, tackling a continuous pest populations is actually more complex, requiring more resources and a long-term, regional commitment (for details, see [10,11,14]).

Looking at F_{ST} values reported in previous studies on *G. p. gambiensis*, the one observed in the present work between Missira and the Niayes populations was ten times higher than those observed along 260 km on the Mouhoun river in Burkina Faso [17], and two times higher than the values observed in Guinea between Loos islands and the continent [64]. These values are of the same order of magnitude as those observed between the two different taxa, *G. p. gambiensis* and *G. palpalis palpalis* (DK, PS, unpublished data). It is also noteworthy that some of the microsatellite loci used on the individuals in this study amplified poorly, a behaviour not recorded in earlier studies of *G. p. gambiensis* [17,19,64]. This may be an additional argument for genetic divergence, since it is known that in different taxa polymorphisms in sequences flanking the microsatellite may occur [69] leading then to mismatches in the primer binding sites. If gene flow between the sites occurs then shared haplotypes of the mitochondrial gene COI would be expected. In fact, we observed no haplotypes shared between Missira and the Niayes region. Future work should include more detailed examination of

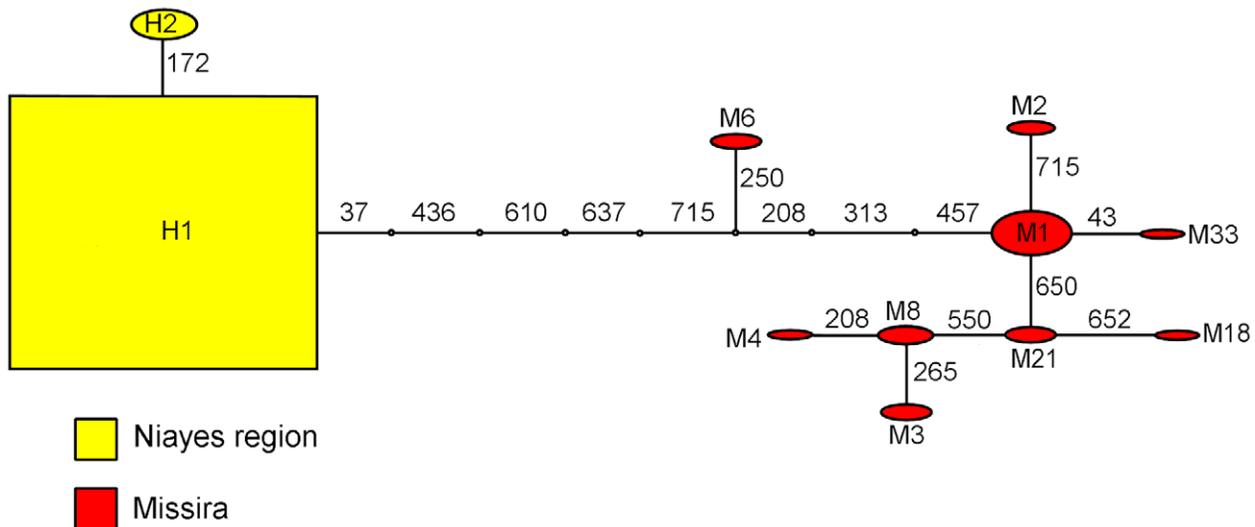


Figure 4. Haplotype trees for 738 nucleotides of COI from *G. p. gambiensis*. Nodes are labelled with the name of one of the individuals having a haplotype at that node. All the Niayes haplotypes are included in the nodes labelled H1 and H2. Node size is proportional to the number of haplotypes at that node. Abbreviations: H: Dakar Hann, M: Missira. The numbers on the branches indicate the position of the nucleotide in the alignment which is mutated.

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Table 2. Diversity statistics for the studied populations based on *COI* sequences.

