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# Molecular identification of tsetse fly (Diptera: Glossinidae) species based on mitochondrial DNA (COII and CytB) sequences

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**Tsetse fly (Diptera: Glossinidae) anti-vector measures are reliant upon accurate identification of species and their subpopulations. Two species were studied, *Glossina palpalis palpalis* and *Glossina morsitans submorsitans* using two mitochondrial DNA: cytochrome oxidase subunit II (COII) and cytochrome b (CytB). Sequencing data were used to perform phylogenetic analysis of the two reared species together with other *Glossina* species' sequences from the DNA data base. For each gene, members of the same species group, *palpalis* or *morsitans* demonstrated a common ancestry and closer relatedness by belonging to one cluster. Within each species group members of the same species clustered together, an indication of common ancestry and relatedness too. Inspite of the few mixed clusters, the pattern produced in the phylogenetic trees can provide a good guide to support any other method of *Glossina* identification. It was recommended that evaluations be made to validate other genetic markers that can produce better resolutions to identify tsetse fly species using phylogenetic tree.**

**Key words:** Trypanosomiasis, *Glossina palpalis palpalis*, *Glossina morsitans submorsitans*, cytochrome oxidase II, cytochrome b, neighbour joining tree.

## INTRODUCTION

Tsetse flies (Diptera: Glossinidae) are the vectors of trypanosomes, the causative agents of 'sleeping sickness' or human African trypanosomosis (HAT) in humans and 'nagana' or African animal trypanosomosis

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(AAT) in livestock in Sub-saharan Africa. Many consider HAT as one of the major neglected tropical diseases and AAT as the single greatest health constraint to increased livestock production (Vreysen et al., 2013). The tsetse flies belong to the order Diptera, family Glossinidae, and genus *Glossina* (Leak, 1999). *Glossina* ('tongue fly', in reference to the prominent proboscis) species are arranged in three subgenera: *Austenina*, *Nemorhina*, and *Glossina* that correspond roughly with group of species found in different ecological settings. The subgenera often are cited by their group names: the *fusca* group (Austenina), the *palpalis* group (*Nemorhina*), and the *morsitans* group (*Glossina*) (McAlpine, 1969; Potts, 1973; Gouteux, 1987). The three subgenera or species groups consist of 33 currently recognized species and subspecies (Gooding and Krafsur, 2005). The major human and animal disease vectors are members of the *palpalis* a riverine, and *morsitans*, a savannah species group (Aksoy et al., 2001). The *fusca* species group are predominantly inhabitants of tropical forests and, as such, rarely come into contact with and feed on domestic animals and humans but they certainly contribute to the maintenance of the reservoir of infection in wild animals (Jordan, 1988; Abila et al., 2008).

Trypanosomiasis is caused by the flagellated protozoa of the genus *Trypanosoma.* Variability in vector competence of AAT or HAT depends on the species of the vector as well as the trypanosomes (Geiger et al., 2005). Economic losses in cattle production are estimated at US $1 to 1.2 billion and total agricultural losses caused by AAT are estimated at US$ 4 to 4.75 billion per year (Geiger et al., 2005). It has been predicted that an area of Africa larger than Europe will remain infested and under the threat of trypanosomiasis for the foreseeable future (Geiger et al., 2005). Area-wide integrated pest management (AW-IPM) defined as the integrated use of control tactics against an entire tsetse population within a delineated area (Klassen, 2005) is a highly recommended approach to create tsetse-free zones in Africa. The less controversial interventions under AW-IPM targets parasite and vector controls notably application of the sterile insect technique (SIT) against the vectors (Feldmann and Hendrichs, 2002). Both the parasite and vector control interventions of AW-IPM thus involves genetic control among other strategies. Genetic control aims to alter the reproductive potential of a vector (or parasite) or its vectorial competence such as the sterile insect technique (SIT) (De Deken, www.afrivip.org/sites/default/files/10_tsetse_references.pdf). AW-IPM usually includes genetic analysis to determine the degree of isolation of the populations of any of the vectors species (Vreysen et al., 2013).

