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The internal transcribed spacer 1 sequence polymorphism brings updates to tsetse species distribution in the northern Cameroon: Importance in planning efficient vector control

Steve Feudjio Soffack[1] | Tito Tresor Melachio Tanekou[2,3,4] | Oumarou Farikou[5] | Ginette Irma Kame Ngasse[6] | Mureille Carole Tchami Mbagnia[1] | Murielle Wondji[3,4] | Charles S. Wondji[3,4] | Adly M. M. Abd-Alla[7] | Anne Geiger[8] | Gustave Simo[9] | Flobert Njiokou[1]

[1]Laboratory of Parasitology and Ecology, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon
[2]Department of Microbiology and Parasitology, Faculty of Science, University of Bamenda, Bamenda, Cameroon
[3]Centre for Research in Infectious Diseases (CRID), Yaoundé, Cameroon
[4]Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool, UK
[5]Faculty of Health Science, University of Bamenda, Bamenda, Cameroon
[6]Centre for Research on Health and Priority Pathologies, IMPM, Yaoundé, Cameroon
[7]Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Vienna, Austria
[8]UMR177, Institut de Recherche pour le Développement (IRD)-CIRAD, Montpellier, France
[9]Molecular Parasitology and Entomology Unit, Department of Biochemistry, Faculty of Science, University of Dschang, Dschang, Cameroon

Correspondence
Tito Tresor Melachio Tanekou, Department of Microbiology and Parasitology, Faculty of Science, University of Bamenda, Bamenda, Cameroon.
Email: tresor.melachio@crid-cam.net; Tresor.Melachio@lstmed.ac.uk

Flobert Njiokou, Laboratory of Parasitology and Ecology, Faculty of Science, University of Yaoundé I, PO BOX 812, Yaoundé, Cameroon.
Email: njiokouf@yahoo.com

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Abstract

Vector control remains one of the best strategies to prevent the transmission of trypanosome infections in humans and livestock and, thus, a good way to achieve the elimination of human African trypanosomiasis and animal African trypanosomiasis. A key prerequisite for the success of any vector control strategy is the accurate identification and correct mapping of tsetse species. In this work, we updated the tsetse fly species identification and distribution in many geographical areas in Cameroon. Tsetse flies were captured from six localities in Cameroon, and their species were morphologically identified. Thereafter, DNA was extracted from legs of each tsetse fly and the length polymorphism of internal transcribed spacer-1 (ITS1) region of each fly was investigated using PCR. ITS1 DNA fragments of each tsetse species were sequenced. The sequences obtained were analysed and compared to those available in GenBank. This enabled to confirm/infirm results of the morphologic identification and then, to establish the phylogenetic relationships between tsetse species. Morphologic features allowed to clearly distinguish all the tsetse species captured in the South Region of Cameroon, that is, *Glossina palpalis palpalis*, *G. pallicera*, *G. caliginea* and *G. nigrofusca*. In the northern area, *G. morsitans submorsitans* could also be distinguished from *G. palpalis palpalis*, *G. tachinoides* and *G. fuscipes*, but these three later could not be distinguished with

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routine morphological characters. The ITS1 length polymorphism was high among most of the studied species and allowed to identify the following similar species with a single PCR, that is, *G. palpalis palpalis* with 241 or 242 bp and *G. tachinoides* with 221 or 222 bp, *G. fuscipes* with 236 or 237 bp. We also updated the old distribution of tsetse species in the areas assessed, highlighting the presence of *G. palpalis palpalis* instead of *G. fuscipes* in Mbakaou, or in sympatry with *G. morsitans submorsitans* in Dodeo (northern Cameroon). This study confirms the presence of *G. palpalis palpalis* in the Adamawa Region of Cameroon. It highlights the limits of using morphological criteria to differentiate some tsetse species. Molecular tools based on the polymorphism of ITS1 of tsetse flies can differentiate tsetse species through a simple PCR before downstream analyses or vector control planning.

KEYWORDS
distribution maps, molecular tools, tsetse identification, vector control