Population	Number of individuals sequenced	Number of Haplotypes	Polymorphic Sites	Hd	Pi	K (variance)
Missira	34	9	10	0.777 (3.87×10 ⁻³)	0.00271 (2×10 ⁻⁷)	1.996 (1.333)
Sebikotan	32	2	1	0.063 (3.33×10 ⁻³)	0.00008 (0×10 ⁻⁷)	0.063 (0.019)
Diacsaw Peul	30	1	0	0.000 (0.0000)	0.00000 (0×10 ⁻⁷)	0.000 (0.000)
Dakar Hann	27	2	1	0.142 (7.43×10 ⁻³)	0.00019 (0×10 ⁻⁷)	0.142 (0.045)
Pout	25	2	1	0.380 (8.33×10 ⁻³)	0.00051 (0×10 ⁻⁷)	0.380 (0.139)
Total	148	11	15	0.483 (2.39×10 ⁻³)	0.00441 (2×10 ⁻⁷)	3.257 (2.849)

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potential cryptic species within the distribution area of *G. p. gambiensis* since such cryptic species have been suspected and shown to exist in closely related taxa [70,71].

In case the Government of Senegal decides to integrate the sterile insect technique (SIT) as part of the AW-IPM (area-wide integrated pest management) approach, it would be advisable that prior to the release of sterile males, experiments be conducted to assess mating compatibility between the *G. p. gambiensis* from the Niayes and those which should be released if they do not originate from the same area (e.g. from Burkina Faso). Although previous studies with *G. p. gambiensis* originating from Mali and Burkina Faso revealed no mating barriers between these populations (G. Mutika, personal communication), nor did another study between *G. p. gambiensis* and *G. p. palpalis* [72], these tests would provide evidence that genetic differentiation of the Niayes population has not been accompanied by pre-mating barriers, that would threaten the success of the programme. Similarly, it may also be recommended to release sterile flies coming from Burkina Faso in the Niayes to assess their behaviour and performance (dispersal, dispersion, mobility, lifespan, mating frequency etc.) in the natural habitat. Indeed, the particular eco-system of the Niayes, the fragmented nature of the preferred vegetation types of *G. p. gambiensis* (mango-tree plantations, *Euphorbia spp.* hedges) and an annual precipitation below 400–500 mm might have induced the development of some level of xerotolerance of these relatively small populations [73,74].

The second question that has relevance for a future AW-IPM programme was to know whether within the Niayes region, the four populations showed any genetic differentiation, or if they constituted a single, panmictic (i.e random mating) population. Low haplotype diversity of the populations of this area is in agreement with a previous Single Strand Conformation Polymor-

phism-based study on mitochondrial DNA haplotypes in the Niayes region of Senegal [75]. The Niayes population is probably a remnant population, possibly of small size and therefore likely to lose rare haplotypes by genetic drift more rapidly than larger populations. Many ancestral *G. p. gambiensis* haplotypes have probably been lost from the Niayes region, leaving just two haplotypes remaining today. The low diversity was also observed at microsatellite loci with lower genetic diversity than in Missira. The three different markers used in this study generally showed good agreement in the differentiations observed, as can be seen by the high and significant correlations coefficients computed between them.

The only apparent discrepancy between microsatellites on one hand and COI based and morphometrics based distances on the other hand probably comes from the variance expected for paired F_{ST} estimates and the small sample sizes, the effect of which is probably increased by historical events such as the probable bottleneck that affected the Dakar-Hann population. This may explain why only microsatellite data suggest that the population from Dakar Hann is so much differentiated from all the others, including the geographically closest ones. From an operational point of view however, the population of Dakar Hann is probably also isolated from the three others, since the bottleneck signature was still visible, suggesting very limited exchanges (if any) with the other populations. Field data also corroborate this since the population of Dakar Hann is located within an animal park of this huge city which can be seen as an isolated refuge for this tsetse population. This further suggests that an elimination operation may probably be implemented on this population.

The results also suggest that the samples from Diacsaw Peul, Sebikotan and Pout, although showing some genetic differentiation, are not completely isolated from each other. This is also consistent with ecological data since it appears that the maximum distance between the forest patches of this area is less than 2km (data not shown.).

