Vector Competence of *Glossina palpalis gambiensis* for *Trypanosoma brucei* s.l. and Genetic Diversity of the Symbiont *Sodalis glossinidius*

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Tsetse flies transmit African trypanosomes, responsible for sleeping sickness in humans and nagana in animals. This disease affects many people with considerable impact on public health and economy in sub-Saharan Africa, whereas trypanosomes' resistance to drugs is rising. The symbiont *Sodalis glossinidius* is considered to play a role in the ability of the fly to acquire trypanosomes. Different species of *Glossina* were shown to harbor genetically distinct populations of *S. glossinidius*. We therefore investigated whether vector competence for a given trypanosome species could be linked to the presence of specific genotypes of *S. glossinidius*.

Glossina palpalis gambiensis individuals were fed on blood infected either with *Trypanosoma brucei gambiense* or *Trypanosoma brucei brucei*. The genetic diversity of *S. glossinidius* strains isolated from infected and noninfected dissected flies was investigated using amplified fragment length polymorphism markers. Correspondence between occurrence of these markers and parasite establishment was analyzed using multivariate analysis.

Sodalis glossinidius strains isolated from *T. brucei gambiense*–infected flies clustered differently than that isolated from *T. brucei brucei*–infected individuals. The ability of *T. brucei gambiense* and *T. brucei brucei* to establish in *G. palpalis gambiensis* insect midgut is statistically linked to the presence of specific genotypes of *S. glossinidius*. This could explain variations in *Glossina* vector competence in the wild. Then, assessment of the prevalence of specific *S. glossinidius* genotypes could lead to novel risk management strategies.

Introduction

Tsetse flies are medically and agriculturally important vectors that transmit African trypanosomes, the causative agents of sleeping sickness in human and nagana in animals. Following a long period of increase, the human African trypanosomiasis seems now to be decreasing (Barrett 2006). However, this debilitating disease still affects a wide range of people in sub-Saharan Africa (WHO 2006) and is invariably fatal if untreated. Nagana is estimated to cost African agriculture US$4.5 billion per year (Reinhardt 2002). Until now, drugs were unsatisfactory, some being toxic and all being difficult to administer (Barrett 2006). Furthermore, resistance is increasing (Matovu et al. 2001). However, DB289 (diamidine derivative), a new orally administered drug, which is in stage III trials, will offer some benefits if registered, but it is suitable only for early-stage disease. For the late-stage disease, new medications are most urgently required (Barrett 2006). So, the investigation for novel strategies must continue and among them are better risk management strategies and alternative vector-based strategies, such as the engineering of insects capable of blocking the transmission of the parasite (Rio et al. 2004).

To be transmitted, the parasite must first establish in the insect midgut following an infective blood meal and then mature in the salivary glands or the mouthparts, depending on the trypanosome species (Vickerman et al. 1988; Van den Abbeele et al. 1999). Tsetse flies are normally refractory to trypanosome infection with typically less than 50% infection under ideal laboratory conditions. Field infection rates rarely exceed 10% of the fly population. Furthermore, many infected flies fail to produce mature parasites and therefore never become infective (Moloo et al. 1986; Dukes et al. 1989; Frézil and Cuisance 1994; Maudlin and Welburn 1994; Jamonneau et al. 2004). This ability to acquire the parasite, favor its maturation, and transmit it to a mammalian host is known as "vector competence," which depends on both *Glossina* and trypanosome species. The "*morsitans* group" was shown to be the major vector for trypanosomes in the subgenus *Nannomonas*, whereas the "*palpalis* group" is a poor vector (Kazadi 2000). Furthermore, the *morsitans* group is the vector of *Trypanosoma brucei rhodesiense*, the causative agent of the East African human trypanosomiasis, whereas the *palpalis* group is the vector of *Trypanosoma brucei gambiense*, responsible for the Western and Central African human trypanosomiasis (Hoare 1972).

Tsetse flies harbor 3 different symbiotic microorganisms (Aksoy 2000) among which *Sodalis glossinidius* (Cheng and Aksoy 1999; Dale and Maudlin 1999) is suspected to be involved in the vector competence of *Glossina* by favoring parasite installation in the insect midgut through a complex biochemical mechanism involving the production of N-acetyl glucosamine (Maudlin and Ellis 1985; Welburn and Maudlin 1999). This sugar, resulting from hydrolysis of pupae chitin by a *S. glossinidius*–produced endochitinase, was reported to inhibit a tsetse-midgut lectin lethal for the procyclic forms of the trypanosome (Welburn and Maudlin 1999; Dale and Welburn 2001). The specific removal of *S. glossinidius* from tsetse fly midguts significantly decreases longevity, indicating involvement of mutualistic interactions (Dale and Welburn 2001). The recently reported full-length sequencing of the complete genome (Toh et al. 2006) and extrachromosomal DNA (Darby et al. 2005) showed that *S. glossinidius* display active mechanisms of cellular interactions and is an intermediate between free-living and obligate intracellular bacteria evolving toward specific interaction with *Glossina* (Darby et al. 2005; Toh et al. 2006).