BACKGROUND

Human African trypanosomiases (HATs or sleeping sickness) and animal African trypanosomiases (AAT) are infectious diseases caused by protozoan parasites of the genus *Trypanosoma* that are still prevalent in Africa. They are mainly transmitted by dipteran flies of the genus *Glossina* also called tsetse flies. With the efforts of World Health Organisation (WHO), national control programmes and different actors, HAT has regressed to the lowest records of 992 and 663 new cases in 2019 and 2020, respectively (WHO, 2021). Diagnosis and treatment of cases have been the main strategy to fight sleeping sickness but were principally hindered by the difficult access to most cases in at risk areas (Tirados et al., 2015). Regarding AAT, 3 billion livestock are lost per year due to these diseases (Cecchi & Mattioli, 2009; Gao et al., 2020). The control of AAT has been less coordinated and has mainly relied on farmer-based chemotherapy and chemoprophylaxis (Steverding, 2008; Van den Bossche et al., 2000). Moreover, cattle movements and contact with wild animals in tsetse infested areas increase their risk of infection with trypanosomes (Kasozi et al., 2021). Therefore, tsetse vector control, as a good additive strategy of fighting HAT and AAT has been acknowledged for its great contribution (Meyer et al., 2016; Ndung'u et al., 2020). In Cameroon, around seven new HAT cases still occur each year, 90% of these being recorded in Campo focus in the South Region. The other active foci include Bipindi (also in the South) and Yocadouma in the East Region (FAO & WHO, 2022). Animal trypanosomiases are more widespread across the country, and trypanosomes were detected in tsetse flies and mammals from many areas (Farikou et al., 2010; Mamoudou et al., 2016; Ngomtcho et al., 2017; Simo et al., 2015).

Tsetse flies are widely spread in sub-Saharan Africa and occupy about 10 million km^2 across 38 countries (Kuzoe & Schofield, 2004). The control strategies used have deeply evolved, to increase their impact on tsetse populations and their suitability for environment. Indeed, first large-scale control strategies involved ground spraying with dichlorodiphenyltrichloroethane, game destruction or bush clearing, that were later criticised for negative side effects for human and the environment (Kabasenche & Skinner, 2014; Özkara et al., 2016; Smies, 1980). Other techniques and/or tools were also developed, including sequential aerial spraying, which have mostly been efficient in the savannah (Kgori et al., 2006) and may be less effective against riverine tsetse flies because of the canopy (De Deken & Bouyer, 2018), insecticide-treated traps and targets (Kuzoe & Schofield, 2004) and insecticide-treated cattle used as live baits (Okello et al., 2021; Vale et al., 1999). Most of these techniques were shown suitable for small or large-scale tsetse control programmes, but in most of the cases, they could not complete the eradication of the targeted tsetse population: indeed, after significant reduction of tsetse densities, the control efforts were released and tsetse recolonized the treated areas from small number of residual individuals or migration from neighbouring areas (Brightwell et al., 1997; Hargrove, 2003; Vale et al., 1999). Currently, complete sustainable eradication of tsetse fly populations was only achieved, after combining a technique using mass-rearing alive flies, that is, the sterile insect technique (SIT) to the other existing methods (Politzar and Cuisance, 1984; Vreysen et al., 2000, 2014). Moreover, other innovative fly engineering techniques based on paratransgenesis of their symbionts are under development, to provide tsetse flies harbouring symbionts that they can transmit to offspring and that produce factors capable of impairing trypanosome development (Cheng and Aksoy, 1999; De Vooght et al., 2012, 2014). Also, as recently suggested, both SIT and paratransgenesis can be used in combination for a better effect (Demirbas-Uzel et al., 2018). Although these living fly-based techniques are useful in areas with difficult access to human, they are unfortunately species-specific, that is, cannot be transferred from a targeted engineered species to another allopatric one on the field, or from sterile males that can mate with virgin females from different incompatible species in SIT programme. The clear identification of a target tsetse population is, thus, a prerequisite to any downstream study or dedicated vector control action (Leak et al., 2008).

Although morphological characteristics allowed the classification of 31 tsetse species into three distinct groups, it remains very difficult to clearly distinguish some tsetse species from the same group

(Augustinos et al., 2018). For instance, *G. palpalis palpalis*, *G. tachinoides* and *G. fuscipes* not only share identical morphological characteristics but also can be in sympatry in some bio-ecological settings. Molecular tools using a simple PCR have been investigated to help accurate identification of these species. Indeed, the interspecific polymorphism at the internal transcribed spacer-1 (ITS1) region has proven highly discriminatory for the majority of *Glossina* taxa from sub-Saharan Africa (Augustinos et al., 2018; Dyer et al., 2008; Shaida et al., 2018).

This study aimed to update the tsetse fly species distribution in many geographical areas in Cameroon by analysing the length polymorphism of ITS1 of different species.

MATERIALS AND METHODS

Study areas

The tsetse samples collected came from the HAT foci of Bipindi and Campo (South Region), Doumé (East Region) and the AAT foci of Dodéo and Mbakaou (Adamawa Region), and Sora Mboum (North Region). We also obtained flies from the HAT focus of Mouhoun in Burkina Faso that were used as comparative group (Figure 1).

Bipindi (3°2′ N, 10°22′ E) is in the south equatorial rainforest, 70 km from the Atlantic coast, with dense hydrographic network of fast-running streams. Biotopes are highly suitable for the development of tsetse flies, with the presence of wild animals as source of blood meal (Massussi et al., 2009; Njiokou et al., 2006). Bipindi is a historical focus of HAT known since 1920 where at least three species/sub-species of tsetse flies were reported, that is, *Glossina palpalis palpalis*, *G. pallicera* and *G. nigrofusca* (Tchouomene-Labou et al., 2013).

