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1 Short Communication

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3 **Microsatellite genotyping reveals diversity within populations of *Sodalis***
4 ***glossinidius*, the secondary symbiont of tsetse flies**

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6 Running title: *Sodalis glossinidius* genotyping using microsatellite assay

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25 **Abstract**

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27 The aim of this study was to develop a PCR-based microsatellite genotyping method for
28 identifying genetic diversity in *Sodalis glossinidius*, a symbiont associated with tsetse fly
29 infection by trypanosomes causing human and animal trypanosomiasis. Allelic polymorphism
30 at three loci, investigated on 40 fly gut extracts, evidenced eight alleles and the existence of
31 five genotypes. This novel approach was shown to be efficient and suitable for routine large-
32 scale genotyping of *S. glossinidius* present in the biologically complex tsetse fly extracts; it
33 could favor progress in the fields of diagnosis, epidemiology, population genetics, and
34 fly/symbiont/trypanosome interactions.

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48 **Keywords:** *Sodalis glossinidius*, symbiont, microsatellites, genotyping, tsetse fly, Nagana,

49 sleeping sickness

50 1. Introduction

51 Tsetse flies transmit trypanosomes responsible for human African trypanosomiasis (HAT) as
52 well as the animal disease (Nagana). HAT affects a wide range of people in sub-Saharan
53 Africa and is fatal if untreated, and Nagana is estimated to cost African agriculture US\$4.5
54 billion per year (Reinhardt, 2002). The available drugs are unsatisfactory (Barrett, 2006),
55 while resistance is increasing (Matovu et al., 2001). Consequently, investigations for novel
56 strategies must continue. Trypanosome transmission needs the parasite to establish in the
57 tsetse fly midgut and to mature into an infective form before being transmitted to a novel
58 mammalian host (Van den Abbeele et al., 1999). Tsetse flies harbor three different symbiotic
59 bacteria among which *Sodalis glossinidius* is suspected of being involved in the fly's vector
60 competence (Cheng and Aksoy, 1999; Dale and Welburn, 2001; Welburn and Maudlin,
61 1999). Recently, this bacterium was shown to be associated with infection of field populations
62 of tsetse flies by trypanosomes (Farikou et al., 2010). Furthermore, genetic diversity was
63 evidenced in *S. glossinidius* populations from insectary flies, suggesting that fly infection by
64 trypanosomes could be favored by the presence of symbiont-specific genotype(s) (Geiger et
65 al., 2007). Thus, a large-scale evaluation of the *S. glossinidius* genetic diversity in field
66 populations of flies is needed. The AFLP technique previously used on DNA extracted from
67 insectary fly hemolymph is not well adapted to routinely typing field samples because the
68 hemolymph volume of such individual fly is too low, and the gut extracts, including the
69 bacteria, are too complex. We therefore aimed to develop a Variable Number Tandem Repeat
70 (VNTR) approach known to be highly discriminant (Le Flèche et al., 2006; Lindstedt, 2005;
71 Whatmore et al., 2006). As the microsatellite approach has never been used to investigate the
72 genetic diversity of *S. glossinidius*, we had to determine whether: a) it would be appropriate to
73 distinguish genetically distinct *Sodalis* strains, b) the microsatellite approach could work on
74 complex biological extract samples, and c) it would be suitable for routine analyses.

75 2. Materials and methods

76 *Glossina palpalis gambiensis* individuals were chosen from flies that were field sampled in
77 different areas of Burkina Faso. Pupae were collected from these flies. After adult emergence,
78 the population was maintained in a level 2 containment insectary at 23°C and 80% relative
79 humidity, without any selection. Individuals used in the present work were randomly chosen.
80 The flies were dissected in a drop of sterile 0.9% saline solution. Midguts from each fly were
81 then separately transferred into microfuge tubes containing ethanol (95°) for further symbiont
82 analyses. The instruments were carefully cleaned after the dissection of each fly to prevent
83 contamination. The microfuge tubes were stored at -20°C until use.

