

## Two Tsetse Fly Species, *Glossina palpalis gambiensis* and *Glossina morsitans morsitans*, Carry Genetically Distinct Populations of the Secondary Symbiont *Sodalis glossinidius*

Anne Geiger,\* Gérard Cuny, and Roger Frutos

UMR 17, IRD-CIRAD, CIRAD TA 207/G, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France

Received 16 February 2005/Accepted 1 August 2005

**Genetic diversity among *Sodalis glossinidius* populations was investigated using amplified fragment length polymorphism markers. Strains collected from *Glossina palpalis gambiensis* and *Glossina morsitans morsitans* flies group into separate clusters, being differentially structured. This differential structuring may reflect different host-related selection pressures and may be related to the different vector competences of *Glossina* spp.**

Tsetse flies are vectors of African trypanosomes, the causative agents of sleeping sickness in humans and nagana, a tropical disease of cattle. African trypanosomiasis in humans is reemerging and has considerable impact on public health and economic development in sub-Saharan Africa (13, 35), whereas African trypanosomiasis in animals costs \$4.5 billion per year.

To be transmitted, the parasite must first establish itself in the insect midgut and undergo a subsequent maturation process into the salivary gland or the mouthparts, depending on the species of trypanosome (28, 30). Factors involved in establishment are largely unknown, and only a small proportion of flies develop mature infection and transmit the disease (16). Variability in vector competence depends on the species of *Glossina* and trypanosomes. *Glossina morsitans* is a good vector of *Trypanosoma congolense* (10, 18, 29), whereas *Glossina palpalis* is a poor vector (12, 18, 22). Conversely, *Glossina palpalis* is the main vector of *Trypanosoma brucei gambiense* (11), the causative agent of African trypanosomiasis in humans, whereas *Glossina morsitans* is not (7, 15).

Tsetse flies harbor three different symbionts (3), among which *Sodalis glossinidius* (1, 4, 5) is considered to be involved in vector competence (14) and to favor the establishment of the parasite in the insect midgut (32, 34). This role is still discussed (14, 19, 27, 31, 33). To investigate whether vector competence could be related to genetic diversity, we conducted an amplified fragment length polymorphism (AFLP) analysis of *S. glossinidius* strains from two species of *Glossina*.

Hemolymph of 20 *G. palpalis gambiensis* and 19 *G. morsitans morsitans* female flies was individually collected in phosphate-buffered saline. The bacteria were separated from insect cells by differential centrifugation (6). DNA was extracted from these bacteria and from the reference strain, *S. glossinidius* type strain M1 (5), using the DNeasy tissue kit.

The identity of the bacteria, including strain M1, was assessed by amplification of a specific PCR fragment using prim-

ers GPO1 F and GPO1 R (4, 5, 20) and analysis of the 16S rRNA gene as previously described (9). For each fly, sequencing of different clones did not show any difference, suggesting that only one bacterial strain was present or was the main component of a population. Bacterial DNA was digested with EcoRI and MseI. Double-stranded oligonucleotide adaptors (Table 1) were ligated to the restriction fragments. Pre-amplification was performed with nonselective primers. Amplification was performed using the first PCR products as template and five selective primer combinations (I to V [Table 1]). PCR products labeled with different markers were separated on a two-dye, model 4200 LI-COR automated DNA sequencer. Infrared images were analyzed using the AFLP-Quantar program. Only clear and unambiguous bands ranging between 150 and 500 bp were considered. DNA from strain M1 was used as a control to avoid artifactual polymorphism. The presence/absence of fragments was scored in a binary matrix. A similarity matrix (Jaccard coefficient) was calculated, and an un-

TABLE 1. Double-stranded oligonucleotide adaptor sequences and combinations of primers used for AFLP selective amplification

Direction or no.	EcoRI primer	MseI primer
Adaptors		
Forward	5'-CTCGTAGACTGC GTACC-3'	5'-GACGATGAGTCC TGAG-3'
Reverse	5'-AATTGGTACGCA GTCTAC-3'	5'-TACTCAGGACTC AT-3'
Combinations of primers <sup>c</sup>		
I	<i>EcoRI</i> -AG <sup>*a</sup>	<i>MseI</i> -0
II	<i>EcoRI</i> -AG <sup>*a</sup>	<i>MseI</i> -C
III	<i>EcoRI</i> -C <sup>*b</sup>	<i>MseI</i> -0
IV	<i>EcoRI</i> -C <sup>*b</sup>	<i>MseI</i> -C
V	<i>EcoRI</i> -0	<i>MseI</i> -C <sup>*b</sup>

<sup>a</sup> Primer labeled with infrared dye IRD800.