To conclude, the use of genetic and morphometric markers has been instrumental in the decision-making process of selecting and developing of an appropriate intervention strategy to create a sustainably zone free of *G. p. gambiensis* and Trypanosomosis in the Niayes region of Senegal. In the near future, it should be encouraged to carry out such studies prior to the selection of target areas or the choice of control strategies, and these should be part of the overall collection of baseline data (see ref [76] for recent review). In addition, the results obtained here suggest that efforts should be made to look for other genetic discontinuities in *G. palpalis* s.l. distribution that may be indicative of the presence of cryptic species.

Table 3. Geometric morphometrics: reclassification scores of individuals in their population of origin.

	Proportion of individuals correctly reclassified in their population (%)
Diacsaw Peul	15/20 (75)
Dakar Hann	16/21 (76)
Sebikotan	14/18 (77)
Missira	29/34 (85)
Pout	13/18 (72)

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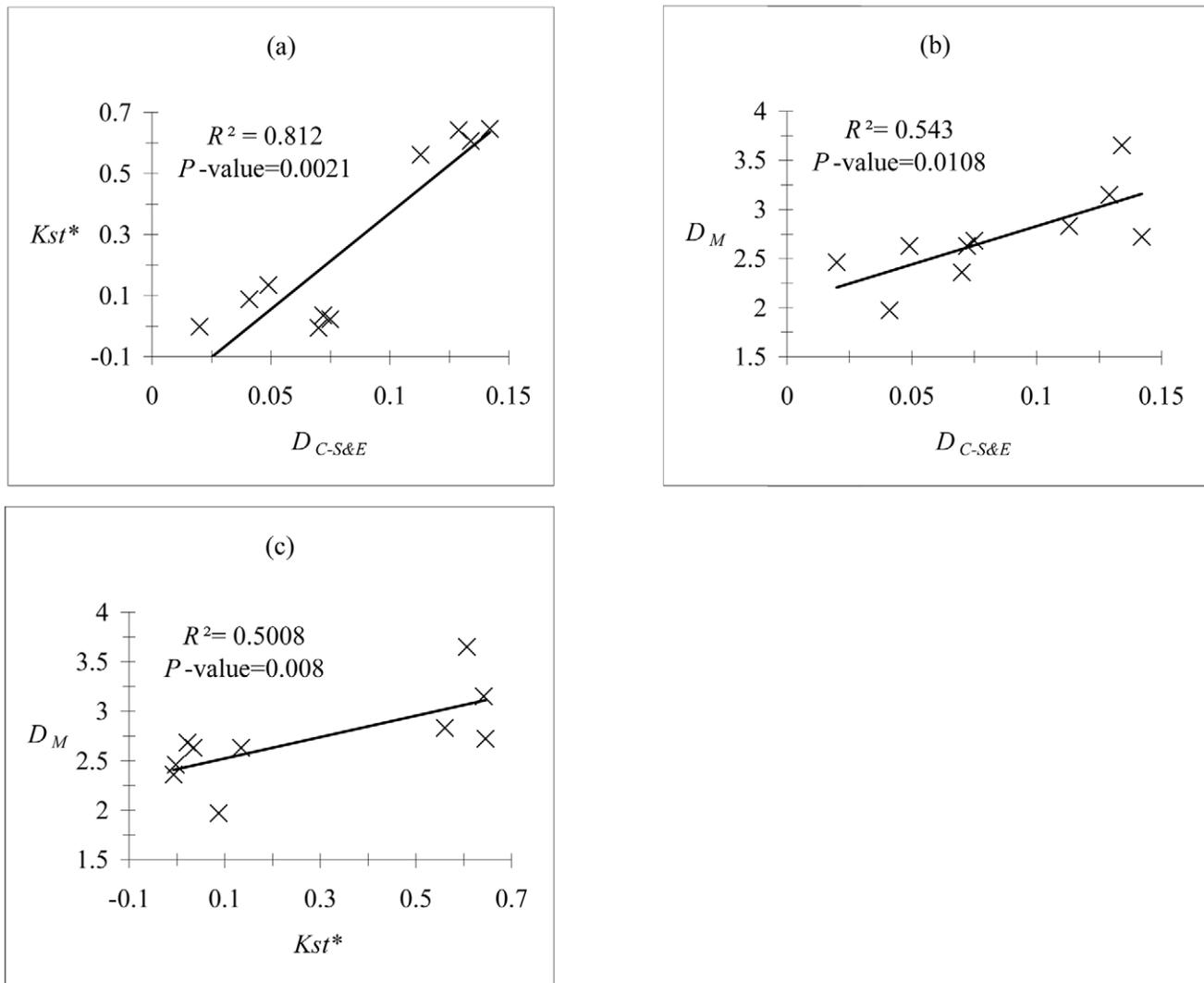


