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Microsatellite DNA markers reveal genetic differentiation among populations of *Glossina palpalis gambiensis* collected in the agro-pastoral zone of Sideradougou, Burkina Faso

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Abstract

Intraspecific genetic variability of *Glossina palpalis gambiensis* in the area of Sideradougou, Burkina Faso, was studied using polymorphic microsatellite DNA markers. This genetic study was combined with other epidemiological information on the same tsetse: blood-meal identification, dissection of tsetse and molecular characterization of the trypanosomes detected. There was significant genetic differentiation among flies caught only a few kilometers apart, within the same riverine habitat. These distinct subpopulations were also differentially infected by trypanosomes. In part of the study area, a Factorial Correspondence Analysis undertaken on the genotypes allowed us to detect a Wahlund effect, suggesting the presence of tsetse originating from different source populations coming from two distinct drainage systems. The apparent structuring of populations of *G. palpalis gambiensis* is discussed relative to appropriate strategies to control African Trypanosomosis.

Keywords: Burkina Faso, epidemiology, *Glossina palpalis gambiensis*, microsatellite, population genetics, trypanosomosis.

Introduction

Genetic variation among vector populations probably affects the transmission of many parasitic diseases at a macro-geographic level (Lanzaro & Warburg, 1995), but little information is available to assess the spatial scale of this phenomenon. Recent developments in techniques for assessing variation at highly polymorphic microsatellite DNA markers (Tautz & Renz, 1984; Estoup *et al.*, 1993; Weber & Wong, 1993) have provided us with new tools to investigate the question. *Glossina palpalis gambiensis* VanderPlank 1949, is a riverine West African tsetse fly that transmits trypanosomes causing both human and animal African Trypanosomoses. Improving our understanding of the genetics of the vectorial capacity of tsetse is clearly a worthy endeavour (Janssen & Wijers, 1974; Maudlin, 1980; Reifenberg *et al.*, 1997; Kazadi *et al.*, 1998). Similarly, genetic studies may provide useful information on the potential for large-scale tsetse control particularly in terms of Sterile Insect Technique (Cheng & Aksoy, 1999; Dale *et al.*, 1995; Vreysen *et al.*, 1998).

Tsetse were eradicated from the agro-pastoral zone of Sideradougou, south-west Burkina Faso, 15 years ago through the combined use of insecticide-impregnated screens and Sterile Insect Technique (Politzar & Cuisance, 1984). In recent years, tsetse have reinvaded the area, and now once again inhabit the gallery along major rivers. As part of a larger multidisciplinary work to understand the key factors determining the presence of tsetse flies (de La Rocque & Cuisance, 1997), we undertook basic studies on the relationships between microgeographic genetic variation in a tsetse species and other risk factors related to disease transmission.

We had previously isolated three microsatellite polymorphic loci from a genomic bank of captive-bred *G. palpalis gambiensis* (Solano *et al.*, 1997). Here we report the use of these loci to characterize tsetse captured in the area of Sideradougou, in relation to the microgeographic epidemiology of trypanosomosis.

Results

Entomology

A total of 620 *G. palpalis gambiensis* was caught with apparent densities of eight to thirty-one flies/trap/day in the west, and up to five in the east. The mean sex ratio of the tsetse was 1.4 males per female in the west, and it was 0.6 in the east (Fisher's exact test: $P = 0$).

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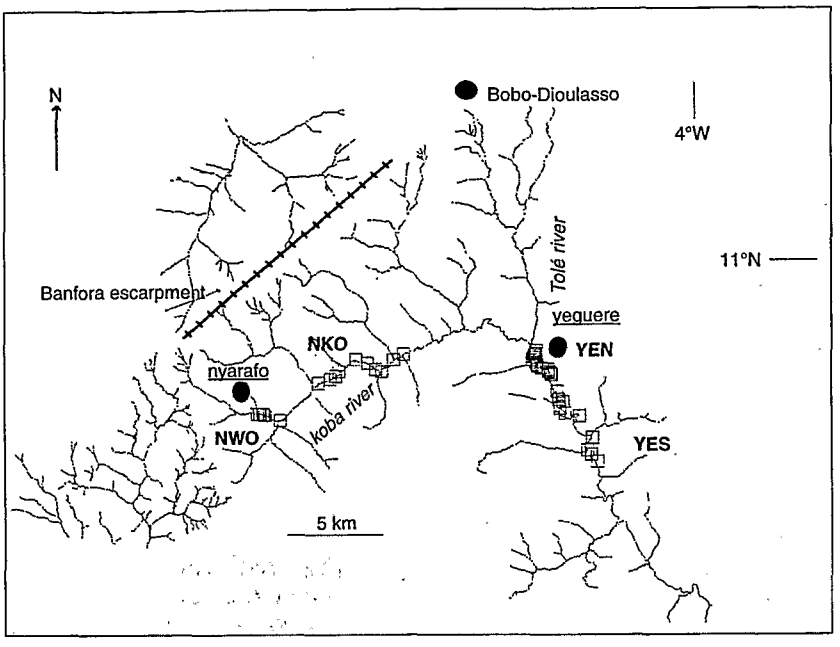