Molecular marker applications are being used to differentiate between tsetse fly species, their subpopulations and their evolutionary relationships. A study of genetic relationships of 13 species of the genus *Glossina* inferred from mitochondrial [cytochrome oxidase 1 (CO1), NADH dehydrogenase 2 (ND 2) and 16S] and nuclear (internal transcribed spacer 1 of rDNA, that is, ITS 1] sequences was conducted and reported by Dyer et al., (2008). Abila et al., (2008) studied levels of genetic differentiation between Ugandan *Glossina fuscipes fuscipes* populations based on COII and CytB, hence the markers are expected to be useful in species identification. An assessment of the possibility of applying sequence analysis of the region coding for CytB as a method of species identification in the field of forensic science was reported by Branicki et al. (2003). DNA originating from individuals in major phyla of vertebrates revealed that the technique is a very sensitive and reliable method for species identification (for vertebrates) and confirms that analysis can be carried out even when there is no reference sample, and the sequences obtained can be assessed through analysis of their similarity to cytochrome b sequences present in the DNA databases. Several genetic markers from mitochondrial DNA [cytochrome oxidase gene (COI, COII, 12S mtDNA)] and nuclear ribosomal DNA (16S rRNA, 28S rRNA) have been used in identification, population genetics and evolutionary studies of different families of myiasis-causing (maggot infestation) flies (Otranto and Stevens, 2002).

Conventionally, tsetse fly species identification is by the use of morphological characteristics in the form of identification keys (Buxton, 1955; Gouteux, 1987; FAO, 1992). This classical species identification relies on minor morphological differences, often challenging for field workers. Other systems of tsetse fly identification have been developed in the last few decades. Wing morphometrics can distinguish between species and subpopulations of tsetse flies and reportedly has the advantage of simplicity of data acquisition and low cost (Rohlf and Marcus, 1993; Solano et al., 1999; Patterson and Schofield, 2005; Camara et al., 2006; Leak et al., 2008; Getahun et al, 2014). For cuticular hydrocarbons identification method, the examination of the potentially stimulating methylalkanes [(via gas chromatography (GC) patterns)] provide reasons for the reproductive isolation of closely related species from each other (Sutton and Carlson, 1997; Getahun et al, 2014) and is used in classification and population studies. While, the phylogenetics method is expected to give greater accuracy when fully developed, a combination of identification methods is expected to give more accurate identification than the single methods. It was proposed that in control programs that involve Sterile Insect Technique (SIT) as suggested for a *G. f. fuscipes* SIT program in Ethiopia, morphological classification alone is not used to classify such populations (Dyer et al., 2011).

In this study mitochondrial DNA (mtDNA) sequence data (COII and CytB) from laboratory reared species were used to determine the sequence relationship between laboratory reared *Glossina palpalis palpalis* and *Glossina morsitans submorsitans* together with sequences

of other tsetse fly species obtained from GenBank databases, to evaluate the possibility of using the phylogenetic relationship to identify and characterize the species and eventually provide information to support Area Wide-Integrated Pest Management (AW-IPM). Previous studies have used different genetic markers to demonstrate that the phylogenetic relationship between the three species groups *palpalis*, *morsitans* and *fusca* are in concordance with their distinct morphological classification and to show genetic differences (and similarities) between different geographical populations of individual species (Patterson and Schofield, 2005; Dyer et al., 2008; Abila et al., 2008; Dyer et al., 2011). The research questions to be answered in this study are: (a) with the COII sequences from the sample *G. palpalis palpalis* and *G. morsitans submorsitans* and their homologous species sequences from the GenBank, can we correctly identify the two species using a phylogenetic tree? and (b) can we do the same based on CytB sequences?

## MATERIALS AND METHODS

### Laboratory reared tsetse flies used in the study

The two tsetse fly species studied include *G. p. palpalis* and *G. m. submorsitans* which are among the four species of economic importance out of 11 species in Nigeria (FAO, 1992; Leak, 1999). The tsetse flies were collected from the laboratory colony maintained at the Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Reseach (NITR), Kaduna, Nigeria. NITR was established in 1947 as West African Institute for Trypanosomiasis Research (WAITR) (NITR, 2008). However, the increasing need of tsetse flies for research and sterile insect control (SIT) requiring mass dispersal of sterile males necessitated their laboratory rearing. The foundation stock for establishment of a new colony is with the pupae. The *G. palpalis palpalis* colony originated from a waterway in Ija Gwari National Park in Suleija, Niger State, a suburb of Abuja, Nigeria's capital. Evidence of pupae collections from Suleija area was reported by Abubakar et al. (2010). Tsetse flies from NITR colony were used in a SIT project in Nigeria. Pupae from NITR colony of *G. palpalis palpalis* was used for mass-rearing of the species at the International Atomic Energy Agency (IAEA) in Seibersdorf, Austria for the Biological Control (BICOT) project in Nigeria. While, the project office was in Vom, Plateau State, the fly control site was in Lafia Local Government Area, Nassarawa State, Nigeria (Oluwafemi, 2008). IAEA laboratory supplied *G. palpalis palpalis* material to the project through weekly air shipments to Kano, Nigeria in 1988 to 1990. The IAEA colony was continued at a small size until 2009 when it was transferred to Centre International La recherché agronomique pour le développement (CIRAD) insectary in Montpellier, France, while IAEA continued rearing the related species *G. palpalis gambiensis*. For the other laboratory reared species in this study, *G. morsitans submorsitans*, the foundation pupae came from Centre International de Recherche-Développement sur l'Elevage en zone Subhumide (CIRDES), Bobo Dioulasso, Burkina Faso that was set up in 1972. The *G. p. palpalis* of the CIRDES colony originated with pupae that was collected from Samorogouan about 200 km to Bobo Dioulasso in 1980 to 1981. It was brought to NITR in July, 2010.