Campo (2°20′N, 9°52′ E) is located along the Atlantic coast and the Ntem river that serves as border between Cameroon and Equatorial Guinea. Campo is a hypo-endemic focus of HAT (Penchenier et al., 1999), where around five cases were reported per year between 2012 and 2018 (Records of the national sleeping sickness control programme). Campo is also located in the equatorial rainforest with dense hydrographic network including several rivers and swampy areas that offer suitable biotopes for tsetse flies' development. At least four tsetse fly species/sub-species were recently reported in the area, that is, *Glossina palpalis palpalis*, *G. pallicera*, *G. caliginea* and *G. nigrofusca* (Grébaut et al., 2016; Melachio Tanekou et al., 2022).

Doumé (4°5′N; 13°27′ E) is a hypo-endemic HAT located in the East Region of Cameroon, in the forest degraded by very intensive logging and the setting of coffee plantations. Two main rivers are found in the area, the Doumé and its main tributary, the Mala, both with vast marshy areas along. The only tsetse fly sub-species recently reported there is *Glossina fuscipes fuscipes* (Mbida Mbida et al., 2009).

Dodéo (7°26′N; 12°00′ E) and Mbakaou (6°18′N; 12°47′ E) are located in the Faro et Déo and Djérem Divisions in the Adamawa Region, respectively. The Adamawa Region of Cameroon is a mountainous area of 72,000 km^2, at the transition between the southern forest and the northern Sahelian areas. With intensive cattle rearing, it offers suitable environments for tsetse development (Tanenbe et al., 2010). Many tsetse fly species/sub-species were previously reported in these areas, that is, *G. fuscipes fuscipes*, *G morsitans submorsitans*, *G. tachinoides*, *G. fusca congolensis*, and *G. palpalis palpalis* (Farikou et al., 2022; Kame-Ngasse et al., 2018; Shaida et al., 2018).

Sora-Mboum (7°47′N, 15°00′ E) is savannah grassland located in Mayo Rey Division of North Region in Cameroon. Livestock breeding is the main socio-economic activity of rural inhabitant and people always complain about the decimation of their livestock by bovine trypanosomiasis. Tsetse fly species/sub-species reported in the area include *G. tachinoides* and *G. morsitans submorsitans* (Rageau & Adam, 1953).

The Mouhoun River basin in Burkina Faso (4°26′N, 11°13′W) is mainly a forest gallery in a savannah area, that is highly fragmented by

FIGURE 1 Tsetse flies sampling areas in Cameroon and Burkina Faso.

cotton farms and where *Glossina palpalis gambiensis* and *G. tachinoides* share the same biotopes (Kone et al., 2010).

Tsetse fly collections

Tsetse flies used in this study were sampled during independent studies in Cameroon (May 2011 in Bipindi, June 2012 in Doumé, May 2017 in Dodéo, July 2019 in Campo, March 2020 in Sora-Mboum and October 2020 in Mbakaou) and in the Mouhoun basin in Burkina Faso (May 2020). Tsetse flies were caught using pyramidal traps (Gouteux & Lancien, 1986) that were installed in suitable biotopes for tsetse development and visited once a day for two to four consecutive days. Geographical coordinates were recorded for each trap using a global positioning system device. The flies captured were collected from traps every day and brought to the field laboratory for morphological identification. Then, they were individually stored in Eppendorf tubes containing 95% ethanol at room temperature on the field and at -20°C once in the laboratory, till DNA extraction and molecular analyses.

Morphologic identification

The flies' species were identified first from their localization, that is, using old distribution maps, and with routine time-friendly characters like the size, the general colour, the presence of a dorsal longitudinal whitish line on the abdomen or the presence of a dorsal and central circular spot on the first abdominal segment, and the colour of the last three tarsal segments of fore and hind legs (Pollock, 1982). The sex of each fly was also identified from the presence of phallus for males.

After morphological identification, tsetse legs were separated from each fly and put in 1.5-µL microtubes containing 95% ethanol for further molecular analyses. In the field, tubes containing tsetse legs were kept at room temperature and in the laboratory, they were stored at -20°C.

DNA extraction

DNA was extracted from three legs of each fly using cetyl trimethyl ammonium bromide (CTAB) as described by Maniatis et al. (1982). Briefly, the legs were crushed with a pestle in CTAB buffer (CTAB 5%; 1 M Tris pH 8; 0.5 M EDTA pH 8; 5 M NaCl). The mixture obtained was incubated at 60°C for 30 min. Thereafter, an equal volume of 24/1 chloroform/isoamylic alcohol was added in each microtube, and the mixture was centrifuged at 10,000*g* for 15 min. The aqueous phase was collected and transferred to a clean microtube, and an equal volume of isopropanol was added for nucleic acid precipitation. This was done through centrifugation at 10,000*g* for 15 min. The pellet was washed twice with 70% ethanol and air-dried before its re-suspension in 100 µL nuclease-free distilled water. DNA extracts were stored at -20°C until use.