84 DNA was extracted from midgut using the classical protocol (Navajas et al., 1998). Briefly,
85 tissues were homogenized with a pestle in a cetyl trimethyl ammonium bromide (CTAB)
86 buffer (CTAB 2%; 0.1 M Tris, pH 8; 0.02 M EDTA pH 8; 1.4 M NaCl) and incubated at
87 60°C for 30 min. The DNA was extracted from the lysis mixture with chloroform/isoamyllic
88 alcohol (24/1; V/V) and precipitated by adding isopropanol (V/V). After centrifugation
89 (10,000 g, 15 min), the pellet was rinsed with 70% ethanol, air-dried, and resuspended in
90 distilled sterile water. The DNA samples were stored at -20°C until PCR amplification
91 processing.

92 *S. glossinidius* microsatellite repeat sequences were selected in a data bank (GenBank,
93 accession number AP008232) (Toh et al., 2006) using the Vector NTI software; primer pairs
94 were designed for the sequences flanking such regions and tested to ensure that they
95 specifically amplify *S. glossinidius* and not host DNA. Table 1 shows the primers selected to
96 perform the present study.

97 One µl of DNA was used for subsequent DNA amplification. Polymerase chain reactions
98 (PCRs) were carried out using a DNA thermal cycler (PE Applied Biosystems, Foster City,
99 CA, USA) in 20-µl final volumes, containing 4 pmol of each primer, 0.2 mM of each

100 deoxyribonucleotide, 1X incubation buffer with 1.5 mM MgCl₂ (Quantum Appligène, Ilkirch,
101 France) and 0.5 units of Taq DNA polymerase (Quantum Appligène). Samples were first
102 denatured at 94°C for 3 min and then processed through 40 cycles consisting in a denaturation
103 step at 94°C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 1
104 min. The final elongation step was lengthened to 5 min at 72°C. PCR products were checked
105 by 2% agarose gel electrophoresis and visualized under UV light after ethidium bromide
106 staining (0.5 µg/ml). Allele bands were then resolved in nondenaturing acrylamide gels
107 (10%), after loading 2-5 µl PCR products, and revealed by ethidium bromide staining.

108 In order to assess whether the amplicons actually corresponded to *S. glossinidius*
109 microsatellites, bands representing the different alleles were purified using the Wizard DNA
110 Clean Up System (Promega Corporation, Madison, WI, USA). Purified PCR fragments were
111 cloned into pGEM-T Easy Vector (Promega Corporation). Clones of each cloned allele were
112 purified with the Wizard Plus SV Minipreps DNA Purification System Kit (Promega,
113 Charbonnière, France) and sequenced by GATC Biotech (Konstanz, Germany), allowing the
114 allele size to be determined as well as the exact number of microsatellite repeats (GenBank
115 accession numbers HQ842611-HQ842618).

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125 3. Results

126 Figure 1 shows an example of an electrophoretic separation on a 10% polyacrylamide gel of
127 different alleles; it demonstrates the development of the experimental process to be
128 successful. Table 1 summarizes the characteristics of the three microsatellite markers selected
129 to investigate the polymorphism within the *S. glossinidius* samples from 40 *G. palpalis*
130 *gambiensis* flies, and to test the genotyping method: sequence of the repeats, part of the
131 flanking sequences used as primers, their location on the *S. glossinidius* genome, and finally
132 the number and size of the alleles evidenced at each locus. The repeat sequence of the ADNg
133 2/5 marker is (AC). At this locus, two alleles that differ in the number of repeats, either five
134 or seven, were identified among the 40 samples; their size, including the flanking sequences,
135 was 176 and 180 bp, respectively. At the locus carrying the ADNg 21/22 marker, four alleles
136 were characterized that differ in the number of repeats, six, seven, nine, or 11, of the repeated
137 unit (GCC), and in their total length, 163, 166, 172, and 178 bp, respectively. Finally, two
138 alleles were identified at the ADNg 15/16 locus; they differed in the number, five or seven, of
139 their (AGG) unit repeat, and in their size, 106 or 112 bp. The combination of alleles amplified
140 at the three loci on each sample (fly midgut extract) characterizes the genotype of the *Sodalis*
141 strain the fly harbors. Significant diversity was evidenced among the *S. glossinidius* strains
142 from the 40 tsetse flies. Table 2 presents the combination of alleles, identified by their size
143 expressed as base pairs, for the 40 strains. Five allelic combinations, i.e., five genotypes, were
144 found, one of which was much more frequent (62.5%) than the four others (22.5, 5, 5, and 5%
145 respectively).