Tsetse flies depend on warm, vertebrate, antibiotic-free blood as their sole food. Traditionally, tsetse flies are made to feed on live animals (Nash et al., 1968). At NITR the flies in a production cage were allowed to feed on the blood of a restrained rabbit or goat. An improved system is where the tsetse flies are fed through membranes resembling host skin made of silicone rubber or of agar and parafilm, overlying blood pools poured onto grooved glass plates (Bauer and Wetzel, 1976). The blood is usually collected from abattoirs. This system which is more acceptable to animal welfare groups and more practical (Feldmann and Hendrichs, 2002) is not practiced at NITR. Tsetse fly rearing is simplified in the laboratory because only two developmental stages need to be considered for management - the adult and pupal stages.Tsetse fly reproduction is viviparous as the female gives birth to live offspring. The larva is nourished within the mother and larviposited at an advanced stage of development. The average female lifespan is 100 to 120 days with an average of about 4 pupae per female under laboratory conditions which is less than the optimal yield under natural conditions. A total of 24 teneral tsetse flies were collected from the laboratory, 12 flies for each specie with equal number of males and females, although sex was not considerd in the identification. Sample collection and laboratory analysis were done between May and June, 2012. Tsetse flies were preserved in 95% ethanol and stored in a freezer following collection before use based on a procedure described for sample preservation for DNA extraction for insects (Schauff, 1986).

### Molecular methods

DNA extraction and PCR was done at the Molecular Biology Laboratory of the National Veterinary Research Institute (NVRI), Vom, Jos, Plateau State, Nigeria. Each whole body of the 12 *G. p. palpalis* and 12 *G. m. submorsitans* was used for DNA extraction based on the recommendation that whole body should be used for mitochondrial DNA and only legs for microsatellites analysis (Leak et al., 2008). Homogenate was obtained by grinding a whole fly body in a mortar with pestle, and 1000 µl of phosphate buffer saline (PBS) was added to make a solution. Extraction of genomic DNA was performed using the Zymo Research (ZR) Tissue and Insect DNA MicroPrep$^{TM}$ (Zymo Research Corp. USA) based on manufacturer's instructions. PCR Amplification was initiated for the 400 bp region of the mtDNA cytochrome oxidase II (COII) using the forward and reverse universal invertebrate primer pairs mtD13:COIIF–5'AATATGGCAGATTAGTGCA3' and mtD15: COIIR – 5'TCATAAGTTCARTATCATTG3'. Similarly, amplification was initiated for the 500 bp region of the cytochrome b (CytB) gene using the forward and reverse universal invertebrate primer pairs mtD26: CytB2F – 5'TATGTACTACCATGAGGACAAATATC3' and mtD28: CytB2R– 5'ATTACACCTCCTAATTTATTAGGAAT3' (Simon et al., 1994). Polymerase chain reactions (PCR) were performed in a 25 µl reaction mixtures containing 1 µl of template DNA, 2.5 µl 10X PCR buffer, 0.8 mM dNTP, 2 mM $MgCl_2$, 0.4 µM of each primer, 1 µl of BSA (Bovine Serum Albumin) and 1 unit of AmpliTaq Gold (Applied Biosystems). Gene Amp PCR$^{R}$ System 9700 was used for the amplification reactions. Thermal cycler conditions consisted of an initial denaturation step at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1 min. Reactions were terminated with a final extension at 72°C for 5 min (Abila et al., 2008). PCR products were analyzed by electrophoresis on 2% agarose gel and visualized under ultra violet light.