PCR-amplification of ITS1 region of tsetse flies

The ITS1 DNA fragment of each tsetse fly was amplified as described by Dyer et al. (2008) using species diagnostic PCR with forward (TGGACTTCGGATTAAGTACAACA) and reverse (TCATTATGCGCTA TTAAGGTAAGC) primers. Each amplification was performed in a total PCR volume of 30 µL containing 12.9 µL of double-distilled water, 3 µL of 10X PCR buffer (Bioline), 1 µL of 10 mM dNTPs mixture, 2 µL of each 10 µM primer, 3 µL of 50 mM $MgCl_2$ (final concentration 2.5 mM), 0.5 U (0.1 µL) of BIOTaq DNA polymerase (Bioline, Ohio, USA) and 6 µL of DNA extract. The amplification reaction comprised an initial denaturation step at 94°C for 5 min followed by 30 amplification cycles. Each of these cycles included a denaturation step at 94°C for 30 s, an annealing step at 58°C for 30 s and an extension step at 72°C for 1 min. A final extension was performed at 72°C for 10 min.

PCR products were resolved on 13% polyacrylamide gel, that was stained with ethidium bromide and visualised under UV illumination. Molecular weight markers (Hyperladders 25 bp and 100 bp [Bioline]) were used to estimate the size of different fragments obtained.

Purification, sequencing and identification of ITS1 fragments

Amplified PCR products of samples that showed a single band on the polyacrylamide gel were cleaned up with Exonuclease I and Shrimp Alkaline Phosphatase (Exo-SAP, New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocol. These products were sent for Sanger sequencing with primers in both directions. The sequencing was performed by a commercial company (Microsynth Seqlab, Germany). After sequencing, the forward and reverse sequences for each sample were corrected using Chromas software Version 2.6.6 (https://technelysium.com.au/wp/chromas/) and aligned to obtain consensus sequences using BioEdit V7.1.9 software (Hall, 1999). Clean sequences were subjected to nucleotide blast search (Blastn) in the GenBank nucleotide collection (nr) database (https://blast.ncbi.nlm.nih.gov/) for comparison with sequences pre-existing in this database and species attributed to each individual fly was the one presenting high similarity (Identity > 98%, E-value <<< 0.001) with the query sequence submitted.

Phylogeny analysis

All sequences were aligned in BioEdit (Hall, 1999) using the ClustalW algorithm for phylogenetic analyses. The evolutionary history was inferred in MEGA 11 software (Tamura et al., 2021) using the neighbour joining method (Saitou & Nei, 1987) based on the Jukes-Cantor model. The optimal tree was computed and bootstrap values supporting the nodes were computed as the percentage of trees in which associated taxa clustered together. ITS1 sequences of *G. tachinoides* (EU591936.1), *G. palpalis palpalis* (FJ767881.1), *G. palpalis gambiensis* (EU591931.1) and *G. fuscipes fuscipes* (HQ387131.1) were downloaded and used as reference in the tree construction.

RESULTS

Tsetse fly species identified using morphological characters

Overall, 660 tsetse flies (Table 1) used in this study belonged to eight different putative species/subspecies according to their morphological characteristics (Figure 2).

Identification of tsetse species using PCR-based method

Among the 660 tsetse adults that were collected and identified morphologically, ITS-1 fragment of 649 tsetse flies was successfully amplified. Length polymorphism of these fragments revealed a total of six DNA profiles (Figure 3). Five of these profiles had a single band while one had two bands. The sequencing of 43 amplicons representing the five profiles with a single band confirmed the polymorphism obtained on the gel, with sizes ranging from 149 to 247 base pairs (Table 2).

Molecular identification of *G. palpalis palpalis*

All the 100 *G. palpalis palpalis* collected in Campo displayed the profile A (latter attributed to the subspecies), and the sequencing of one individual gave an exact size of 247 bp which was 100% similar to *G. palpalis palpalis* sequences in GenBank (accession number FJ767881.1 for example). Also, 12 *G. palpalis palpalis* from Bipindi shown the same profile A, but with 246 bp after sequencing of three individuals and 99.59% of similarity.

TABLE 1 Tsetse species identified based on their morphological features.