Figure 5. Mantel correlation tests (P -values) between the different markers used in this study. Mantel correlation tests and percentages of variance explained (R^2) between (a) Cavalli-Sforza and Edwards ($D_{C-S\&E}$) computed from microsatellite data and Kst^* computed from COI mitochondrial data, (b) $D_{C-S\&E}$ and Mahalanobis distance (D_M) computed from morphometric data, and (c) Kst^* and D_M , for tsetse flies from the five different samples from Senegal (10 distances). doi:10.1371/journal.pntd.0000692.g005

Supporting Information

Alternative Language Abstract S1 Translation of the abstract into French by PS.

Found at: doi:10.1371/journal.pntd.0000692.s001 (0.03 MB DOC)

Table S1 Effective population size estimates according to the different methods described.

Found at: doi:10.1371/journal.pntd.0000692.s002 (0.02 MB XLS)

Table S2 Probabilities of detecting bottleneck in each population according to microsatellite mutation model.

Found at: doi:10.1371/journal.pntd.0000692.s003 (0.02 MB XLS)

References

- Solano P, Bouyer J, Itard J, Cuisance D (2009a) Cyclical vectors of Trypanosomiasis. In: Lefèvre PC, Chermette J, Blancou R, Uilenberg G, eds. Infectious and Parasitic Diseases of Livestock, Lavoisier Tec&Doc. Chap 12. pp 153–181. in press.
- Budd L (1999) Economic analysis. In: DFID-funded tsetse and trypanosomiasis research and development since 1980. Chatham, UK, Department for International Development: Livestock Production Programme, Animal Health Programme/Natural Resources Systems Programme.

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Author Contributions

Conceived and designed the experiments: PS BS MJBV MTS MJD TDM JB. Performed the experiments: DK SR NAD BS MTS HD LG. Analyzed the data: PS SR NAD HD LG MJD TDM JB. Contributed reagents/materials/analysis tools: DK SR BS MJBV MTS HD LG. Wrote the paper: PS DK NAD MJBV MJD TDM JB.