<sup>b</sup> Primer labeled with infrared dye IRD700.

<sup>c</sup> 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. A zero indicates that no base was added. \*, primer labeled for detection.

\* Corresponding author. Mailing address: UMR 17, IRD-CIRAD, CIRAD TA 207/G, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France. Phone: 33 4 67 59 39 25. Fax: 33 4 67 59 39 20. E-mail: Anne.Geiger@mpl.ird.fr.

TABLE 2. AFLP markers generated on *S. glossinidius* strains from *G. palpalis gambiensis* using five primer pair combinations

No.	Primer pair <sup>c</sup>	Total no. of markers	No. of polymorphic markers	% Polymorphic markers
I	<i>EcoRI</i> -AG <sup>*a</sup> / <i>MseI</i> -0	24	5	20.8
II	<i>EcoRI</i> -AG <sup>*a</sup> / <i>MseI</i> -C	15	3	20
III	<i>EcoRI</i> -C <sup>*b</sup> / <i>MseI</i> -0	55	7	12.7
IV	<i>EcoRI</i> -C <sup>*b</sup> / <i>MseI</i> -C	35	6	17.1
V	<i>EcoRI</i> -0/ <i>MseI</i> -C <sup>*b</sup>	36	3	8.3
Total		165	24	14.5

<sup>a</sup> Primer labeled with infrared dye IRD800.

<sup>b</sup> Primer labeled with infrared dye IRD700.

<sup>c</sup> 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. A zero indicates that no base was added. \*, primer labeled for detection.

weighted neighbor-joining tree (8, 26) was built using DARwin version 4.0 (25).

Five combinations of primers (Table 1) were used to perform AFLP analysis on 39 *S. glossinidius* strains, which generated a variable number of AFLP markers depending on the primer pair (Table 2 and 3). One hundred sixty-five markers were selected for both genetic distance calculation and cluster analysis. About 14.5% of the markers from *G. palpalis gambiensis* bacterial strains were polymorphic (Table 2), whereas polymorphism was found in only 6% of those from *G. morsitans morsitans* symbionts (Table 3).

The dendrogram representing the cluster distribution of reference strain M1 and 39 *S. glossinidius* strains sampled from *G. palpalis gambiensis* and *G. morsitans morsitans* is shown in Fig. 1. Strains from *G. palpalis gambiensis* are distributed within three clusters (I, II, and III) associated with high bootstrap values (i.e., 75 to 100). Three other clusters (IV, V, and VI), with low bootstrap values, can be distinguished and correspond to strains from *G. morsitans morsitans*. Reference strain M1 branches separately. Populations of *S. glossinidius* isolated from *G. palpalis gambiensis* and *G. morsitans morsitans* are genetically distinct. Furthermore, populations of *S. glossinidius* from *G. palpalis gambiensis* are strongly structured in genetically distinct groups, whereas populations from *G. mor-*