Tsetse fly species	Study sites (ecological niche)	Number of individuals
Glossina fusca congolensis	Dodéo (Forest gallery)	8
G. morsitans submorsitans	Sora-Mboum (Savannah grassland)	28
	Dodéo (Forest gallery)	14
G. fuscipes fuscipes	Mbakaou (Forest gallery)	7
	Doumé (Forest area)	54
	Dodéo (Forest gallery)	1
G. nigrofusca nigrofusca	Campo Forest area	12
G. pallicera pallicera	Campo (Forest area)	20
	Bipindi (Forest area)	1
G. palpalis palpalis	Bipindi (Forest area)	12
	Campo (Forest area)	100
G. caliginea	Campo (Forest area)	22
G. tachinoides	Sora-Mboum (Savannah grassland)	156
	Dodéo (Forest gallery)	184
	Mouhoun (Forest gallery)	41
Total	/	660

Molecular identification of *G. tachinoides*

Of the 156 *G. tachinoides* from Sora-Mboum, 125 displayed the profile B (attributed to *G. tachinoides*) on electrophoresis gel and 31, two bands products. The sequencing of four amplicons of the profile B gave as exact sequence size of 222 bp and sequences were 99.55% similar to *G. tachinoides* (accession number EU591936.1 for example) in GenBank (Table 2).

All 184 identified as *G. tachinoides* in Dodeo displayed the profile A (of *G. palpalis palpalis*) on the gel. Six of these amplicons sequenced showed sequences of 246 bp that were highly similar (100% identity) to that of *G. palpalis palpalis* in GenBank, instead of *G. tachinoides* (85% identity).

Among the 41 *G. tachinoides* from the Mouhoun area in Burkina Faso, 36 displayed the profile B, and five displayed profile (F) on the electrophoresis gel. The sequencing of two amplicons of the profile B confirmed they were *G. tachinoides* with 221 bp and 100% similarity to *G. tachinoides* sequences in GenBank, while the sequence of the profile F amplicon was of 167 bp and identical (100%) to *G. palpalis gambiensis* sequences present in GenBank (accession number EU591931.1 for example).

Molecular identification Glossina morsitans submorsitans

Of the 28 *G. morsitans submorsitans* from Sora-mboum, 13 showed ITS1 PCR products of profile B (*G. tachinoides*) while 15 showed the two-band profile as already observed for some *G. tachinoides* from the same locality. The sequencing of five samples matching with profile B provided sequences of 222 and 221 bp, with 99.55% and 98.65% identity with *G. tachinoides* ITS1 sequences present in GenBank, respectively (accession number EU591936.1 for example).

All 14 tsetse flies identified as *G. morsitans submorsitans* in Dodéo showed ITS1 products of profile A on electrophoresis gel. The sequencing of four of these PCR products generated sequences having 246 bp that had 100% identity with those of *G. palpalis palpalis* found in GenBank (accession number FJ767881.1 for example).

Molecular identification of *Glossina fuscipes fuscipes*

All the 54 *G. fuscipes fuscipes* from Doumé displayed the profile C (attributed to *G. fuscipes fuscipes*) and the sequencing results revealed a size of 236 or 237 bp, and a 100% similarity with *G. fuscipes fuscipes* sequences present in GenBank (accession number HQ387131.1 for example). These results confirmed those of the morphological identification.

FIGURE 2 Routine morphological characteristics used for fast identification of some tsetse fly species (a, dorsal whitish circular spot used for *Glossina caliginea*; b, funnel shape used for *G. palpalis palpalis* and *G. tachinoides*, but also present on *G. palpalis gambiensis* and *G fuscipes*; c, yellow colour used for *G. pallicera*; d, black colour of last tarsal segments of hind limbs used to distinguish *G. morsitans submorsitans* from the other sympatric species with all black tarsal segments (e)).

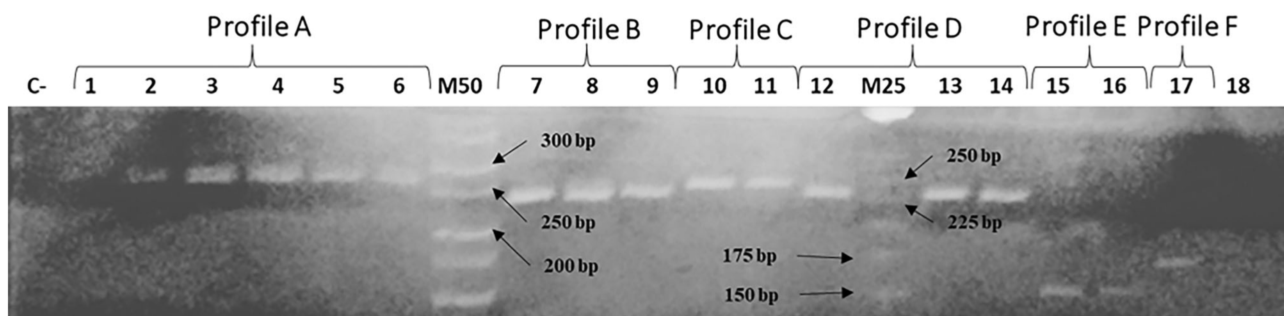

FIGURE 3 Polyacrylamide gel showing DNA fragments resulting from the amplification of ITS1 fragment of flies belonging to different tsetse species (*Glossina palpalis palpalis*: 1–6, *G. tachinoides*: 7–9; *G. fuscipes fuscipes*: 10 and 11, *G. pallicera*: 12–14, *G. caliginea*: 15 and 16, *G. palpalis gambiensis*: 17; M50 and M25 are 50 bp lanes and 25 bp lanes molecular weight markers, respectively).