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150 **4. Discussion**

151 One of the challenges in the study of *S. glossinidius* is the difficulty encountered when
152 attempting to detect genetic diversity among strains. Previous studies have inferred
153 phylogenetic relatedness among strains based on similarity at the 16S rDNA gene (Aksoy et
154 al., 1997). However, single-gene phylogenetics are unreliable for resolving close relationships
155 (Baldo et al., 2006). As shown in the introduction, to progress in the understanding of the role
156 played by the symbiont *S. glossinidius* in the tsetse's capacity to transmit trypanosomes, the
157 availability of an efficient and accurate genotyping tool is needed; the previously used AFLP
158 method (Geiger et al., 2007) is not well suitable for routine genotyping of symbiont
159 populations from field flies: an alternative and effective new method had to be developed.

160 From a general point of view, it appears that the *S. glossinidius* developed microsatellite
161 method fulfils the performance criteria for a genotyping assay (Bricker and Ewalt, 2005), i.e.,
162 typing ability, reproducibility, stability, and discriminating power which is even better when
163 the investigation is extended to several loci. Substantial genetic diversity was therefore
164 observed among the 40 flies: eight alleles at the three loci investigated. These eight alleles
165 were shown to be combined differently in the 40 individuals and resulted in the identification
166 of five genotypes. It should be noted that the approach is actually quite appropriate to genetic
167 diversity analyses of microorganisms present in complex biological samples such as *S.*
168 *glossinidius* included in tsetse flies midgut extracts. It requires a small quantity of biological
169 material, and the alleles can be analyzed by simple polyacrylamide gel electrophoresis;
170 standardization and quality control are easy to achieve, and the recorded data can be easily
171 coded. The proposed technical approach will be useful for large scientific uses, genetic
172 population structure, epidemiology etc. It could possibly also be used for detecting mixed
173 infections: given that *S. glossinidius* is haploid, the detection of more than one band (one

174 allele) at a given locus will mean that more than one *S. glossinidius* genotype is present in the
175 sample analyzed and the existence of a mixed infection can be assessed in the fly sampled.
176 To conclude, the microsatellite genotyping process we have adapted is an important tool for
177 routine and effective diagnosis of *Sodalis* strains and will advance the elucidation of the role
178 played by the symbiont in the vector competence of the tsetse fly, and finally in human and
179 animal trypanosomiasis.

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197 **Conflict of interest statement**

198 There is no conflict of interest with respect to funding or any other issue.

199

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291 **Figure legends**

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293 **Figure 1. Example of fragment length polymorphism at the ADNg 21/22 locus in 18**

294 *Sodalis glossinidius* samples.

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296 The alleles have been separated by electrophoresis on a 10% polyacrylamide gel on the basis

297 of the size of their corresponding DNA sequence. Lane 1 corresponds to the "no template"

298 negative control. Lanes 2-19 correspond to *S. glossinidius* samples. Here three alleles have

299 been separated into three sizes: 163 (4 strains), 172 (13 strains), and 178 (1 strain) base pairs.