Figure 1. Map of the study area and geographical location of the traps (□) and subsamples (NWO, NKO, YEN, YES).

Epidemiological results

From 215 dissected flies, mean parasitological infection rates differed significantly between the areas: 20.3% (west) and 3.5% (east) (Fisher's exact test: $P = 0.0024$).

Using PCR to characterize the trypanosomes in the flies, some infections in the west could be characterized as *T. vivax* (40% of infected flies) or *T. congolense* «forest type» (6%). Surprisingly large numbers of infections (54%) did not react with any primers. These infections were localized only in the midgut of the flies. Because reptiles constituted the main food source (eight of twenty-one identified bloodmeals), this lead us to hypothesize that reptilian trypanosomes could account for these many infections. To date, specific primers for reptilian trypanosomes are not generally available (but see Gouteux & Gibson, 1996). Tsetse in both areas also fed on Suidae, followed by other species including cattle and man.

Infection rates were very low in the East, only two infected *G. palpalis gambiensis* were found; these harboured *T. vivax*.

Genetic analyses at the scale of the agropastoral zone

Among 201 analysed tsetse females, a total of twelve and seventeen alleles was recorded, respectively, at loci *Gpg55.3* and *Gpg19.62*. Seven subsamples (i.e. populations) were defined according to their geographical location and year of capture (Fig. 1).

No significant linkage disequilibrium was detected between loci in any of the subsamples.

The overall *Fis* value (0.163) across these two first microsatellite loci indicated a strong departure from panmixia,

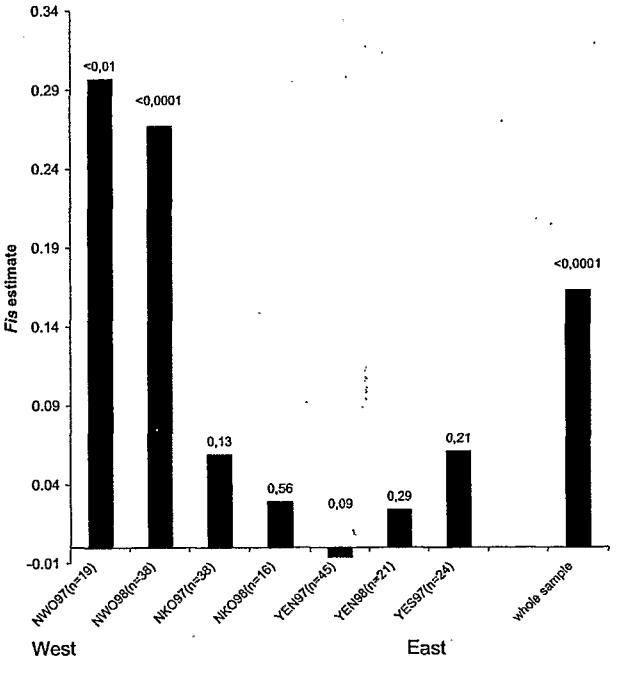

Figure 2. Graph showing within-sample heterozygote deficiency and its significance in each subsample. The subsamples are presented sequentially from the west to the east of the area and are plotted against the *Fis* estimate. Above each sample is represented the probability associated with the test of significance of the *Fis*. The number of females of each subsample is indicated in parentheses.

but this observation was dependent on location. Positive values of *Fis* (within sample heterozygote deficiency) were common only in the west. In the east, we could not reject the null hypothesis of panmixia (Fig. 2).