No direct correlation was found between the presence of *S. glossinidius* and the ability of the insect to acquire

Key words: *Sodalis glossinidius*, *Trypanosoma brucei*, *Glossina palpalis*, AFLP, genetic diversity, vector competence, sleeping sickness.

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Trypanosoma congolense (Geiger, Ravel, et al. 2005). However, *Glossina palpalis gambiensis* (*palpalis* group) and *Glossina morsitans morsitans* (*morsitans* group) were shown to harbor genetically distinct populations of *S. glossinidius* (Geiger, Cuny, et al. 2005), suggesting that vector competence might be linked to given genotypes of *S. glossinidius* rather than a mere presence/absence. We demonstrate here that the ability of *T. brucei gambiense* and *Trypanosoma brucei brucei* to establish in *G. palpalis gambiensis* insect midgut is statistically linked to the presence of specific genotypes of *S. glossinidius*.

Materials and Methods

Insect, Trypanosomes, and Bacterial Reference Strain

Glossina palpalis gambiensis flies originate from flies field collected in different areas of Burkina Faso. Pupae were collected from these flies. Following adult emergence, the population was maintained in a level 2 containment insectary at 23 °C and 80% relative humidity (Geiger, Ravel, et al. 2005) without any selection. Individuals used in the present work were randomly chosen for infection experiments. *Trypanosoma brucei gambiense* A005 was isolated from human in 1989 in Fontem, Cameroon (Dukes et al. 1989) and maintained as a procyclic form in Cunningham's medium supplemented with 20% fetal calf serum. Procyclic forms for infection were taken from cultures displaying logarithmic growth and washed 3 times with phosphate-buffered saline (PBS). The resulting pellet was then resuspended in PBS. *Trypanosoma brucei brucei* was isolated from cattle in Sideradougou, Burkina Faso. A stabilate of *T. brucei brucei* was thawed and injected intraperitoneally into BALB/C mice. Murine infections were monitored by examining samples of tail blood by phase contrast microscopy at ×400 until the parasitemia reached 3×10^7 to 1×10^8 trypanosomes/ml. The reference *S. glossinidius* type strain M1 was purchased from National Collection of Industrial and Marine Bacteria (Aberdeen, United Kingdom) and grown as previously described (Geiger, Cuny, et al. 2005).

Infection of *G. palpalis gambiensis*

Procyclic *T. brucei gambiense* (14.10^8) were mixed with 100 ml of defibrinated and decomplemented bovine blood and offered immediately to teneral flies through a silicone membrane. Feeding time was restricted to 30 min at 35 °C, and the viability of the trypanosomes was checked by phase contrast microscopy before and after this single blood meal. Infection with *T. brucei brucei* was performed by feeding teneral flies on the bellies of infected mice (3×10^7 to 1×10^8 trypanosomes/ml). Flies failing to feed were removed. Engorged flies were placed in cages and maintained by feeding on uninfected rabbits until the end of the experiment.

Dissection and DNA Extraction from Organs

Flies were dissected 48 days postinfection, and infection was assessed by examination of midguts and salivary glands by phase contrast microscopy. Midguts and salivary glands from each fly were collected separately and processed as previously described (Geiger, Ravel, et al. 2005).

Isolation of *S. glossinidius* Strains

Hemolymph was collected prior to dissection in 200 µl of PBS from a cut leg joint, by gently applying pressure on the abdomen of flies. Hemolymph insect cells were pelleted by low-speed centrifugation (i.e., 1,500 g for 10 min at 25 °C), and the supernatant was collected. This step was repeated 3 more times to isolate pure *S. glossinidius* bacteria free of insect cells (Dale et al. 2001). DNA was extracted from *S. glossinidius* using the DNeasy tissue kit (Qiagen, Courtaboeuf, France).

Polymerase Chain Reaction Detection of Trypanosomes and Bacteria

Detection of *T. brucei brucei* and *T. brucei gambiense* was performed on hemolymph-extracted DNA using primers TBR1 and TBR2 (Moser et al. 1989). Specific polymerase chain reaction (PCR) detection of *S. glossinidius* was performed on midgut chelex-extracted DNA, bacteria isolated from hemolymph, and the M1 reference strain as previously described (Cheng and Aksoy 1999; Geiger, Ravel, et al. 2005). As previously described (Geiger, Cuny, et al. 2005), the 1,100-bp PCR product corresponding to *S. glossinidius* 16S rDNA was cloned into pGEM-T Easy (Promega, Charbonnieres, France). For each fly, several recombinant plasmids were sequenced and compared with the reference sequence of *S. glossinidius* isolated from *Glossina palpalis palpalis*, which belongs to the *palpalis* group as *G. palpalis gambiensis* (Aksoy et al. 1997; Chen et al. 1999) (GenBank accession number U64867).