3. Touré SM (1973) Lutte contre *Glossina palpalis gambiensis* dans la région des niayes du Sénégal. Rev Elev Méd vét Pays trop 26: 339–347.
4. Challier A, Eyraud M, Lafaye A, Laveissière C (1977) Amélioration du rendement du piège biconique pour glossines (Diptera: Glossinidae) par l'emploi d'un cône inférieur bleu. Cah ORSTOM sér Ent Méd Parasitol 15: 283–286.
5. Vreysen MJB, Saleh KM, Ali MY, Abdulla AM, Zhu ZR, et al. (2000) *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the Sterile Insect Technique. J Econ Entomol 93: 123–135.
6. Cuisance D, Itard J, Solano P, Desquesnes M, Frézil JL, et al. (2003) Trypanosomoses. Méthodes de lutte. In: Editions Médicales Internationales Tec et Doc. Principales maladies infectieuses et parasitaires du bétail. Europe et Régions chaudes. Paris, France: Lavoisier. pp 139–165.
7. Kgori PM, Modo S, Torr SJ (2006) The use of aerial spraying to eliminate tsetse from the Okavango Delta of Botswana. Acta Trop 99: 184–199.
8. Bouyer J, Solano P, Cuisance D, Itard J, Frézil JL, et al. (2009a) Control methods in Trypanosomosis. In: Lefèvre PC, Chermette J, Blancou R, Uilenberg G, eds. Infectious and Parasitic Diseases of Livestock, Chap 127 Lavoisier Tec&Doc, in press.
9. Torr SJ, Solano P (2009) Olfaction in tsetse hosts interactions. In: Knols B, Takken W, eds. Olfaction in vector hosts interactions. Netherlands: Wageningen University, In press.
10. Klassen W (2005) Area-wide integrated pest management and the sterile insect technique. In: Dyck VA, Hendrichs J, Robinson AS, eds. Sterile insect technique, Principles and practice in area-wide integrated pest management. Dordrecht, The Netherlands: Springer. pp 39–68.
11. Vreysen M, Robinson AS, Hendrichs J (2007) Area-Wide Control of Insect Pests, from research to field implementation. Dordrecht, The Netherlands: Springer.
12. Hargrove JW (2003) Tsetse eradication: sufficiency, necessity and desirability. UK: Centre for Tropical Veterinary Medicine, University of Edinburgh.
13. Food and Agriculture Organization of the United Nations (2005) Glossary of phytosanitary terms. ISPM, Publication Number 5, International Plant Protection Convention (I PPC). Rome, Italy: FAO.
14. Hendrichs J, Vreysen MJB, Enkerlin WR, Cayol JP (2005) Strategic options in using sterile insects for area-wide integrated pest management. In: Dyck VA, Hendrichs J, Robinson AS, eds. Sterile insect technique, Principles and practice in area-wide integrated pest management. Dordrecht, The Netherlands: Springer. pp 563–600.
15. Solano P, de La Rocque S, de Meeûs T, Cuny G, Duvallet G, et al. (2000) Microsatellite DNA markers reveal genetic differentiation among populations of *Glossina palpalis gambiensis* in the agropastoral zone of Sideradougou, Burkina Faso. Insect Mol Biol 9: 433–439.
16. Gooding RH, Krafur ES (2005) Tsetse genetics: contributions to biology, systematics, and control of tsetse flies. Annu Rev Entomol 50: 101–123.
17. Bouyer J, Ravel S, Dujardin JP, de Meeûs T, Vial L, et al. (2007) Population structuring of *Glossina palpalis gambiensis* (Diptera: Glossinidae) according to landscape fragmentation in the Mouhoun river, Burkina Faso. J Med Entomol 44: 788–795.
18. Bouyer J, Balenghien T, Ravel S, Vial L, Sidibé I, et al. (2009b) Population sizes and dispersal patterns of tsetse flies: rolling on the river? Mol Ecol 18: 2787–2797.
19. Solano P, Ravel S, Bouyer J, Camara M, Kagbadouno MS, et al. (2009b) Population Structures of Insular and Continental *Glossina palpalis gambiensis* in Littoral Guinea. PLoS Negl Trop Dis 3(3): e392. doi:10.1371/journal.pntd.0000392.
20. Kagbadouno M, Camara M, Bouyer J, Hervouet JP, Morifaso O, et al. (2009) Tsetse elimination: its interest and feasibility in the historical sleeping sickness focus of Loos islands, Guinea. Parasite 16: 29–36.
21. Solano P, Duvallet G, Dumas V, Cuisance D, Cuny G (1997) Microsatellite markers for genetic population studies in *Glossina palpalis* (Diptera: Glossinidae). Acta Trop 65: 175–180.
22. Luna C, Bonizzoni MB, Cheng Q, Aksoy S, Zheng L (2001) Microsatellite polymorphism in the tsetse flies (Diptera: Glossinidae). J Med Entomol 38: 376–381.
23. Baker MD, Krafur ES (2001) Identification and properties of microsatellite markers in tsetse flies *Glossina morsitans* sensu lato (Diptera: Glossinidae). Mol Ecol Notes 1: 234–236.
24. Wright S (1965) The interpretation of population structure by *F*-statistics with special regard to system of mating. Evolution 19: 395–420.
25. Nagylaki T (1998) Fixation indices in subdivided populations. Genetics 148: 1325–1332.
26. Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. Evolution 38: 1358–1370.
27. Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate *F*-statistics. J Hered 86: 485–486.