Table 1. Matrix of pair-wise *Fst* measured between the seven studied samples

	NWO97	NKO97	NKO98	NW098	YEN97	YES97	YEN98
NWO97	/	0.0211	−0.0086	−0.0085	**0.0590**	**0.0597**	**0.0831**
NKO97		/	0.0158	**0.0610**	**0.0810**	**0.0713**	**0.1399**
NKO98			/	0.0119	0.0213	0.0236	0.0627
NWO98				/	**0.0995**	**0.1077**	**0.1163**
YEN97					/	−0.0093	0.0354
YES97						/	0.0355
YEN98							/

Significant values after Bonferonni procedure are in bold (Bonferonni level is $\alpha' = \alpha/21$; $\alpha = 0.05$; so $\alpha' = 0.0024$).

The subsamples are presented from the west (four subsamples) to the east (three subsamples). Their names show the location and the year of capture. NOW = Nyarafo Wood; NKO = near Nyarafo on the Koba river; YEN = north of Yeguere; YES = south of Yeguere.

The overall *Fst* value was highly significant (*Fst* = 0.059, $P < 0.0001$) when measured at the scale of the entire area. This indicated genetic differentiation between subsamples (Table 1). The highest values of *Fst* were observed between western and eastern subsamples. Comparisons within each year were also significant (1997: *Fst* = 0.051, $P < 0.0001$; 1998: *Fst* = 0.072, $P < 0.0001$). When samples were pooled according to origin (i.e. west and east), the *Fst* estimate was 0.07 ($P < 0.0001$). This value suggests an exchange rate equivalent to three to four reproducing flies per generation (according to the formula: $Nm = (1 - Fst)/4*Fst$, of the measure of gene flow in an island model at mutation/drift equilibrium; Wright, 1969).

Genetics on the flies collected in Nyarafo, in the west

A strong *Fis* value was found in the samples collected in the gallery forest close to Nyarafo village in the western part. The third locus (*Gpg69.22*) was then used to amplify DNA from the tsetse collected in this Western part, i.e. NWO97, NKO97 and NWO98, which represent a total of ninety-seven females. Additional data at this locus confirmed the previously found strong *Fis* value in this Nyarafo area (across the three loci *Fis* = 0.20, $P < 0.0001$).

A Factorial Correspondence Analysis was then undertaken on the multilocus genotypes of the three loci to detect a possible Wahlund effect (population subdivision). After removal of flies carrying rare alleles, several groups were readily apparent in the plot of results from the FCA. Group C defined by axis 2 contained only six flies. It can be seen in Fig. 3 that axis 1 (which represented 16% of the total variance out of 14 axes) helped to more or less arbitrarily define two groups, A and B. These two groups may be seen as belonging to a single group, but the following suggest that a big proportion of group A's individuals and a big proportion of group B's individuals do not belong to the same population. Indeed, the mean *Fis* values for each locus decreased when calculated separately for the two groups A and B (Table 2). The mean value (*Fis* = 0.034) in the two separated groups A and B was no longer

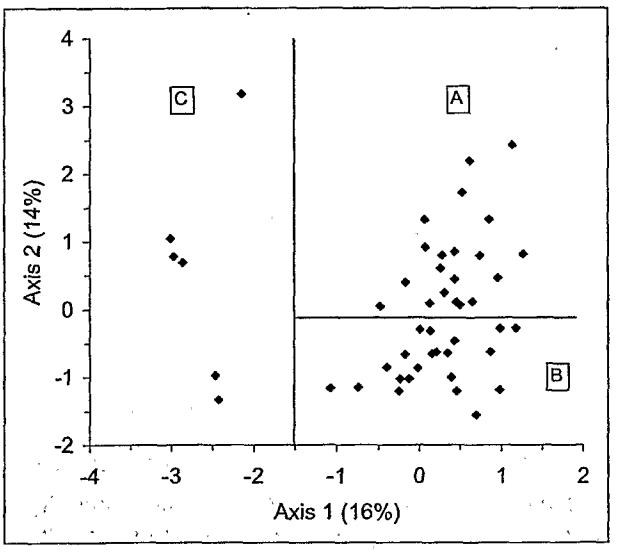

Figure 3. Results of the Factorial Correspondence Analysis undertaken on the flies collected in the western part (Nyarafo).