AFLP Analysis

It was performed as previously described (Geiger, Cuny, et al. 2005). Bacterial DNA was digested with *Eco*RI and *Mse*I (New England Biolabs, Ipswich, MA) at 37 °C in a total reaction volume of 20 µl. After 3 h of digestion, restriction fragments were precipitated with 1/2 volume of ammonium acetate 7.5 M and 2.5 volume of absolute ethanol and spinned for 20 minutes at 13,000 g. The pellet was dissolved in 10 µl sterile water. Double-stranded oligonucleotide adaptors (table 1), composed of a unique sequence and an overhang complementary to the restriction sites (*Eco*RI/*Mse*I), were then ligated to the restriction fragments for 16 h at 15 °C under the following conditions: 10 µl restriction fragments, 5 pmol *Eco*RI adapter, 50 pmol *Mse*I adapter, 2 µl ligase buffer 10× (New England Biolabs), 400 U T4 DNA ligase (New England Biolabs), and sterile distilled water up to 20 µl. Ligation was terminated by a 2-fold dilution in sterile water. Preamplification (nonselective PCR) was performed with 4 µl of diluted ligation product, 10 pmol *Eco*RI + 0 primer, 10 pmol *Mse*I + 0 primer, 10 mM dNTPs, 1× PCR buffer with 15 mM MgCl2, and 0.25 U of Taq DNA polymerase (QBiogene) in a total reaction volume of 20 µl. Amplification (selective PCR) was performed using the first PCR products (20-fold diluted) as template. Reaction was run under the same conditions as for preamplification, except for the primers. Five selective primer combinations (N° I, II, III, IV, and V) were used for each sample (table 1). Each combination used 1 primer labeled

Table 1
Adaptors and Combinations of Primers Used for AFLP Selective Amplification

N°	*Eco*RI Primers	*Mse*I Primers
Adaptors		
Forward	5′-CTCGTAGACTGCGTACC-3′	5′-GACGATGAGTCCTGAG-3′
Reverse	5′-AATTGGTACGCAGTCTAC-3′	5′-TACTCAGGACTCAT-3′
Combinations of primers[a]		
I	*Eco*RI-AG[b,c]	*Mse*I-0
II	*Eco*RI-AG[b,c]	*Mse*I-C
III	*Eco*RI-C[b,d]	*Mse*I-0
IV	*Eco*RI-C[b,d]	*Mse*I-C
V	*Eco*RI-0	*Mse*I-C[b,d]

NOTE.—''0'' indicates that no base was added.
[a] Letters after the restriction enzyme name represent the nucleotide base(s) added to each primer to select only a subset of the fragments using PCR amplification.
[b] Primer labeled for detection.
[c] Primer labeled with infrared dye (IRD) IRD800.
[d] Primer labeled with infrared dye (IRD) IRD700.

with infrared dye IRDye 700 (IRD700) or IRDye 800 (IRD800) (Biolegio BV, Nijmegen, The Netherlands). After selective amplification, PCR products labeled with different markers were pooled in loading buffer (95% deionized formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, and 1 mg/ml bromophenol blue), denatured for 3 min at 95 °C, and transferred to ice before loading. Sample-loading volume was 0.6–0.8 μl depending on the banding intensity produced by the specific primer combination, and each mixture was separated, in a 3 h run at 1500 V, on a 6.5% (wt/vol) Long Ranger polyacrylamide gel, using 1× Tris-borate–EDTA buffer (Bio-Rad, Hercules, CA), on a 2-dye, model 4200 LI-COR–automated DNA sequencer.

AFLP Data Analysis

Infrared images of the band patterns were analyzed using the semiautomated scoring program AFLP-Quantar (version 1.05; KeyGene products B.V., Wageningen, The Netherlands). Images were normalized using molecular size markers. Only clear and unambiguous bands ranging between 150 and 500 bp were considered. A similarity matrix using the Jaccard coefficient was calculated, and an unweighted Neighbor-Joining tree (Saitou and Nei 1987; Gascuel 1997) was built using DARwin 4.0 (Roumagnac et al. 2004). The robustness of the tree was assessed by bootstrap analysis using the same software (1,000 replicates).

Statistical Analysis

A database was established considering the presence or the absence of the 25 polymorphic AFLP markers (M1–M25) generated on *S. glossinidius* isolated from 151 *G. palpalis gambiensis* flies that previously fed on different trypanosome bloods (table 2). Seven markers (M2, M3, M8, M9, M21, and M24 on one hand and M18 on the other hand) were removed from the analysis because of their redundancy with M1 and M14, respectively (same presence or absence of the markers in all the flies). A correspondence analysis (COA) was performed to study the relationship between genetic diversity of *S. glossinidius* and trypanosome infection. This COA gave a loading plot for the AFLP markers (statistical variables) defined by the first 2 eigenvalues and a score plot of the samples according to the fly-feeding groups. COA was performed using ADE-4 multivariate analysis and graphical display software (Thioulouse et al. 1997).