28. Goudet J (2005) HIERFSTAT, a package for R to compute and test hierarchical *F*-statistics. Mol Ecol Notes 5: 184–186.
29. R-Development-core-team (2008) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing, <http://www.R-project.org>, ISBN 3-900051-07-0.
30. De Meeûs T, Goudet J (2007) A step-by-step tutorial to use HierFstat to analyse populations hierarchically structured at multiple levels. Infect Genet Evol 7: 731–735.
31. Goudet J, Raymond M, De Meeûs T, Rousset F (1996) Testing differentiation in diploid populations. Genetics 144: 1933–1940.
32. Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICROCHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4: 535–538.
33. Brookfield JFY (1996) A simple new method for estimating null allele frequency from heterozygote deficiency. Mol Ecol 5: 453–455.
34. De Meeûs T, Teriokhin AT, GJ F (2009) MultiTest V.1.2, a program to binomially combine independent tests and performance comparison with other related methods on proportional data. BMC Bioinformatics, in press.
35. Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5: 150–163.
36. Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: model and estimation procedures. Am J Hum Genet 19: 233–257.
37. Takezaki N, Nei M (1996) Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics 144: 389–399.
38. Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Montpellier (France): Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II.
39. Vitalis R, Couvet D (2001) ESTIM 1.0: a computer program to infer population parameters from one- and two-locus gene identity probabilities. Mol Ecol Notes 1: 354–356.
40. Bartley D, Bagley M, Gall G, Bentley B (1992) Use of linkage disequilibrium data to estimate effective size of hatchery and natural fish populations. Conserv Biol 6: 365–375.
41. Hill WG (1981) Estimation of effective population size from data on linkage disequilibrium. Genet Res 38: 209–216.
42. Waples RS (1991) Genetic methods for estimating the effective size of cetacean populations. In: Hoelzel AR, ed. Genetic ecology of whales and dolphins, International Whaling Commission (Special Issue No. 13). pp 279–300.
43. Peel D, Ovenden JR, Peel SL (2004) NeEstimator Version 1.3: software for estimating effective population size. Queensland Government, Department of Primary Industries and Fisheries.
44. Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conserv Genet 7: 167–184.
45. Weir BS (1979) Inferences about linkage disequilibrium. Biometrics 35: 235–254.
46. England PR, Cornuet JM, Berthier P, Tallmon DA, Luikart G (2006) Estimating effective population size from linkage disequilibrium: severe bias in small samples. Conserv Genet 7: 303–308.
47. Pudovkin AI, Zaykin DV, Hedgcock D (1996) On the potential for estimating the effective number of breeders from heterozygote excess in progeny. Genetics 144: 383–387.
48. Luikart G, Cornuet JM (1999) Estimating the effective number of breeders from heterozygote excess in progeny. Genetics 151: 1211–1216.
49. Balloux F (2004) Heterozygote excess in small populations and the heterozygote-excess effective population size. Evolution 58: 1891–1900.
50. Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144: 2001–2014.
51. Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. J Hered 90: 502–503.
52. Nei M, Chesser RK (1983) Estimation of fixation indices and gene diversities. Ann Hum Genet 47: 253–259.
53. Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Stat 6: 65–70.
54. De Meeûs T, McCoy KD, Prugnolle F, Chevillon C, Durand P, et al. (2007) Population genetics and molecular epidemiology or how to “débûsquier la bête”. Infect Genet Evol 7: 308–332.
55. Simon C, Frati F, Bechenbach A, Crespi B, Liu H, et al. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc America 87: 651–701.
56. Dyer NA, Lawton SP, Ravel S, Choi KS, Lehane MJ, et al. (2008) Molecular phylogenetics of tsetse flies (*Diptera: Glossinidae*) based on mitochondrial (CO1, 16S, ND2) and nuclear ribosomal DNA sequences, with an emphasis on the *palpalis* group. Mol Phylog Evol 49: 227–239.
57. Rozas J, Rozas R (1995) DnaSP, DNA sequence polymorphism: an interactive program for estimating population genetics parameters from DNA sequence data. Comput Appl Biosci 11: 621–625.
58. Nei M (1973) Molecular Evolutionary Genetics. New York: Columbia Univ. Press.
59. Tajima F (1993) Measurement of DNA polymorphism. In: Takahata N, Clark AG, eds. Mechanisms of Molecular Evolution. Sunderland, Massachusetts: Sinauer Associates Inc., pp 37–59.
60. Hudson RR, Boos DD, Kaplan ND (1992) A statistical test for detecting geographic subdivision. Mol Biol Evol 9: 138–151.
61. Tajima F (1983) Evolutionary relationship of DNA sequences in finite populations. Genetics 105: 437–460.