From the 12 tsetse flies for each species, amplified DNA samples from the PCR products were used for nucleotide sequencing. A total of 44 amplicons were thus used for sequencing. For *G. p. palpalis,* they comprised of 24 amplicons: 12 amplicons for COII and 12 amplicons for CytB, hence all the 12 samples of each gene amplified. For *G. m. submorsitans* they comprised of 20 amplicons: 10 amplicons for COII and 10 amplicons for CytB, hence there was no amplification for 2 DNA samples for COII and CytB, respectively. Sequencing of the 44 amplicons resulted in 88 reactions as

**Table 1.** BLAST result of G*lossina palpalis palpalis* COII.

| Sample | Base pairs | BLAST result | | | |
|---|---|---|---|---|---|
| | | Matching species | Gene | % Maximum identity | Accession number |
| P1a-NITR | 341 | *Glossina f. fuscipes* hap 16 | COII | 93 | EU559620 |
| P3a-NITR | 284 | *Glossina f. fuscipes hap 7* | COII | 94 | GU296752 |
| P8a-NITR | 345 | *Glossina f. fuscipes hap 16* | COII | 93 | EU 559620 |
| P9a-NITR | 350 | *Glossina f. fuscipes hap 16* | COII | 94 | EU559620 |
| P10a-NITR | 345 | *Glossina f. fuscipes hap 7* | COII | 93 | GU296752 |

**Table 2.** BLAST result of *Glossina palpalis palpalis* COII.

| Sample | Base pairs | BLAST Result | | | |
|---|---|---|---|---|---|
| | | Matching species | Gene | % Maximum Identity | Accession Number |
| P1b-NITR | 375 | *Glossina morsitans* | CytB | 91 | KC177594 |
| P2b-NITR | 224 | *Glossina f. fuscipes hap 20* | CytB | 93 | EU562281 |
| P4b-NTR | 200 | *Glossina f. fuscipes hap 20* | CytB | 93 | EU562281 |
| P5b-NITR | 277 | *Glossina morsitans* | CytB | 95 | KC177594 |
| P6b-NITR | 268 | *Glossina f. fuscipes hap 16* | CytB | 92 | EU562277 |
| P8b-NITR | 374 | *Glossina morsitans* | CytB | 91 | KC177594 |
| P9b-NITR | 379 | *Glossina morsitans* | CytB | 90 | KC177594 |
| P10b-NITR | 426 | *Glossina morsitans* | CytB | 89 | KC177594 |

sequencing was done in the forward and reverse directions to minimize errors. Sequencing was performed by Macrogen USA, Rockville, Maryland using ABI 3730XL (Applied Biosystems) automated sequencer following standard manufacturer's protocols.

**BLAST and phylogenetic analysis of mtDNA sequences for relationship between the species**

Only sequences that yielded greater than 200 bp were selected for basic local alignment search tool (BLAST) and to generate the neighbour joining trees. All the forward sequences were removed from the analysis as they did not meet this criterion. All the sequences that met this criterion fell within the reverse (minus) sequences but they were converted to forward (plus) sequences using a converter programme, Reverse Complement (The Bioinformatics Organization Inc., 2000). Among them, duplicate sequences with 100% homology were removed before BLAST and phylogenetic analysis. Such duplicate homologous sequences were not submitted to the GenBank. BLAST was done using BLASTN 2.2.27 (Zhang et al., 2000) at the database of the National Centre for Biotechnology Information (NCBI) (http//www.ncbi.nlm.nih.gov/BLAST), that is, GenBank. The sequences were aligned with CLUSTAL W (Thompson et al., 1997) with default parameters and scrutinized and edited using MEGA version 4.1 (Tamura et al., 2007). Sequences from the database that best matched that of the samples were collected for phylogenetic analysis. The MEGA 4.1 Kimura-2 parameter model simultaneously calculates the genetic distances between pairs of sequences and constructs a neighbour joining tree (Saitou and Nei, 1987). The bootstrap test of phylogeny (Hillis and Bull, 1993) option of MEGA 4.1 was selected and used.

The sample and GenBank database sequences of the species were combined in MEGA 4.1 to generate neighbour joining tree separately for the two genes. The following numbers of the sequences were used for the trees: *G. p. palpalis* COII (5), *G. p. palpalis* CytB (8), *G. m. submorsitans* COII (3) *G. m. submorsitans* CytB (3). For the neighbour joining tree, COII sequences of *G. p. palpalis* (5) and *G. m. submorsitans* (3) were used together with COII of *G. f. fuscipes* (2) from the GenBank. This was followed by generating neighbour joining tree for CytB together for *G. p. palpalis* (8) and *G. m. submorsitans* (3), as well as *G. f. fuscipes* (2) and *G. morsitans* (1) from GenBank database. A similar combination of sample DNA sequences and database sequences was used to generate neighbour joining tree for the identification of blow flies (*Cordylobia anthropophaga*), another insect of medical, veterinary and forensic importance (Ogo et al., 2012). DnaSP version 5 was used to generate the haplotypes, an index of DNA polymorphism (Librado and Rozas, 2009).