A permutation test was performed to verify that the distribution of the variables (AFLP markers) depended on the samples (flies). A classical Fisher test was applied only on the more informative variables.

Table 2
Glossina palpalis gambiensis* Infections by Either *Trypanosoma brucei brucei* Or *Trypanosoma brucei gambiense

	Blood meal Infection						
	No Trypanosome	*T. brucei gambiense*			*T. brucei brucei*		
Midgut infection (establishment)	Not infected	Not infected	Infected		Not infected	Infected	
Number of individuals	20	68	23		27	13	
Salivary gland infection			Immature infection	MI		Immature infection	MI
Number of individuals			23	0		8	5
Infection group sample number	C 1–20	GNI 21–88	GI 116–138		BNI 89–115	BI 139–151 (with MI: 140, 144, 146, 150, 151)	
Number of dissected flies	20	91				40	

NOTE.—Mature infection, MI; C, control.

Results

Tsetse Flies Infections

Out of 91 midguts isolated after flies' infection with *T. brucei gambiense*, 23 (25.3%) bore the parasite (GI) and 68 (74.7%) did not display any trypanosome (GNI) (table 2). No parasite was found in the salivary gland indicating that these 23 flies were displaying an immature infection (table 2). Out of 40 flies dissected after feeding on *T. brucei brucei*–infected mice, 13 (32.5%) were infected with *T. brucei brucei* (BI), whereas 27 (67.5%) did not display any infection (BNI) (table 2). The salivary glands from the 13 infected individuals were screened for the presence of trypanosomes. Five individuals (individuals 140, 144, 146, 150, and 151) (38.50%) displayed *T. brucei brucei* in the salivary glands (maturation stage) (table 2). The remaining 8 *T. brucei brucei*–infected flies (61.50%) had no parasite in the salivary glands (immature infection) (table 2). No trypanosome was detected in hemolymph samples.

Detection and Characterization of Bacterial Strains

The expected 1.2-kb *S. glossinidius*–specific PCR product (Cheng and Aksoy 1999; Geiger, Ravel, et al. 2005) was detected in all noninfected and infected dissected midguts (whatever the trypanosome subspecies) (fig. 1*A*) and hemolymph samples (fig. 1*B*). It was also detected for the reference strain but not for *Escherichia coli* DNA used as a negative control. This, combined with amplification and sequencing of a part of 16S rDNA (data not shown), confirms the identity of the bacterial strains analyzed as *S. glossinidius*.

Polymorphism of Observed AFLP Markers

All 5 primer combinations (table 1) generated a variable number of AFLP markers depending on the primer's pair (table 3). A total of 166 consistent markers were selected for both genetic distance calculation and cluster analysis. In all, 15.1% were polymorphic. The *Eco*RI-AG (IRD800)/*Mse*I-0 primer pair generated 19–23 fragments depending on the strains, whereas the *Eco*RI-AG (IRD800)/*Mse*I-C, *Eco*RI-C (IRD700)/*Mse*I-0, *Eco*RI-C (IRD700)/*Mse*I-C, and *Eco*RI-0/*Mse*I-C (IRD700) primer pairs generated 12–15 fragments, 48–55 fragments, 29–35 fragments, and 33–38 fragments, respectively. All fragments ranged from 150 to 500 bp. Variation occurred in the number of markers and patterns generated by the different primer pairs but also in the polymorphism rate, which varied from 12.7% up to nearly 20% (table 3).

Genetic Diversity of *S. glossinidius* Strains

Bacterial strains from *G. palpalis gambiensis* are spread into 2 main clusters, cluster I and II, separated by a high bootstrap value of 96 (fig. 2 and table 4). Cluster I can also be separated into subclusters IA and IB associated with a lower bootstrap value of 62 (fig. 2 and table 4). Cluster IA, IB, and II comprise 87, 10, and 54 strains, respectively (fig. 2 and table 4). These 3 clusters contain several subclusters characterized by low bootstrap values indicative of a low genetic diversity (fig. 2). With respect to infected flies, cluster IA gathers 10 *S. glossinidius* strains from *G. palpalis gambiensis* infected by *T. brucei brucei* (BI) out of a total of 13 (76.92%). It also contains *S. glossinidius* samples from 7 *G. palpalis gambiensis* individuals infected by *T. brucei gambiense* (GI) out of 23 (30.43%). The smaller