62. Templeton AR, Crandall KA, Sing CF (1992) A Cladistic-Analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA-sequence data.3. Cladogram Estimation. *Genetics* 132: 619–633.
63. Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Mol Ecol* 9: 1657–1659.
64. Camara M, Caro-Riño H, Ravel S, Dujardin JP, Hervouet JP, et al. (2006) Genetic and morphometric evidence for isolation of a tsetse (Diptera: Glossinidae) population (Loos islands, Guinea). *J Med Entomol* 43: 853–860.
65. Rohlf F J (1990) Rotational fit (Procrustes) methods. In: Rohlf FJ, Bookstein FL, eds. *Proceedings of the Michigan Morphometrics Workshop*. Ann Arbor, USA: University of Michigan Museums.
66. Rohlf FJ (1996) Morphometric spaces, shape components and the effects of linear transformations. In: Marcus LF, Corti M, Loy A, Naylor GJP, Slice D, eds. *Advances in Morphometrics, Proc 1993 NATO-ASI on Morphometrics, Series A, Life Sciences*. Plenum Publication, New York, USA. pp 117–129.
67. Good P (2000) *Permutation tests: a practical guide to resampling methods for testing hypotheses*. New York, USA: Springer.
68. Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Res* 27: 209–220.
69. Jarne P, Lagoda PJJL (1996) Microsatellites, from molecules to populations and back. *Trends Ecol Evol* 11: 424–429.
70. Gooding RH, Solano P, Ravel S (2004) X chromosome mapping experiments suggest occurrence of cryptic species in the tsetse fly, *Glossina palpalis palpalis* (Diptera: Glossinidae). *Can J Zool* 82: 1902–1909.
71. Dyer NA, Furtado A, Cano J, Ferreira F, Odete Afonso M, et al. (2009) Evidence for a discrete evolutionary lineage within Equatorial Guinea suggests that the tsetse fly *Glossina palpalis palpalis* exists as a species complex. *Mol Ecol* 18: 3268–3282.
72. Vreysen MJB, Van der Vloedt AMV (1990) The effect of interspecific hybridization and gamma radiation on the reproductive biology of *Glossina palpalis palpalis* and *G. p. gambiensis*. *Ann Soc belge Méd Trop* 70: 145–158.
73. Morel PC, Touré SM (1967) *Glossina palpalis gambiensis* Vanderplank 1949 (Diptera) dans la région des Niayes et sur la Petite Côte (République du Sénégal). *Rev Elev Méd vét Pays trop* 20: 571–578.
74. Touré SM (1974a) Notes sur quelques particularités dans l'habitat de *Glossina palpalis gambiensis* Vanderplank 1949 (Diptera: Glossinidae) observées au Sénégal. *Rev Elev Méd vét Pays trop* 27: 81–91.
75. Marquez JG, Vreysen MJB, Robinson AS, Bado S, Krafsur ES (2004) Mitochondrial diversity analysis of *Glossina palpalis gambiensis* from Mali and Senegal. *Med Vet Entomol* 18: 288–295.
76. Solano P, Ravel S, de Meeus T (2010) How can tsetse population genetics contribute to African Trypanosomiasis Control? *Trends in Parasitol*, in press.