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1 **Table 1. *S. glossinidius* microsatellite markers, PCR primers and allelic polymorphism**

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4	Marker	Repeat sequence ^a	Primer sequences (5'-3')	Location at bp ^b	Number of alleles	Size of the alleles (bp)
5						
6	ADNg 5/2	(AC) _{x 5, 7}	GGCCGGTATTCTAACCGAC	4115043-4115222	2	176/180
7			AACTGCCAGGCATCCATTAC			
8	ADNg 21/22	(GCC) _{x 6, 7, 9, 11}	GAGCAAATCTCCCAGCACAT	1450588-1450759	4	163/166/172/178
9			TTCTTGTCCTCAACCCATC			
10	ADNg 15/16	(AGG) _{x 5, 7}	ATACGGCGAAGCAATGAGAC	3250160-3250283	2	106/112
11			CAGCCTCTAAGCGCTCAACTC			
12						

13 ^a and number of repeats recorded at each locus (5 or 7 at ADNg 5/2, etc); ^bGenBank accession number AP008232

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1 **Table 2. Combinations of three alleles, one from each of the three loci, recorded among**
 2 **the 40 *S. glossinidius* strains sampled; number and percentage of strains carrying each**
 3 **combination**

6	ADNg 2/5	ADNg 21/22	ADNg 15/16	Number and (%)
7				of strains
9	180*	172	112	25 (62.5%)
10	176	163	112	9 (22.5%)
11	180	178	112	2 (5%)
12	180	166	112	2 (5%)
13	176	163	106	2 (5%)

14
 15 *alleles identified by their size (number of base pairs) – see also Table 1 for details

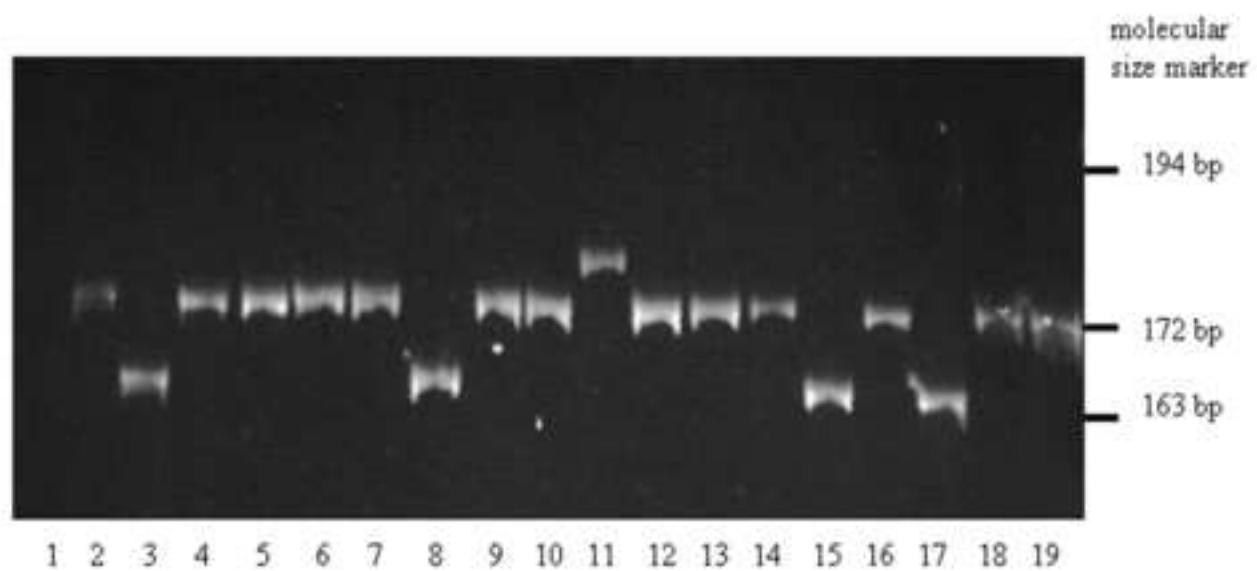


Figure 1