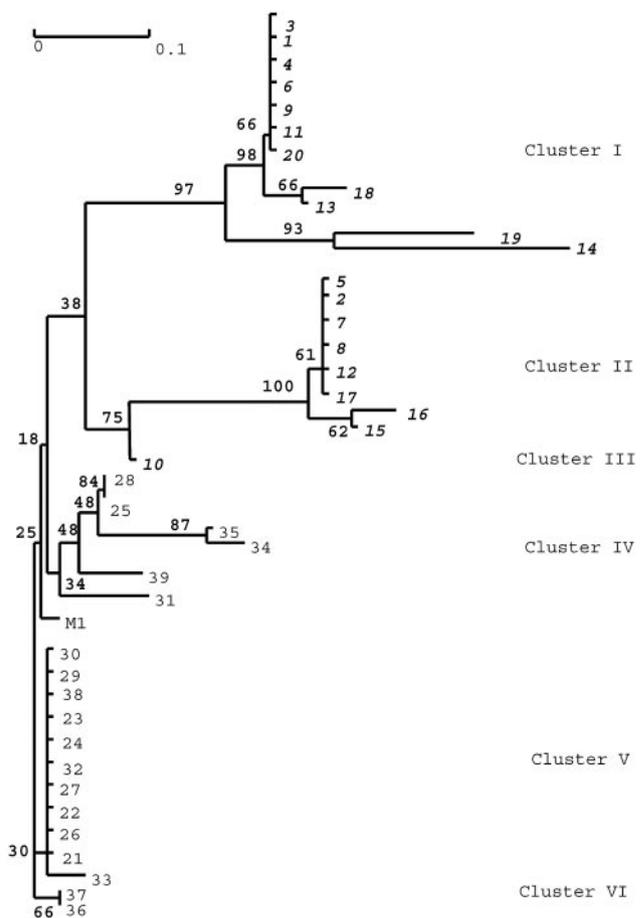


FIG. 1. Unweighted neighbor-joining tree representation of the genetic diversity of *S. glossinidius* strains from *G. palpalis gambiensis* and *G. morsitans morsitans*. Scale indicates genetic distance. Numbers at nodes represent bootstrap values (1,000 replicates). Genetic similarities were calculated using the Jaccard coefficient based on the 29 polymorphic band positions observed for five AFLP primer pairs. The tree was constructed with DARwin version 4.0 (25). Numbers represent *S. glossinidius* strains as follows: 1 to 20, strains from *G. palpalis gambiensis*; 21 to 39, strains from *G. morsitans morsitans*; M1, *S. glossinidius* reference strain M1.

TABLE 3. AFLP markers generated on *S. glossinidius* strains from *G. morsitans morsitans* using five primer pair combinations

No.	Primer pair <sup>c</sup>	Total no. of markers	No. of polymorphic markers	% Polymorphic markers
I	<i>EcoRI</i> -AG <sup>*a</sup> / <i>MseI</i> -0	24	2	8.3
II	<i>EcoRI</i> -AG <sup>*a</sup> / <i>MseI</i> -C	15	0	0
III	<i>EcoRI</i> -C <sup>*b</sup> / <i>MseI</i> -0	55	6	10.9
IV	<i>EcoRI</i> -C <sup>*b</sup> / <i>MseI</i> -C	35	2	5.7
V	<i>EcoRI</i> -0/ <i>MseI</i> -C <sup>*b</sup>	36	0	0
Total		165	10	6

<sup>a</sup> Primer labeled with infrared dye IRD800.

<sup>b</sup> Primer labeled with infrared dye IRD700.

<sup>c</sup> 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. A zero indicates that no base was added. \*, primer labeled for detection.

*sitans morsitans* are not stringently structured and display limited genetic diversity.

This variation in the structure of populations must be regarded in connection with the specific biology of both *S. glossinidius* and tsetse flies. *Glossina* flies reproduce by adentrophic viviparity, and *S. glossinidius* is vertically transmitted to the intrauterine developing larva (2, 4). Exchange of genetic material between strains of *S. glossinidius* is thus very unlikely, and, furthermore, *G. palpalis gambiensis* and *G. morsitans morsitans* are geographically separated. Vertical transmission, sequence similarity of cloned PCR product, high bootstrap values for cluster I to III, and clear genetic structure of the *S. glossinidius* populations suggest that the bacterial strains analyzed in this work are most likely clonal. The difference in genetic diversity observed between *S. glossinidius* strains from *G. palpalis gambiensis* and *G. morsitans morsitans* might therefore reflect differential host-driven selective pressure of closely

related microorganisms, in agreement with hypotheses on the origin and evolution of *S. glossinidius* (24).

Vector competence is a major difference between *G. palpalis gambiensis* and *G. morsitans morsitans* (7, 10, 12, 15, 17, 18, 21, 22, 23, 29) which relates directly to the suggested role of *S. glossinidius* on the inhibition of trypanocidal insect lectins through the production of *N*-acetylglucosamine (32, 34). *S. glossinidius* in *G. palpalis gambiensis* might have been selected to facilitate the establishment and transmission of the parasite, explaining the high bootstrap values and the structured population. On the other hand, the presence of genetically different populations of *S. glossinidius* in *G. morsitans morsitans* might also be related to its differing vector competence. However, further research is needed to clearly establish the correlation between a given genotype of *S. glossinidius* and vector competence. The demonstration of the existence of genetic diversity in *S. glossinidius* is a first step towards the characterization of natural populations and a better understanding of the tripartite *Glossina-Sodalis-Trypanosoma* interactions most likely involved in the transmission of this deadly reemerging disease.

We are grateful to D. Verhaegen for his help in data analysis. We are particularly grateful to B. Tchicaya and J. Janelle for maintenance and management of the tsetse colonies.

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