## RESULTS

### BLAST of the sequences

Basic local alignment search tool (BLAST) of the DNA sequences of COII and CytB genes of the two species studied revealed only *Glossina* species related to *G. p. palpalis* and *G. m. submorsitans* with percentage identity ranging from 89 to 95% (Tables 1 to 4). No exact sequence of the two species for the two genes were revealed in the GenBank to be used for the phylogenetics identification of the sample species hence only the related species were compared. This also implies that this is the first time that nucleotide database deposits

**Table 3.** Blast result of *Glossina morsitans submorsitans* CytB.

| Sample | Base pairs | BLAST result | | | |
|---|---|---|---|---|---|
| | | Organism | Gene | % Maximum Identity | Accession number |
| M3b-NITR | 294 | *Glossina morsitans* | CytB | 95 | KC177594 |
| M5b-NITR | 417 | *Glossina morsitans* | CytB | 93 | KC177594 |
| M9b-NITR | 350 | *Glossina morsitans* | CytB | 93 | KC177594 |

**Table 4.** Blast result of*Glossina morsitans submorsitans* COII.

| Sample | Base pairs | BLAST result | | | |
|---|---|---|---|---|---|
| | | Matching species | Gene | % Maximum identity | Accession number |
| M1a-NITR | 339 | *Glossina f. fuscipes* hap 7 | COII | 94 | GU296752 |
| M5a-NITR | 336 | *Glossina f. fuscipes hap 7* | COII | 92 | GU296752 |
| M12a-NITR | 342 | *Glossina f. fuscipes hap 16* | COII | 93 | EU 559620 |

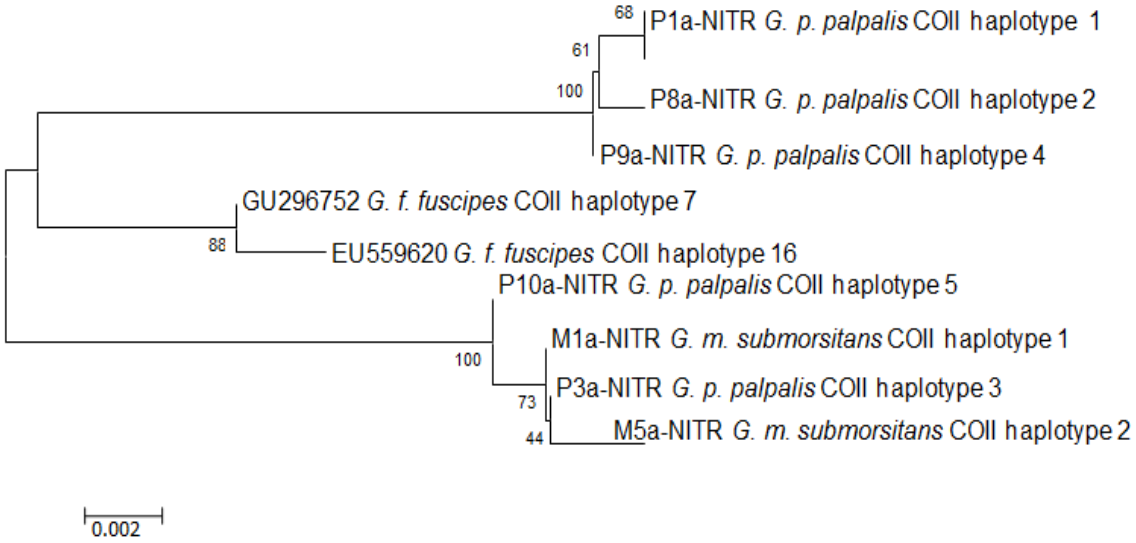


**Figure 1.** Neighbour joining tree based on sequences of three species' (COII) to identify *Glossina palpalis palpalis* and *Glossina morsitans submorsitans*. *G. p. palpalis* and *Glossina fuscipes fuscipes* (GU2967652 and EU559620) are species of the *palpalis* species group and showed a common ancestry while members of the *G. p. palpalis* and *G. f. fuscipes* species clustered according to their species. *G. m. submorsitans* showed a different ancestry from *palpalis* species group as it belongs to the *morsitans* species group. The scale bar indicates the number of nucleotide substitutions per site.

were made for these species sequences for COII and CytB.