Table 2. Decrease of the *Fis* value between the total sample and the two groups (A and B) deduced from the Factorial Correspondence Analysis carried out on the flies from Nyarafo

Fis value	Total sample (A + B)	Sample A-Sample B
Locus *Gpg55.3*	0.09	−0.07
Locus *Gpg19.62*	0.12	0.03
Locus *Gpg69.22*	0.12	0.07
Across loci	0.20	0.034

significant. This means that subsamples A and B did not represent a random subsampling of the original sample. These results (i.e. the FCA and the decrease of the *Fis* in each of the two groups at all loci) were consistent with the hypothesis that the initial *Fis* did not differ from 0 and that there was a Wahlund effect in the sampled flies. No significant linkage disequilibrium could be detected in the sample.

Taking into account these two groups of individuals (A and B) occurring in sympatry in the western side of the area in the sacred wood of Nyarafo, the population differentiation remained significant compared to the eastern zone.

Discussion

In this work, the study of *G. palpalis gambiensis* population structure was undertaken simultaneously with other epidemiological factors, i.e. bloodmeals preferences and molecular identification of the trypanosomes found in these tsetse. Particular attention was made to simplify protocols for DNA extraction, in order to enable such work to be carried out in the field. The combined study of different components of the pathogenic system should allow a better understanding of the trypanosomosis risk in a given area (de La Rocque *et al.*, 1998).

We have found significant cryptic substructuring of local tsetse populations over just a few kilometres in a gallery forest habitat along a given riverine system. The low rates of gene flow suspected between the western and eastern tsetse populations of the Koba river might be explained by the relatively harsh habitat conditions separating these two parts: water is not present during the whole year and the vegetation is sparse. Indeed only a few tsetse were caught in this place during the extensive survey of 1996, and among these hardly any teneral (i.e. young) flies (de La Rocque & Cuisance, 1997). Still comparing western and eastern situations, differences were also found in the sex ratio of the captured tsetse, their infection rates and the identity of the trypanosomes that infected them. These differences confirmed those already reported in another study in the same area on more than 2000 tsetse: 'western' flies infected by trypanosomes were found much more often, and the hypothesis was that these infections could be attributed mainly to reptilian, non pathogenic trypanosomes (de La Rocque *et al.*, 1998; Lefrançois *et al.*, 1998). Conversely, the 'eastern' flies, despite being less infected, fed much more on cattle, and were infected mostly by *T. vivax*, a trypanosome of major veterinary importance (de La Rocque *et al.*, 1998; Lefrançois *et al.*, 1998).

Hence, along the drainage system of the Koba river, genetic structuring of the populations of *G. palpalis gambiensis* was consistent with an 'epidemiological structuring'. Genetically different populations of the vector were associated with different epidemiological patterns in the area. Obviously, various other factors could also play a role in the efficiency of the transmission of the disease, such as cattle distribution and density (Michel *et al.*, 1999), land use, water resources and vegetation (Cuisance & de La Rocque, 1998). Other genetic studies on tsetse have also demonstrated genetic differences among populations, e.g. through isoenzyme analyses of *Glossina pallidipes* in eastern and southern Africa (Nesbitt *et al.*, 1990; Kence *et al.*, 1995; Krafsur *et al.*, 1997), or through the use of the same microsatellite loci between geographically distant populations of *G. palpalis gambiensis* (Solano *et al.*, 1999), however these studies have worked on a macrogeographic scale, or have compared populations from ecologically distinct areas.

In the present study, as a second level of structuring, a highly significant heterozygote deficiency was observed, involving the three microsatellite loci on the flies collected in the Western part, near Nyarafo. It should be noted that, to explain a strong *Fis*, technical and/or biological factors might be involved.

From a technical point of view, the existence of null alleles at microsatellite loci has already been reported, i.e. non-amplification of alleles at microsatellite loci due to mutations in the flanking sequences (Callen *et al.*, 1993; Paetkau & Strobeck, 1995; Dumas *et al.*, 1998). In the present study, both microsatellite loci *Gpg55.3* and *Gpg19.62* were interpreted to be located on the X chromosome. Thus, if null alleles were the cause of the heterozygote deficiencies, we would have expected the occurrence of null males. However, all the males showed one band. Even in females, the frequency of a null allele accounting for the observed deficiency would be so high that null homozygotes (i.e. females showing no PCR product) would have been expected (Brookfield, 1996), which was not the case. Therefore it is not likely that null alleles could explain the observed heterozygote deficiencies.

