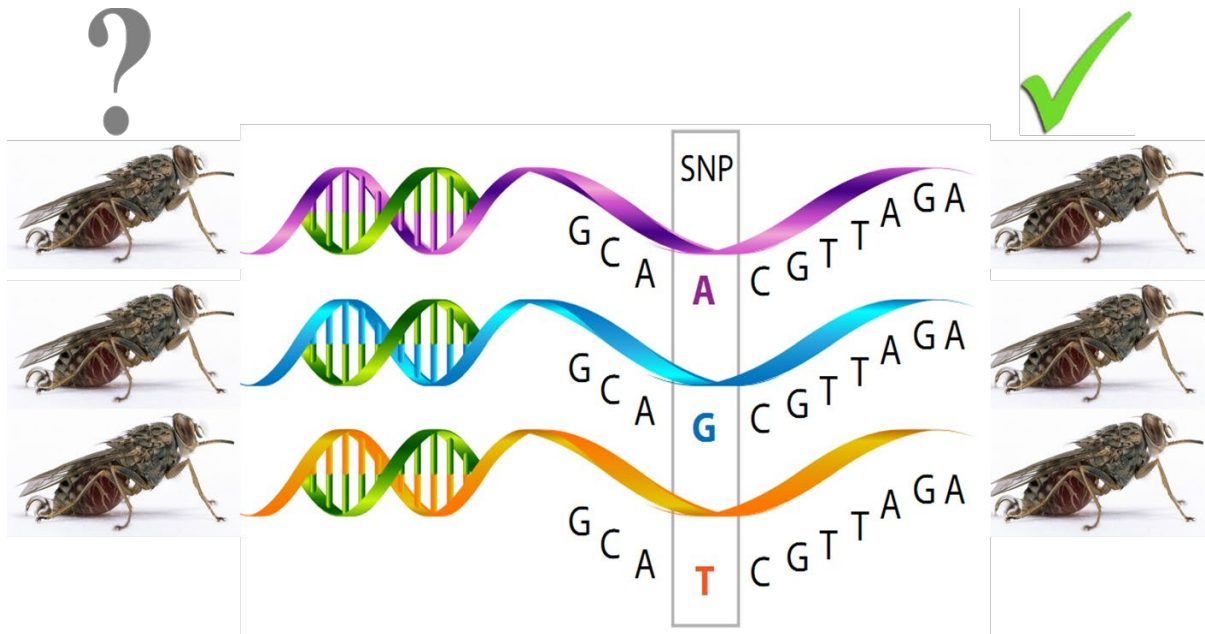


Standard Operating Procedures for Identification of Tsetse Species from Wild Populations and Laboratory Colonies

Version 1.0



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1. INTRODUCTION

Tsetse flies are solely responsible for the cyclical transmission of the trypanosomes that are the causative agents of sleeping sickness or human African trypanosomosis (HAT) and nagana or animal African trypanosomosis (AAT) in livestock. There are about 31 tsetse fly species and sub-species placed in the genus *Glossina* of the family Glossinidae. All are restricted to Sub-Saharan Africa, but only 8-10 of these species are of economic or human health importance. The genus *Glossina* is divided into three distinct taxonomic groups based on morphological characters such as external genitalia of the male flies, their habitat requirements and the preferred hosts.

There are several strategies that have been applied to suppress the vectors to manage trypanosomosis, which include the use of the sequential aerosol technique (SAT), stationary and mobile attractive devices, the live bait technique and the sterile insect technique (SIT) etc. The SIT involves the production of the target insect species in large numbers in rearing factories, and subsequently males are sterilized by irradiation. This is followed by the sustained and systematic release of the sterile males over the target area in numbers large enough to out-compete the wild male population for wild females. Matings of sterile males with virgin wild females result in no offspring, which will lead to a decrease of the population of the target species with each generation.