## Identification of the species using neighbour joining tree

A neighbour joining tree involving four sequences from three species was generated for COII, the sequences are *G. p. palpalis* and *G. m. submorsitans* from this study along with *G. f. fuscipes* (GU296752) and *G. f. fuscipes* (EU559620) from GenBank as revealed from BLAST. The three species clustered differently with a few exceptions, that is, P10a-NITR and P3a-NITR which are *G. p. palpalis* species' group members that appeared in the *G. m. submorsitans* cluster (Figure 1). For CytB, the neighbour joining tree involved four species: *G. p. palpalis* and *G. m. submorsitans* from this study along with *G. f. fuscipes* (EU562277, EU562281) and *G. morsitans* (KC177594) from GenBank and the different

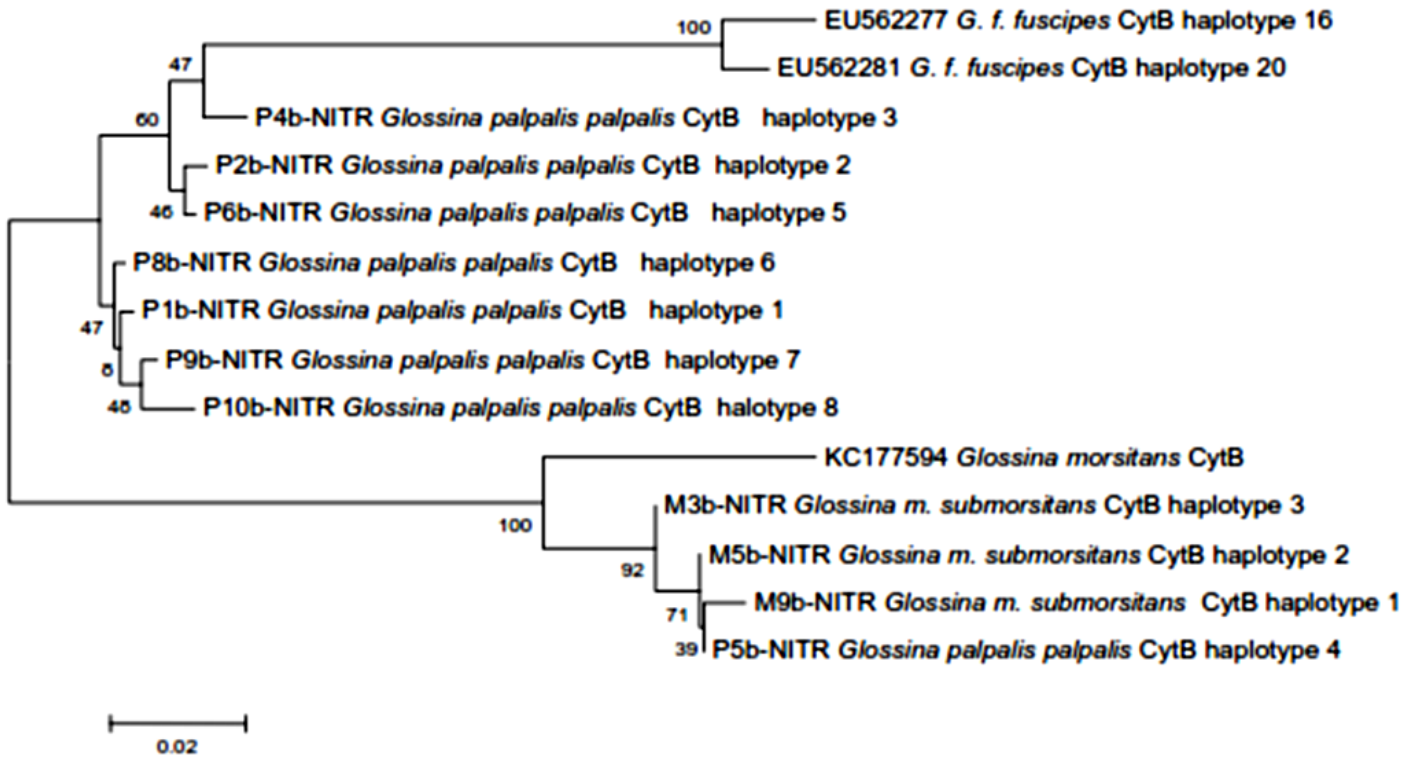


**Figure 2.** Neighbour joining tree based on sequences of four species' (CytB) *to identify G. p. palpalis* and *G. morsitans submorsitans*. *G. p. palpalis* CytB has a common ancestry with *G. f. fuscipes* CytB (EU562277 and EU562281) from GenBank as both are species of the *palpalis* species group. Members of each species also clustered more closely. *G. morsitans* (KC177594) from GenBank also showed a common ancestry with *G. m. submorsitans* as both are species of the *morsitans* species group. Members of each species also clustered more closely. The only exception was the P5b-NITR, a *palpalis* species member that clustered with the *G. m. submorsitans*. The scale bar indicates the number ofnucleotide persite.