Biological reasons could also account for the observed high *Fis* values, such as the presence of a Wahlund effect (sampling two independent gene pools). Indeed the FCA, which was used here as a tool to visualize possible structuring, suggested the occurrence, in sympatry, of two (or more) genetically distinct groups of tsetse in the western part of the area in the sacred wood close to the village of Nyarafo. It is not stated here that all the individuals of group A and all individuals of group B belong to different populations. What appears from the analyses is that the strong original *Fis* found in the total sample comes from a Wahlund effect, because if these two groups did not reflect any biological reality, there is no reason why the *Fis* woud have changed. Here, this Wahlund effect can be the consequence of two phenomena. First, *G. palpalis gambiensis* could represent a complex of (at least) two species with restricted or null gene flow. Second, the area may have been colonized by flies coming from different source populations following the end of tsetse control operations (Politzar & Cuisance, 1984). Our study area is just under Banfora escarpment (see Fig. 1), above which flows the basin of the Comoe river. Two different drainage systems join here, i.e. the Comoe and the Koba rivers (the latter belonging to the Mouhoun basin), which might represent the providers of these different tsetse populations.

Table 3. Characteristics of the three microsatellite loci isolated from *Glossina palpalis gambiensis*

Microsatellite locus	Core microsatellite sequence	Annealing temperature	Number of alleles in the whole sample	Frequency of most common allele
Gpg55.3	$(GT)_{14}(GC)(GT)_4$	50 °C	12	0.79
Gpg19.62	$(GT)_{12}$	50 °C	18	0.49
Gpg69.22	$(TA)_{10}$	48 °C	8*	0.60

*Locus *Gpg69.22* was scored on ninety-seven females from the western area.

Because this tsetse species moves preferentially along riverine networks and needs high humidity to survive (Buxton, 1955), the geography of the area provides the most likely explanation for population substructuring.

It would be of interest to know if the genetic differences at the intraspecific level pointed out in the present work could lead to different vectorial competences (Maudlin, 1980; Reifenberg *et al.*, 1997; Moloo *et al.*, 1998).

The lack of evidence for any linkage disequilibrium is not definitive in our study due to the low number of loci analysed. The third group of the FCA (group C) might also represent individuals having migrated from other localities. However in the absence of clearer information that would come from the analysis of more loci, we cannot draw more precise conclusions. It should be noted that to the author's knowledge, these three microsatellite loci are the only ones that have been described in tsetse flies so far. Nevertheless the information expected from such approaches will contribute in designing future control strategies: genetic studies on vector populations combined with the study of other epidemiological factors will be of help in identifying 'epidemiologically dangerous points' to assess areas at risk for trypanosomosis (Cuisance *et al.*, 1980; de La Rocque, 1997; Hendrickx *et al.*, 2000; B. Bauer & B. Snow, personal communication), and will also help to assess the feasibility of the Sterile Insect Technique in tsetse eradication schemes (Vreysen *et al.*, 1998).

Experimental procedures

Study area

Sideradougou lies in the southern half of Burkina Faso in the Sudan savannah vegetation zone (4°20′W, 10°55′N). Two riverine tsetse species are present in the area: *Glossina palpalis gambiensis* and *G. tachinoides* Westwood 1850. The main drainage system comprises the Koba and the Tolé rivers (Fig. 1). An exhaustive entomological survey was completed in 1996 along 120 km of the main Koba river (de La Rocque, 1997). These data were used to select two areas for detailed investigation in the dry seasons of 1997 and 1998. In each area we deployed biconical (Challier & Laveissière, 1973; Challier *et al.*, 1977) and monoconical (Laveissière & Grébaut, 1990) traps for 2 days, near the village of Nyarafo in the west, and near Yeguere in the east (Fig. 1). These two areas differ in infection rates and in the identity of the trypanosomes in the flies (de La Rocque *et al.*, 1998; Lefrançois *et al.*, 1998).

Field techniques

Each trap site was georeferenced using a GPS and tsetse species, sex and apparent density were recorded.

In the field camp, after removing of the tsetse from the trap, individual *G. palpalis gambiensis* were processed as follows:

- Three legs were removed and stored in dry eppendorf tubes for subsequent genetic analyses.
- The mouthparts, salivary glands and midgut were dissected to detect trypanosome infections by microscope examination (Lloyd & Johnson, 1924).
- The midgut content was collected for subsequent bloodmeal identification (Kaboré *et al.*, 1994).
- When a tsetse was found infected with trypanosomes, each of the three organs was collected separately in eppendorf tubes (dry for the mouthparts, and with 50 µl sterile distilled water for the midgut and salivary glands). This material was used for PCR identification of trypanosomes using DNA primers designed from genomic satellite sequences specific of the different taxonomic groups (Majiwa *et al.*, 1994; Solano *et al.*, 1995).