All the seven tsetse flies identified as *G. fuscipes fuscipes* from Mbakaou and one from Dodéo displayed the profile A, and after sequencing, those ITS1 fragments were 246 bp, expected size of *G. palpalis palpalis* with which they presented 100% similarity. All these height specimens were thus misidentified in the field.

Molecular identification of *Glossina caliginea*

The amplicons of ITS1 region of 22 *G. caliginea* individuals from Campo showed a single profile D (attributed to *G. caliginea*) on the gel. The sequencing showed the exact size of 149 bp and 99.33% similarity with sequences of *G. caliginea* present in GenBank.

TABLE 2 Concordance/discrepancies between morphologic and molecular identification of tsetse flies analysed.

Glossina taxa identified morphologically	Number of flies analysed	ITS1 amplicon profiles	Number of flies per profile	Number sequenced per profile	Sequence size (bp)	Molecular taxon assigned[a]
G. palpalis palpalis (Bipindi)	12	A	12	3	246	*G. palpalis palpalis*
G. palpalis palpalis (Campo)	100	A	100	1	247	*G. palpalis palpalis*
G. tachinoides (Sora-Mboum)	156	B	125	4	222	*G. tachinoides*
		G (two bands)	31	0	/	**NI**
G. tachinoides (Dodéo)	184	A	184	6	246	***G. palpalis palpalis***
G. tachinoides (Burkina Faso)	41	B	36	2	221	*G. tachinoides*
		F	5	1	167	***G. palpalis gambiensis***
G. morsitans submorsitans (Sora-Mboum)	28	B	13	5	221 and 222	***G. tachinoides***
		G (two bands)	15	0	/	NI
G. morsitans submorsitans (Dodéo)	14	A	14	3	246	***G. palpalis palpalis***
G. fuscipes fuscipes (Mbakaou)	7	A	7	2	246	***G. palpalis palpalis***
G. fuscipes fuscipes (Doumé)	54	C	54	3	236 and 237	*G. fuscipes fuscipes*
G. fuscipes fuscipes (Dodéo)	1	A	1	1	246	***G. palpalis palpalis***
G. fusca congolensis (Dodéo)	8	NA	7	/	/	NI
		A	1	1	246	***G. palpalis palpalis***
G. nigrofusca (Campo)	12	B	8	2	228	***G. pallicera***
		NA	4	/	/	NI
G. pallicera (Campo)	20	B	20	3	228	*G. pallicera*
G. pallicera (Bipindi)	2	B	2	1	228	*G. pallicera*
G. caliginea (Campo)	22	E	22	4	149	*G. caliginea*

Abbreviations: bp, base pairs; NA, non-amplified; NI, not identified.
[a]Species that were misidentified with morphology are in bold.

Molecular identification of *Glossina pallicera*

All the 20 *G. pallicera* from Campo and 2 from Bipindi displayed the profile D (attributed to *G. pallicera*), which after sequencing displayed a size of 228 bp, and good similarity (99.56%) to *G. pallicera* in the GenBank, thus confirming the morphologic identification.

Molecular identification of *Glossina fusca congolensis*

Of the eight tsetse flies identified as *G. fusca congolensis*, seven did not show amplification products and one displayed the profile A. The sequencing of these amplicons showed an exact size of 246 bp that was 100% similar to sequences of *G. palpalis palpalis* found in GenBank.

Molecular identification of *Glossina nigrofusca*

Of the 12 *G. nigrofusca* species collected, 8 displayed the profile D (of *G. pallicera*) on electrophoresis gel while 4 did not amplify. Sequencing of two amplicons gave an exact size of 228 bp, and these sequences had 99.56% identity with those of *G. pallicera* from GenBank.

Phylogeny estimated using ITS1 sequences

Analyses performed on all the 43 sequences obtained from the tsetse flies of the palpalis group, plus four sequences downloaded from GenBank as reference showed that the sequences of each of the six tsetse species were highly uniform and formed a single haplotype, thus a total of six haplotypes for the palpalis group (Figure 4).