The application of the SIT requires the mass-rearing of the correct target species. Although in many instances the colony will be established from samples from the targeted area, in other cases they might be imported from a regional facility and this would require confirmation of the correct species status. The morphological identification of especially closely related species of tsetse species has been challenging. These standard operating procedures (SOP) has been developed to enable the quick and easy identification of tsetse species using molecular techniques, thereby facilitating the implementation of the SIT against selected tsetse species in Africa.

This document provides useful information to correctly identify specimens of nine tsetse flies species/subspecies (*Glossina brevipalpis*, *G. palpalis gambiensis*, *G. morsitans morsitans*, *G. m. centralis*, *G. m. submorsitans*, *G. pallidipes*, *G. fuscipes fuscipes*, *G. tachinoides* and *G. swynnertonii*) derived from field collections or laboratory colonies using molecular techniques. These SOP are intended for staff involved in tsetse rearing with sufficient training or experience in molecular biology techniques such as DNA extraction, the Polymerase Chain Reaction (PCR) method, and sequencing. This procedures manual was developed based on the publication Augustinos *et al.*, (in press).

2. SCHEME FOR A STEP BY STEP IDENTIFICATION OF *GLOSSINA* SPECIES

The procedure for the identification of tsetse species will depend on the starting material, and where the samples were collected, which can be:

- (i) live tsetse flies of an unknown species that need to be kept alive
- (ii) dead tsetse flies of an unknown species
- (iii) DNA samples from unknown tsetse species.

For the identification of live tsetse flies, information on the location of collection, the size and the morphology, including the genitalia of the flies, can help to identify several distinct species, i.e. *Glossina brevipalpis*, that are easily distinguished from other species.

For the identification of already extracted DNA of an unknown tsetse species, the DNA quality and concentration can be measured by spectrophotometer (to ensure that an equal quantity of the DNA is used during further processing).

As shown in **Figure 1** if there is a need to keep flies alive, DNA can be extracted from a single leg of a fly and can be used for further processing. Alternatively, DNA can be extracted from the whole body in case the flies do not need to be kept alive

The following steps should be followed for correct identification:

Step 1: Morphological identification of *G. brevipalpis* by naked eye due to its large size, independent of whether live or dead flies. In case of unknown tsetse DNA samples, proceed to step 2.

Step 2: Perform a PCR for nuclear marker ITS1 which has different size/length in some tsetse species. This will help identify the species *G. pallidipes* and *G. f. fuscipes*, as well as groups of other species as shown in **Figure 1**.

Step 3: Depending on the ITS1 band size corresponding to groups of tsetse species, perform a PCR for *Wolbachia* infection (16S rRNA) to distinguish *G. m. centralis* from *G. m. submorsitans*, and *G. m. morsitans* from *G. swynnertoni* and *G. brevipalpis* (in case of unknown DNA as starting material).

Step 4: Perform a PCR for microsatellite A10 to distinguish *G. p. gambiensis* from *G. tachinoides* and microsatellite Gmm14 to distinguish *G. swynnertoni* from *G. brevipalpis* (in case of unknown DNA as the starting material).