The techniques for identification of the trypanosomes by PCR and for the bloodmeal origin of the tsetse are described fully in Lefrançois *et al.*, 1998.

PCR reactions at microsatellite loci

At the CIRDES (Centre International de Recherche-Développement sur l'Elevage en zone Subhumide) laboratory in Bobo-Dioulasso, 300 µl of 5% Chelex® (BIORAD, Hercules, CA, USA) chelating resin were added to each tube containing the legs of the tsetse (Walsh *et al.*, 1991; Dumas *et al.*, 1998). 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 ***g*** for 2 min and were frozen before further handling.

The PCR reactions were carried out in a thermocycler (MJ Research, Watertown, MA, USA) in 50 µl final volume, using 10 µl of the supernatant from the extraction step. Specific primers were designed in the regions flanking the microsatellite core sequences of loci *Gpg55.3* and *Gpg19.62* (as described in Solano *et al.*, 1997). Precise allele sizing on a limited number of samples had already been determined by performing PCR in the presence of (α^{35}-S) dATP, followed by denaturing electrophoresis in 6% acrylamide gel containing 8M urea (Solano, 1998). In the present work, the loci were amplified using the same conditions (annealing temperature was 50 °C) (see Table 3). After PCR amplification, allele bands were routinely resolved on 10% nondenaturing polyacrylamide gels stained with ethidium bromide. Due to logistical constraints, the third locus (*Gpg69.22*) was scored only in Nyarafo (in the western part) in 1997 and 1998. Primers sequences at the three microsatellite loci are described in Solano *et al.*, 1997.

The microsatellite loci *Gpg55.3* and *Gpg19.62* are presumed to be on the X-chromosome, given an absence of heterozygous

males in captive-bred flies (Solano, 1998). We therefore analysed only field-collected females. Captive breeding also demonstrated mendelian inheritance of the alleles at these microsatellite loci and an absence of null alleles (e.g. Callen *et al.*, 1993; Dumas *et al.*, 1998).

Data analysis on microsatellite loci

In each population, Wright's *Fis* (within sample heterozygote deficiency) and *Fst* (measure of population differentiation) were estimated using Weir & Cockerham's (1984) unbiased estimators (f for *Fis*, θ for *Fst*). These estimators were computed with FSTAT v.1.2 software (Goudet, 1995). For random mating (within samples) or random distribution of individuals (between samples), *F*-values were expected to be zero.

For each locus in each sample, and for all loci, the significance of *Fis* (panmixia) was tested using GENEPOP v.3.1 (Raymond & Rousset, 1995). The probability test described by Guo & Thompson (1992) was used, employing a complete enumeration method (Louis & Dempster, 1987) for loci with up to four alleles, and a Markov chain method (Guo & Thompson, 1992) for loci with more than four alleles. Measuring the deficit in heterozygotes simultaneously in several samples, the multisample extension of the score test described by Rousset & Raymond (1995) was then applied. For this test, the alternative hypothesis was 'H1 = heterozygote deficiency'. The measure of *Fis* and its significance were conducted only on the females, because males were haploid at two loci (i.e. they have only one X-chromosome).

The significance of *Fst* (population differentiation) was assessed using 10 000 permutations of female genotypes among samples (FSTAT; Goudet, 1995). The alternative hypothesis here is 'Fst > 0'.

Linkage disequilibrium was tested by the exact test of GENEPOP for genotypic linkage disequilibrium (Raymond & Rousset, 1995).

To evaluate significance when multiple tests were performed, the sequential Bonferroni procedure was applied (see Rice, 1989).

A Factorial Correspondence Analysis (FCA) was undertaken to explore genetic structure within samples. The analysis was carried out on the multilocus genotypes obtained at the three loci: each individual was characterized for each existing allele by the values 2, 1 or 0 whether it had 2 (homozygotes), 1 (heterozygote) or 0 copies of the considered allele. Individuals were then analysed as active variables using FCA (e.g. Ba *et al.*, 1993). The analysis was carried out after eliminating individuals carrying rare alleles (< 5%), because rare alleles tend to hide the existing patterns.

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