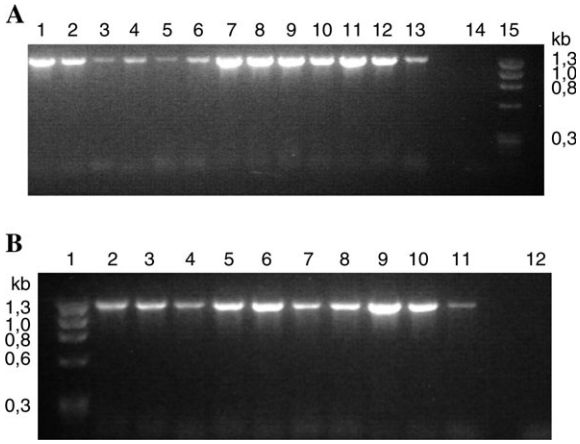

FIG. 1.—PCR detection of *Sodalis glossinidius*. (*A*) PCR detection of *S. glossinidius* from dissected midguts. Lanes 1–6: DNA extracted from 3 infected (1–3) and from 3 noninfected (4–6) midgut of *Glossina palpalis gambiensis* fed on *Trypanosoma brucei brucei*–infected blood meal. Lane 7–12: DNA extracted from 3 infected (7–9) and from 3 noninfected (10–12) midgut of *G. palpalis gambiensis* fed on *Trypanosoma brucei gambiense*–infected blood meal. Lane 13: DNA extracted from *S. glossinidius* type strain M1. Lane 14: negative control (DNA from *E. coli*). Lane 15: molecular size marker. (*B*) PCR detection of *S. glossinidius* from hemolymph. Lane 1: molecular size marker. Lane 2–3: DNA extracted from bacteria isolated from hemolymph of 2 *G. palpalis gambiensis* individuals infected by *T. brucei brucei*. Lane 4–5: DNA extracted from bacteria isolated from hemolymph of 2 noninfected *G. palpalis gambiensis* individuals fed on *T. brucei brucei*–infected blood meal. Lane 6–8: DNA extracted from bacteria isolated from hemolymph of 3 *G. palpalis gambiensis* individuals infected by *T. brucei gambiense*. Lane 9–10: DNA extracted from bacteria isolated from hemolymph of 2 noninfected *G. palpalis gambiensis* individuals fed on *T. brucei gambiense*–infected blood meal. Lane 11: PCR amplification on DNA extracted from *S. glossinidius* type strain M1. Lane 12: negative control (DNA from *E. coli*). Extracted DNA was subjected to polymerase chain reaction amplification using *Sodalis*-specific primer set GPO1F/GPO1R. Although, all samples have been tested using primers GPO1F and GPO1R, only a subset is shown in figure 1. Samples were run on 1.2% agarose gel in Tris Borate Ethylenediamine tetreacetic acid 0.5× at 100 V and stained with ethidium bromide.

Table 3
AFLP Markers Generated on *Sodalis glossinidius* Strains Using 5 Primer-Pair Combinations

Number	Primers Pair	Total Number of Markers	Number of Polymorphic Markers	% Polymorphic Markers
I	*Eco*RI-AG[a,b]/*Mse*I-0	23	4	17.4
II	*Eco*RI-AG[a,b]/*Mse*I-C	15	3	20
III	*Eco*RI-C[a,c]/*Mse*I-0	55	7	12.7
IV	*Eco*RI-C[a,c]/MseI-C	35	6	17.1
V	*Eco*RI-0/*Mse*I-C[a,c]	38	5	13.2
Total		166	25	15.1

NOTE.—Letters after the restriction enzyme name represent the nucleotide base(s) added to each primer to select only a subset of the fragments using PCR amplification. ''0'' indicates that no base was added.

[a] Primer labeled for detection.

[b] Primer labeled with infrared dye IRD800.

[c] Primer labeled with infrared dye IRD700.