species clustered differently with only one exception: P5b-NITR which is a *G. p. palpalis* species that appeared in the *G. m. submorsitans* cluster (Figure 2). It is interesting to note that *G. morsitans* (KC 177594) from GenBank clustered together with *G. m. submorsitans* both of which are species of the *morsitans* species group. The mtDNA sequences of the flies from this study were submitted at the National Centre for Biotechnology Information (NCBI) GenBank database (Accession Numbers *G. p. palpalis* COII KJ013516-20, *G. p. palpalis* CytB KJ013521-28,*G. m. submorsitans* COII KJ207383-5 and *G. m. submorsitans* CytB KJ207386-88) (Table 5). The observed haplotypes are also shown in Table 5.

## DISCUSSION

The objective of the study was to evaluate the utility of the two mitochondrial markers (COII and CytB) in identification of the two species by comparison with homologous database sequences in phylogenetic trees. This study is important because anti-vector measures are reliant upon accurate identification of vector species (Aksoy et al., 2001; Dyer et al., 20011). The BLAST results shown gave good values of percentage identity or homology between the sample species and the species revealed in the GenBank (85 to 95%) while the homology between the sample tsetse fly species for each gene gave 91 to 99%, thus confirming a greater homology within a species than between different species. The availability of homologous species seqences in database is crucial to this identification and characterization method to prove the species' similarity. A bootstrap value of 70% is considered significant evidence for phylogenetic grouping (Hillis and Bull, 1993) and the values obtained in the two neighbour joinng trees met this expectation (Figures 1 and 2). In the phylogenetic analysis, the general pattern observed is that the different species groups – *morsitans* and *palpalis* - clustered differently in the phylogenetic tree for each gene showing that each species group has a common ancestry and relatedness with 100% bootstrap values. Further, within each species group, members of each species clustered together thus demonstrating a closer ancestry than the species outside the cluster. For COII (Figure 1), *G. p. palpalis* clustered together with *G. f. fuscipes* both of which belong to the *palpalis* species group while *G. m. submorsitans* which belong to the *morsitans* species

**Table 5.** Accession Numbers of the mtDNA sequences.

| Isolate | Species | Gene | Haplotype | Accession number |
|---|---|---|---|---|
| P1a-NITR | *G.p. palpalis* | COII | Haplotype 1 | KJ013516 |
| P8a-NITR | | | Haplotype 2 | KJ013518 |
| P3a-NITR | | | Haplotype 3 | KJ013517 |
| P9a-NITR | | | Haplotype 4 | KJ013519 |
| P10a-NITR | | | Haplotype 5 | KJ013520 |
| P1b-NITR | *G. p. palpalis* | CytB | Haplotype 1 | KJ013521 |
| P2b-NITR | | | Haplotype 2 | KJ013522 |
| P4b-NITR | | | Haplotype 3 | KJ013523 |
| P5b-NITR | | | Haplotype 4 | KJ013524 |
| P6b-NITR | | | Haplotype 5 | KJ013525 |
| P8b-NITR | | | Haplotype 6 | KJ013526 |
| P9b-NITR | | | Haplotype 7 | KJ013527 |
| P10b-NITR | | | Haplotype 8 | KJ013528 |
| M1a-NITR | *G. m. submorsitans* | COII | Haplotype 1 | KJ207383 |
| M5a-NITR | | | Haplotype 2 | KJ207384 |
| M12a-NITR | | | Haplotype 3 | KJ207385 |
| M9b-NITR | *G. m. submorsitans* | CytB | Haplotype 1 | KJ207386 |
| M5b-NITR | | | Haplotype 2 | KJ207387 |
| M3b-NITR | | | Haplotype 3 | KJ207388 |

group clustered separately. Within the *palpalis* species group *G. f. fuscipes* species members formed a seperate cluster from the *G. p. palpalis* species. The pattern observed for COII was almost replicated for CytB (Figure 2) where *G. p. palpalis* demonstrated a common ancestry with *G. f. fuscipes* both of which are members of the *palpalis* species group. Within the *palpalis* species group cluster, members of *G. p. palpalis* and *G. f. fuscipes* species formed their own separate clusters. *Morsitans* species group members showed a common ancestry out of which *G. morsitans* (KC 177594) clustered separately from *G. morsitans submorsitans*. This result suggests that the analysis can be used for identification and differentiation of *Glossinas* species since any unknown sample species will cluster with known sequences from the database with respect to species and specie group and make identification possible.