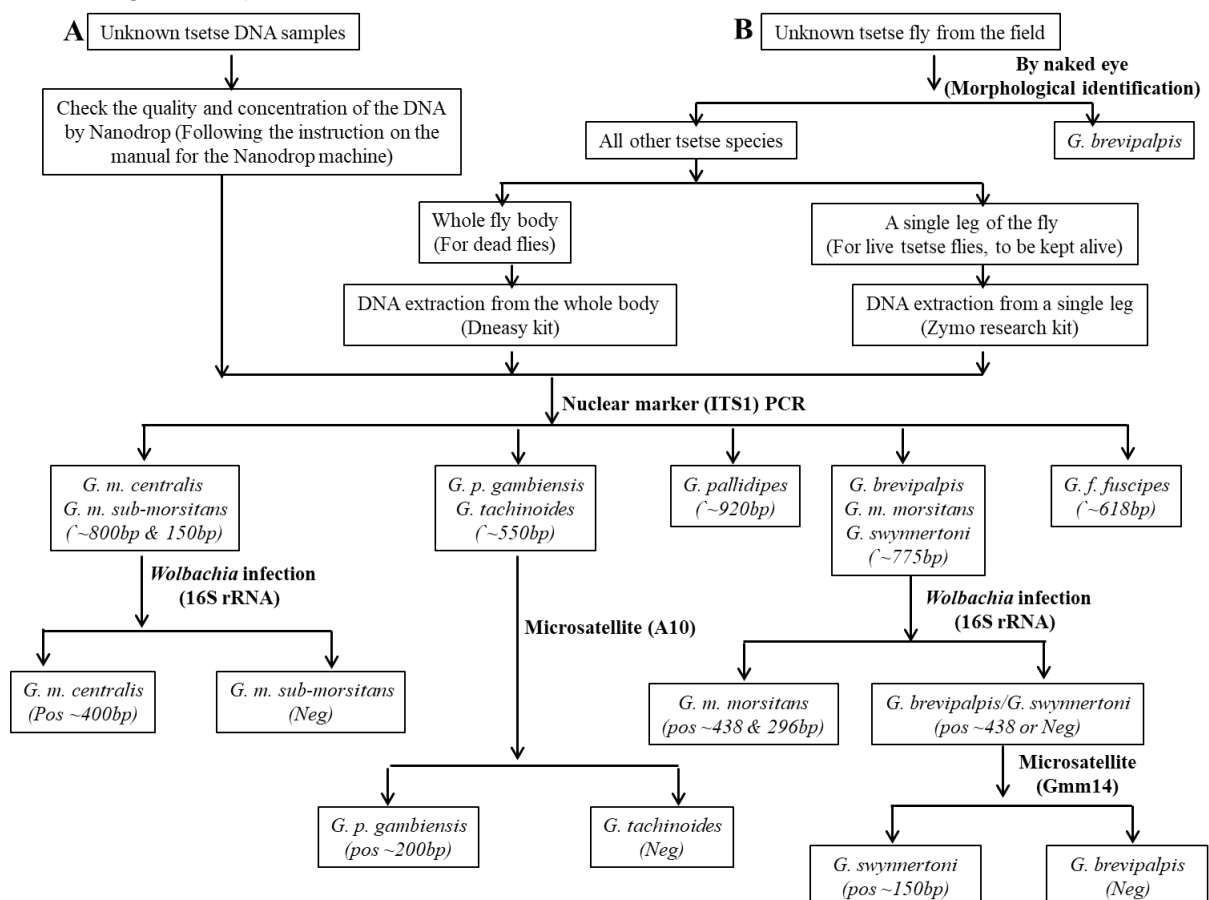


Figure 1. Schematic summary of step by step identification of tsetse species. The starting material can be either DNA samples of unknown tsetse species (A) or unknown tsetse fly specimens (B). Detailed information of the procedures and the expected results are explained below.

3. DNA EXTRACTION FROM TSETSE FLIES

3.1. From the whole body

3.1.1. Purpose

To isolate total DNA from tsetse flies for species identification.

3.1.2. Materials and equipment requirements

This procedure requires the following materials and equipment:

- 1) Fresh or conserved tsetse flies of unknown species preserved in alcohol
- 2) Microtubes
- 3) Microtube holders
- 4) Sterile micropestles
- 5) Liquid nitrogen (N₂)
- 6) Autoclave
- 7) Clean room
- 8) Air conditioning
- 9) Pipette (e.g. Pipetman set)
- 10) Pipette tips (e.g. Pipetman tips)
- 11) Water bath
- 12) Microcentrifuge
- 13) Refrigerator
- 14) Deep freezer
- 15) DNeasy kit
- 16) Spectrophotometer
- 17) Sterile distilled water
- 18) Gloves.

3.1.3. Required skills requirements

Research or laboratory technician with the required skills for molecular biology work, including DNA extraction.