DISCUSSION

Although insecticide-based tools can act non-specifically on all tsetse species present in an area, environmental-friendly methods such as the SIT or paratransgenesis of microbiome are species-specific and require accurate identification of the tsetse population species to

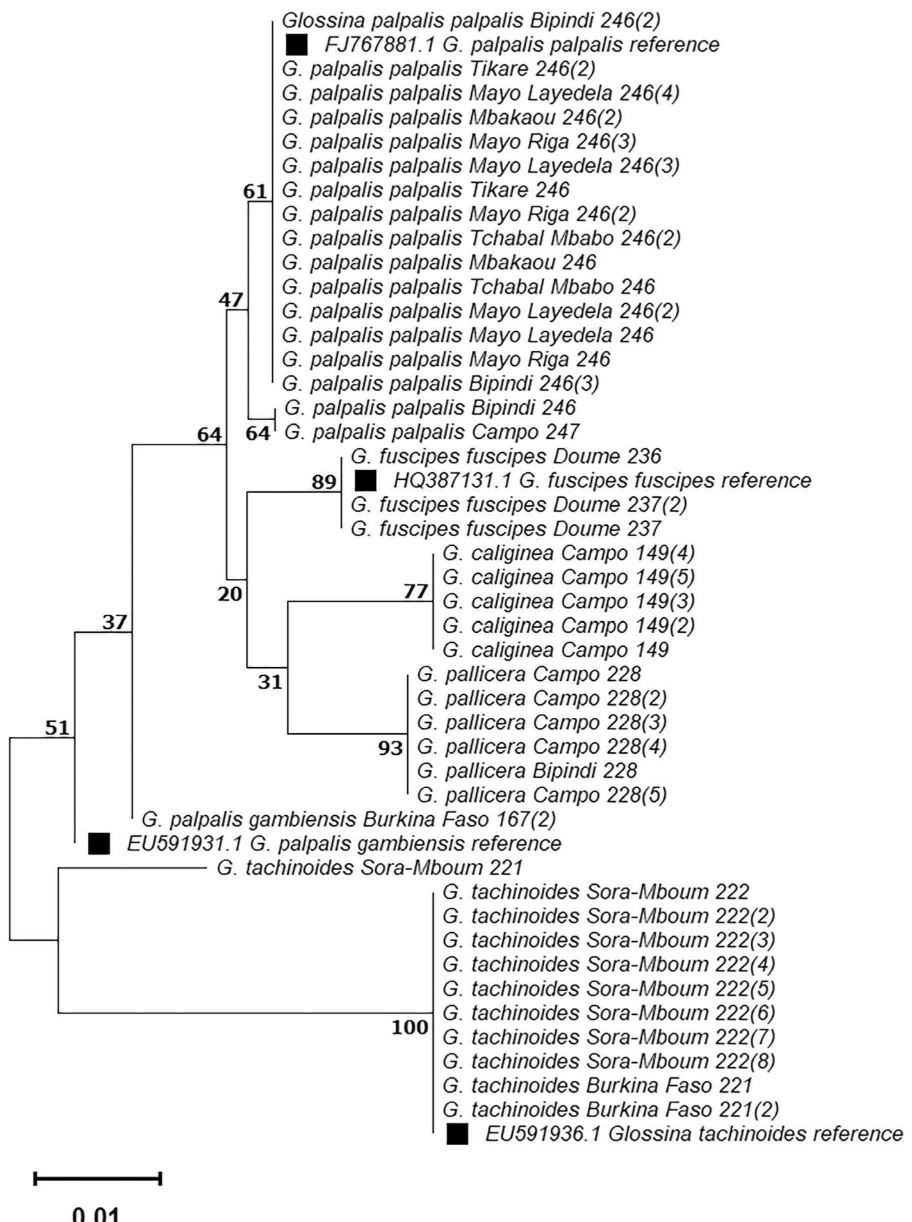

FIGURE 4 Neighbour-joining tree constructed with ITS1 sequences from *Glossina* sp. (samples with a black marker are references downloaded from GenBank, with accession numbers provided; Gpp: *Glossina palpalis palpalis*; Gpg: *G. palpalis gambiensis*; Gcal: *G: caliginea*; Gpal: *G. pallicera*; Gtac: *G. tachinoides*; Gff = *G. fuscipes fuscipes*).

target, and to ensure the species is not in sympatry with another related one in the targeted area.

Tsetse fly identification has mainly relied on morphological characteristics and old geographical records (maps). In this study, morphological characteristics allowed us to distinguish most of tsetse fly taxa captured in different ecological areas in Cameroon, confirming the presence of *Glossina palpalis palpalis*, *G. pallicera*, *G. caliginea* and *G. nigrofusca* in the South Region of the country (Farikou et al., 2010; Grébaut et al., 2016; Melachio Tanekou et al., 2022; Tchouomene-Labou et al., 2013). Moreover, *G. fuscipes fuscipes* was identified in the East Region as previously reported (Mbida Mbida et al., 2009) and *G. tachinoides* and *G. morsitans submorsitans* in the Adamawa Region of Cameroon as reported in 2018 (Kame-Ngasse et al., 2018; Shaida et al., 2018). However, some tsetse specimens that were morphologically identified as *G. tachinoides*, *G. morsitans submorsitans* or *G. fuscipes fuscipes* had ITS1 fragments with sizes not corresponding to those expected for these tsetse species. For instance, 31 of 156 tsetse flies identified as *G. tachinoides* in Sora-Mboum showed a profile with two bands that we suspect to represent *G. morsitans submorsitans*; 15 of 28 flies identified as *G. morsitans submorsitans* from the same area displayed the two-band profile for which we could not perform the Sanger sequencing and that could correspond to *G. morsitans submorsitans*, while the 13 others were confirmed *G. tachinoides* after sequencing. Many specimens morphologically identified as *G. tachinoides*,

