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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
7	
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#### 25 Abstract

The aim of this study was to develop a PCR-based microsatellite genotyping method for identifying genetic diversity in Sodalis glossinidius, a symbiont associated with tsetse fly infection by trypanosomes causing human and animal trypanosomiasis. Allelic polymorphism at three loci, investigated on 40 fly gut extracts, evidenced eight alleles and the existence of five genotypes. This novel approach was shown to be efficient and suitable for routine large-scale genotyping of S. glossinidius present in the biologically complex tsetse fly extracts; it could favor progress in the fields of diagnosis, epidemiology, population genetics, and fly/symbiont/trypanosome interactions. CeRi 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 mammalian host (Van den Abbeele et al., 1999). Tsetse flies harbor three different symbiotic 58 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 populations of flies is needed. The AFLP technique previously used on DNA extracted from 66 67 insectary fly hemolymph is not well adapted to routinely typing field samples because the hemolymph volume of such individual fly is too low, and the gut extracts, including the 68 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. The flies were dissected in a drop of sterile 0.9% saline solution. Midguts from each fly were 80 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/isoamylic 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  $-20^{\circ}$ 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.

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

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

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

#### 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

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

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

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	e markers, PC	R primers	and allelic <b>j</b>	oolymorphism
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2						
3	Marker	Repeat	Primer sequences	Location at bp <sup>b</sup>	Number	Size of the
4		sequence <sup>a</sup>	(5'-3')		of alleles	alleles (bp)
5						
6	ADNg 5/2	(AC) <sub>x 5, 7</sub>	GGCCGGTATTCTAACCGAC	4115043-4115222	2	176/180
7			AACTGCCAGGCATCCATTAC			
8	ADNg 21/22	(GCC) <sub>x 6, 7, 9, 11</sub>	GAGCAAATCTCCCAGCACAT	1450588-1450759	4	163/166/172/178
9			TTCTTGTCCCTCAACCCATC			
10	ADNg 15/16	(AGG) <sub>x 5, 7</sub>	ATACGGCGAAGCAATGAGAC	3250160-3250283	2	106/112
11			CAGCCTCTAAGCGCTCAACTC			
12						

<sup>a</sup> and number of repeats recorded at each locus (5 or 7 at ADNg 5/2, etc); <sup>b</sup>GenBank accession number AP008232

#### NUSCRIPT ACCEPTED

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						
4							
-5							
6	ADNg 2/5	ADNg 21/22	ADNg 15/16	Number and (%)			
7				of strains			
8							
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							

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

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Table 2



Figure 1