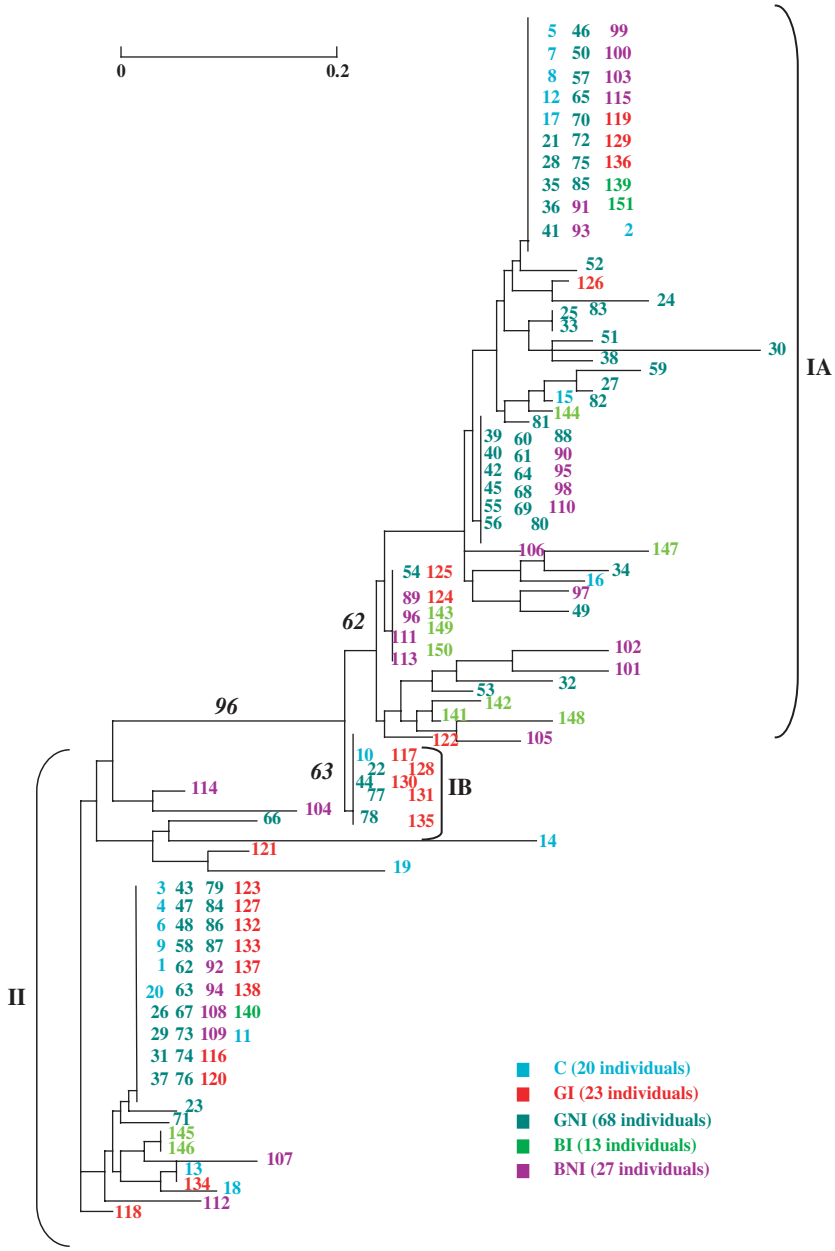

FIG. 2.—Unweighted Neighbor-Joining tree representation of the genetic diversity of *Sodalis glossinidius* strains from infected and noninfected *Glossina palpalis gambiensis* individuals. Scale indicates genetic distance. Numbers at nodes represent bootstrap values (1,000 replicates). Numbers represent *S. glossinidius* strains as follows: 1–20: control strains from *G. palpalis gambiensis* fed on noninfected blood meal (C); 21–88: strains from noninfected flies fed on *Trypanosoma brucei gambiense*–infected blood meal (GNI); 89–115: strains from noninfected flies fed on *Trypanosoma brucei brucei*–infected blood meal (BNI); 116–138: strains from *T. brucei gambiense*–infected flies (GI); 139–151: strains from *T. brucei brucei*–infected flies (BI).

cluster IB comprises only *S. glossinidius* strains isolated from flies infected by *T. brucei gambiense* (GI), that is, 5 individuals out of 23 or 21.73%. Cluster II comprises a majority of bacterial strains from flies infected by *T. brucei gambiense* (GI), that is, 11 individuals out of 23 (47.82%), and a minority of strains from tsetse flies infected by *T. brucei brucei* (BI) (3 individuals out of 13 or 23.07%). *Sodalis glossinidius* strains isolated from noninfected flies (GNI and BNI) are spread over the 3 clusters (fig. 2 and table 4). Cluster IA gathers 43 strains from *T. brucei gambiense* noninfected flies (GNI) out of 68 (63.23%), whereas cluster IB and II comprise 4 strains (5.88%) and 21 strains (30.88%), respectively. *Sodalis glossinidius* strains from flies not infected after exposure to *T. brucei brucei* (BNI) are present in clusters IA and II. Cluster IA gathers 19 BNI strains out of 27 (70.37%), whereas cluster II comprises 8 strains (29.62%). *Sodalis glossinidius* control strains isolated from flies fed on noninfected blood meal (C) are spread over the 3 clusters IA, IB, and II, gathering respectively 8, 1, and 11 strains. These data are summarized in table 4.