G. morsitans submorsitans, or *G. fuscipes fuscipes* in the Adamaoua and North Regions were confirmed *G. palpalis palpalis* after sequencing of their ITS1 sequences. These results are consistent with previous studies that reported a misidentification of wild tsetse species (particularly, *G. palpalis palpalis*, *G. palpalis gambiensis*, *G. tachinoides* and *G. morsitans submorsitans*). They suggest that the morphological characters alone are not sufficient for accurate identification of tsetse fly species (Augustinos et al., 2018; Dyer et al., 2008). The ITS1 length polymorphism detected on a polyacrylamide gel was enough to differentiate the species studied. This tool is cost-effective as there is no need of sequencing and can be cheaper and more friendly with band separation done on agarose gels since the gap between the two closest fragments was up to 14 bp.

Our study also suggests revising tsetse distribution maps, as the one currently used in Cameroon was established in 1953. Indeed, in the northern part of Cameroon, *G. palpalis palpalis* was the only species captured in some areas like in Mbakaou, where *G. fuscipes fuscipes* was originally reported (Rageau & Adam, 1953). Moreover, in other areas, *G. palpalis palpalis* was also captured instead of *G. tachinoides* originally reported, in sympatry with *G. morsitans submorsitans*. These findings may be explained either by misidentification of these species using morphology many decades ago, or by the change in ecological conditions with intensification of cattle breeding and human presence that allowed the installation of the anthropophilic *G. palpalis palpalis*. Although the bigger size and brown colour of tsetse flies of the fusca group allowed us to clearly distinguish them from the other flies, no amplification products were obtained for them. This may be due to mutations in the primer binding sites that prevented their hybridization. Therefore, other sequencing-depending molecular markers such as COI, COII or ND2 may help to improve the identification of some tsetse species (Ngomtcho et al., 2017; Orji et al., 2015; Shaida et al., 2018).

CONCLUSION

Some biological tsetse control tools like the SIT and paratransgenesis are species-specific and require a precise identification of a targeted population to be efficient. Our study shows high rates of misidentification of closely related tsetse fly species on the field using morphological characters. The length polymorphism of the ITS1 region allowed to distinguish *Glossina palpalis palpalis*, *G. tachinoides* and *G. fuscipes fuscipes* and at a lesser extent *G. morsitans submorsitans*. These tsetse fly species are mostly present in sympatry in the northern Cameroon and are not easy to distinguish with routine morphologic characteristics. This tool allowed us to bring updates on the distribution of tsetse fly species in the area.

AUTHOR CONTRIBUTIONS

Steve Feudjio Soffack: Investigation; writing – original draft; methodology; writing – review and editing; software; formal analysis; data curation. **Tito Tresor Melachio Tanekou:** Conceptualization; investigation; methodology; validation; visualization; writing – review and editing; software; data curation; formal analysis. **Oumarou Farikou:** Investigation; methodology; writing – review and editing; data curation. **Ginette Irma Kame Ngasse:** Investigation; writing – original draft; methodology; writing – review and editing; formal analysis; software; data curation. **Mureille Carole Tchami Mbagnia:** Investigation; methodology; writing – review and editing. **Murielle Wondji:** Investigation; methodology; formal analysis; resources. **Charles S. Wondji:** Funding acquisition; writing – review and editing; supervision; resources; validation; project administration. **Adly M. M. Abd-Alla:** Writing – review and editing; validation; methodology. **Anne Geiger:** Methodology; visualization; writing – review and editing. **Gustave Simo:** Conceptualization; validation; writing – review and editing; supervision. **Flobert Njiokou:** Conceptualization; funding acquisition; validation; visualization; writing – review and editing; supervision; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Most of the data that support the findings of this study are presented in this paper; other details are available from the corresponding author upon reasonable request. Representative ITS1 sequences of the tsetse fly species obtained in this study were deposited in GenBank under accession numbers OR600371 to OR600382.

ORCID

Tito Tresor Melachio Tanekou https://orcid.org/0000-0001-5847-758X

Ginette Irma Kame Ngasse https://orcid.org/0000-0003-1410-5156

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