In a study based on internal transcribed spacer 2 (ITS2) genetic marker and different species from the *palpalis*, *morsitans* and *fusca* species groups, comparative morphometric analysis of shape variation in the wings of different tsetse species had revealed close accordance with the phylogenetics of the species indicated by DNA sequences where the different *Glossina* species clustered according to their species groups (Patterson and Schofield, 2005). This is supportive of the results from this study. The resulting clusters from this study also agree with Dyer et al. (2008) that reported a phylogeny which confirms the monophyly (having common ancestry) of the morphologically defined *fusca*, *morsitans* and *palpalis* subgenera in a study that involved mitochondrial (cytochrome oxidase 1, NADH dehydrogenase 2 and 16S) and nuclear (internal transcribed spacer 1 of rDNA) sequences. Dyer et al. (2011) showed the relative power of different genetic markers to support the monophyly and aid characterization of different species and genetic groups of tsetse flies. The nuclear ribosomal internal transcribed spacer 1 (ITS1) provided support for the monophyly of each of the three tsetse fly species groups hence each group is commonly inherited. However, other nuclear and mitochondrial sequence data did not support the monophyly of the morphological subspecies *G. f. fuscipes* or *Glossina fuscipes quanzensis*.

Inspite of the few mixed clusters, the pattern produced in the phylogenetic trees in this study can provide a good guide to support any other method of *Glossina* identification. Further investigation is hereby recommended to include the use of other genetic markers such as mitochondrial cytochrome oxidase 1 (COI), nuclear internal transcribed spacer 1 of rDNA (ITS 1) or ITS 2 and to include controls. The utility of this phylogenetic method in *Glossina* species identification will increase as more deposits of the diferent economically important species and subpopulation are made in the databases to support identification. Though the phylogenetics method has been used for identification of other organisms such as the use of cytochrome b for vertebrates (Branicki et al., 2003) and some other mitochondrial and nuclear ribosomal DNA sequences for blow flies (Otranto and Stevens, 2002; Ogo et al., 2012),

the application of phylogenetics tree as a method for outright identification of tsetse flies has not been reported. It also remains to be established which markers will work best for tsetse flies identification.

The phylogenetic technique can be employed for identification and characterization of species in tsetse fly control programmes to support the conventional morphological technique that employs some standard identification keys, which may be inaccurate and time consuming even for a trained entomologist.

## Conclusion

Accurate characterization and identification of species and their subpopulations is important in the control strategies of tsetse flies. In this study, it was found that it is possible to use the clustering in the neighbour joining tree of the two mtDNA sequences of the species as a means of identification. Members of the same species tended to cluster together implying that they have a common ancestry and relatedness and this can be used for identification if an unknown species is involved. Similarly, species of the same species groups (*palpalis, morsitans*) clustered together, demonstrating their common ancestry and supporting the species' identity also. These findings have demonstrated the earlier species groupings that were based on morphological features. Inspite of the few mixed clusters, the pattern of the trees can be useful as a method of species characterization and identification while it is hoped that evaluation of other genetic markers will give improved results. Since the other tsetse fly identification methods have also associated challenges of accuracy, time, cost and complexity, they can be complemented with the phylogenetics approach for better results. This study will help to promote genetic study, surveillance and control of tsetse fly populations.

**Abbreviations: AAT,** Animal African trypanosomiasis; **BLAST,** Basic local alignment search tool; **COI,** cytochrome oxidase subunit I; **COII,** cytochrome oxidase subunit II; **CytB,** cytochrome b; **DnaSP,** DNA sequence polymorphism; **HAT,** human African trypanosomiasis; **ITS**, internal transcribed spacer; **MEGA,** molecular evolutionary genetic analysis; **mtDNA,** mitochondrial DNA; **NCBI,** national centre for biotechnology information; **NITR,** Nigeria institute for trypanosomiasis (and onchocerciasis) research; **rDNA,** ribosomal DNA; **SIT,** sterile insect technique; **16S,** the large subunit ribosomal RNA (sRNA); **AW-IPM,** area-wide integrated pest management; **PCR,** polymerase chain reactions; **BSA,** bovine serum albumin; **GC,** gas chromatography.

## Conflict of interests

The authors did not declare any conflict of interest.

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