Table 4
Distribution of *Sodalis glossinidius* Strains

	Cluster IA	Cluster IB	Cluster II	Total
Bootstrap	62	63	96	
Strains from *T. b. b.*[a] infected flies (BI)	10	0	3	13
Strains from *T. b. b.* noninfected flies (BNI)	19	0	8	27
Strains from *T. b. b.*–fed flies				40
Strains from *T. b. g.*[b] infected flies (GI)	7	5	11	23
Strains from *T. b. g.* noninfected flies (GNI)	43	4	21	68
Strains from *T. b. g.*–fed flies				91
Control sample (C)	8	1	11	20
Number of *S. glossinidius* strains	87	10	54	151

[a] *Trypanosoma brucei brucei.*
[b] *Trypanosoma brucei gambiense.*

Correspondence between Genetic Diversity of *S. glossinidius* and Trypanosome Infection

The fraction of variance accounted by the first 2 COA eigenvalues was 71%. As shown by the loading plot for the *S. glossinidius* AFLP markers (fig. 3*A*), the first axis CO1 was mainly correlated with the variable M22, which had positive values, and with the variable M1, which had negative values (projections of M1 and M22 on the loading plot were opposite to one another). This axis was also correlated with variables M14 and M23 with less significance. The second axis CO2 was mainly correlated with variables M14 and M23, which had positive values, and with variable M22, which had negative values. The score plot of the samples showed that the first CO1 and the second CO2 axes distinguished the different blood feedings analyzed (fig. 3*B*): 1) the first axis CO1 separated the GI samples, related more with its positive values, from the C, GNI, BNI, and BI samples, related more with its zero value; 2) the second axis CO2 separated the BI samples, related with its positive values, from the other sample groups related more with its 0 value. Considering the most significant AFLP markers in the different fly-feeding groups (table 5), M1 was more frequent in the BI group and less frequent in the GI group. M22 was significantly more frequent in the GI group than in the others. M14 and M23 were more frequent in the infected groups (BI or GI) than in the noninfected groups (C, GNI, or BNI).

Discussion

The data reported here confirm the presence of several genetically distinct clusters of *S. glossinidius* harbored by *G. palpalis gambiensis* species. This structure in the populations of *S. glossinidius* was already observed in *G. palpalis gambiensis* but not in *G. morsitans morsitans*, which harbored a homogeneous population of the S-symbiont (Geiger, Cuny, et al. 2005). Tsetse flies reproduce by adenotropic viviparity, and *S. glossinidius* is vertically transmitted to the intrauterine developing larva (Aksoy et al. 1997; Cheng and Aksoy 1999). The observed genetic diversity may thus reflect differential host-driven selective pressures (Rio et al. 2003; Geiger, Cuny, et al. 2005).

The main conclusion, however, is the nonrandom distribution of *S. glossinidius* genotypes with respect to the fly-feeding groups. *Sodalis glossinidius* strains from *T. brucei brucei*–fed flies (BI and BNI) are significantly associated with the genetically distinct group of genotypes found in cluster IA. Although, the genetic segregation between clusters IA and IB is less significant (i.e., bootstrap values of 62 and 63) than that observed with cluster II (bootstrap of 96), cluster IB, while representing only 6.6% of the *S. glossinidius* samples, comprises exclusively bacterial strains isolated from flies in contact with *T. brucei gambiense* and gathers 22% of the GI bacterial strains. Cluster II, although displaying a more balanced presence of strains from both *T. brucei brucei* and *T. brucei gambiense*–fed flies, is nevertheless characterized by the higher proportion of GI samples. This nonrandom distribution of the *S. glossinidius* genotypes must be considered in the light of the suspected role of *S. glossinidius* in the establishment of trypanosomes.

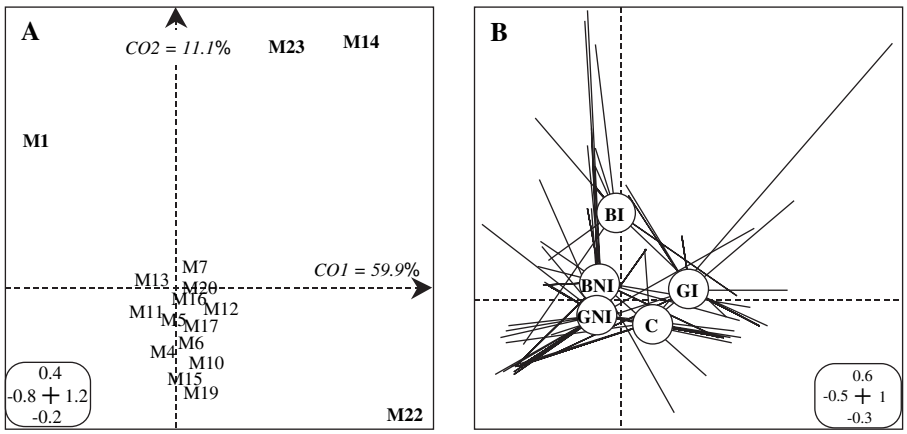

FIG. 3.—*Sodalis glossinidius* diversity and fly–trypanosome infection COA. (*A*) COA-loading plot for the *S. glossinidius* AFLP markers M1–M25. (*B*) Score plot for the flies according to their trypanosome infection group. C = control strains from *Glossina palpalis gambiensis* fed on noninfected blood meal (1–20). GNI = strains from noninfected flies fed on *Trypanosoma brucei gambiense*–infected blood meal (21–88). BNI = strains from noninfected flies fed on *Trypanosoma brucei brucei*–infected blood meal (89–115). GI = strains from *T. brucei gambiense*–infected flies (116–138). BI = strains from *T. brucei brucei*–infected flies (139–151).

Table 5
Frequency of Significant AFLP Markers in the Different Feeding Groups

Glossina palpalis gambiensis Feeding Group	Frequency of *Sodalis glossinidius* AFLP Marker (%)		
	M1	M22	M14 + M23
C + GNI + BNI	65.22 ab	39.13 b	56.52 b
GI	52.17 b	65.22 a	82.61 a
BI	76.92 a	23.08 c	76.92 a

NOTE.—Data in each column followed by same letters are not significantly different (Fisher's test, $P < 0.0001$).

The suspected interaction occurs after the differentiation of blood stream form trypanosomes into the insect-specific procyclic form. Insect-produced trypanocidal lectins specific to the procyclic forms are thought to be inhibited by an N-acetyl glucosamine resulting from the hydrolysis of the pupae chitin by a *S. glossinidius*–produced endochitinase (Welburn and Maudlin 1999). Different *S. glossinidius* genotypes might be associated with differing capabilities of facilitating trypanosome establishment resulting in the observed distribution.

The correspondence observed between *S. glossinidius* groups of markers and successful establishment of both parasites further confirms this nonrandom distribution and suggests that *S. glossinidius* can indeed influence the establishment of trypanosomes in tsetse flies. The M14 and M23 groups of markers are linked to the ability of both parasites to establish in the insect midgut, suggesting the existence of some generic mechanism allowing for infection of *G. palpalis gambiensis* flies with either *T. brucei gambiense* or *T. brucei brucei*. This is compatible with the suggested overall mechanism of facilitation of parasite establishment (Welburn and Maudlin 1999). Furthermore, this observation is also in line with the maternal inheritance of *Glossina* susceptibility for trypanosome infection, owing to the vertical transmission of *S. glossinidius* (Maudlin and Ellis 1985; Aksoy et al. 1997; Cheng and Aksoy 1999). These data also suggest that the involvement of *S. glossinidius* in parasite establishment might not be straightforward and may involve different mechanisms. Indeed, other groups of markers seem to be specifically associated, in an exclusive way, with the establishment of either *T. brucei brucei* or *T. brucei gambiense*. The *S. glossinidius* M1 group of markers is positively linked, that is, significantly higher representation, to successful establishment of *T. brucei brucei*, whereas it is less represented in flies displaying established infection of *T. brucei gambiense*. Conversely, the *S. glossinidius* M22 group of markers is significantly more frequent in flies with established *T. brucei gambiense* and less frequent in flies infected with *T. brucei brucei*. This indicates the existence of more specific mechanisms directly dependent upon the parasite subspecies. Interestingly, no marker discriminates between the noninfected flies, whatever subspecies of trypanosome they have been exposed to. *Sodalis glossinidius* strains isolated from noninfected flies fed either on noninfected blood (C group) or on *T. brucei gambiense* (GNI group)– and *T. brucei brucei* (BNI group)–infected bloods are very close to each other from a genetic standpoint. This further supports the assumption of specific functions associated to the M1, M22, and M14/M23 groups of markers. Interestingly, although the parasite has a negative fitness cost for the flies (Maudlin et al. 1998), genotypes of *S. glossinidius* facilitating parasite infection are maintained in populations of *Glossina*. This implies they may have a positive influence on fitness overcoming the negative effect of parasite infection. Variations in the level of chitinase activity or in the kind of chitinase involved are primary hypotheses. However, data reported here do not allow for identification of this mechanism, and other phenomena might be involved. The very recent developments in the genomics of *S. glossinidius* (Darby et al. 2005; Toh et al. 2006) open a way to further investigate these tripartite insect–parasite–symbiont interactions through comparative and functional genomics. Comparing the genome or the transcriptome of the strains specifically associated with the establishment of *T. brucei gambiense* would definitely help unravelling the mechanisms of facilitation involved.

The differential presence of *S. glossinidius* genotypes could explain variations in vector competence of *Glossina* for *T. brucei* s.l. in the wild (Frézil and Cuisance 1994; Lefrançois et al. 1998). Furthermore, *T. brucei brucei* can be transmitted both by the *palpalis* group and the *morsitans* group unlike *T. brucei gambiense*, which is only transmitted by members of the *palpalis* group (Moloo and Kutuza 1988; Richner et al. 1988). This work provides orientations for a better understanding of vector competence and tripartite interactions but also specific genetic markers to assess the prevalence in tsetse flies of *S. glossinidius* genotypes facilitating the establishment of *T. brucei gambiense*, allowing thus for the development of tools for epidemiological survey and risk mapping and for the development of risk management and vector control strategies aiming at eradicating this deadly disease.

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