3.1.4. Procedure

1. For live tsetse flies from colonies kept in the laboratory, select the flies randomly (preferably 10 days old) and proceed to step 3.
4. For tsetse flies conserved in ethanol (or other solutions for conserving specimens, e.g. propylene glycol), wash off the alcohol and replace it with distilled water, then wash off the water and dry the fly on tissue paper.
5. Put each individual fly in a separate microtube, label each tube with a serial number.
6. Insert one sterile micropestle in each microtube.
7. Add liquid nitrogen to the microtube to freeze the fly and the micropestle, and immediately grind the fly using the micropestle to obtain homogenized fly tissue as a fine powder.
8. Proceed with the DNA extraction using DNeasy® kit following the supplier instructions: http://mvz.berkeley.edu/egl/inserts/DNeasy_Blood_&_Tissue_Handbook.pdf

9. After eluting the DNA, quantify the DNA concentration using a Nano-drop spectrophotometer to ensure extraction of an equal DNA quantity from each fly (for more information see <http://www.nanodrop.com/nucleicacid.aspx>).
10. The DNA samples can be stored in a refrigerator until required for the next step.

3.2. From a single leg

3.2.1. Purpose

This method is designed to identify the species of live tsetse flies that need to be kept alive.

3.2.2. Scope

The following materials and equipment are needed.

1. Live tsetse flies
2. Microtubes
3. Sterile micropestles
4. Fine scissors
5. Benzene flame
6. Ethanol 70%
7. Ice box
8. Plastic Petri dishes
9. Alcohol proof marker
10. Fine forceps
11. Liquid nitrogen (N₂)
12. Autoclave
13. Clean room
14. Air conditioning
15. Pipette (e.g. Pipetman set)
16. Pipette tips (e.g. Pipetman tips)
17. Centrifuge
18. Refrigerator
19. Deep freezer
20. ZR-96 Quick-gDNA extraction kit
21. Nano-drop spectrophotometer
22. Gloves
23. Cylindrical plastic tubes (6 cm long with 4 cm in diameter, with netting from both sides) for holding the live fly.

3.2.3. Prerequisites

Research or laboratory technician with the required skills for molecular biology work including DNA extraction.

3.2.4. Required skills

Research or laboratory technician with the required skills for molecular biology work, including DNA extraction.

3.2.5. Procedure

1. Collect the unknown live tsetse flies.
2. Expose the flies to cold air at 4°C for 2-3 minutes until they become immobile; place the flies in plastic Petri-dishes on ice.
3. Sterilise fine forceps using 70% ethanol and benzene flame and cut off an intermediate leg; place the fly in a small tube and the leg in a microtube; label both the small tube and the microtube with the same code and continue doing this for all flies to be analysed.
4. Hold the flies in a rearing room at the recommended temperature and humidity conditions (70-80% relative humidity, 24 ± 1°C temperature and 12 h photo-phase).
5. Insert one sterile micropestle in each microtube that contain a leg.
6. Add liquid nitrogen to the microtube to freeze the fly leg and the micropestle and immediately grind the fly leg using the micropestle to obtain a fine powder.
7. Proceed with the DNA extraction using ZR-96 Quick-gDNA extraction kit following the supplier instructions: <http://www.zymoresearch.com/downloads/dl/file/id/19/d3010i.pdf>.
8. After eluting the DNA, quantify the DNA concentration using a Nano-drop spectrophotometer to ensure extraction of an equal DNA quantity from each fly to use equal quantity of the DNA in the PCR reaction (for more information see <http://www.nanodrop.com/nucleicacid.aspx>).
9. The DNA samples can be stored in a freezer (20°C) until required for the next step.

4. PCR IDENTIFICATION OF TSETSE SPECIES

A combination of different molecular markers can be applied to distinguish tsetse species as described below.

4.1. Identification of tsetse species using the nuclear marker ITS1

4.1.1. Purpose

This method is used to identify and distinguish tsetse species and subspecies.

4.1.2. Scope

This procedure is designed to correctly identify specimen of tsetse species collected in the field or from laboratory colonies.

4.1.3. Prerequisites

The following materials and equipment are needed:

- 1) DNA of the tsetse samples extracted in Section 3
- 2) Clean room
- 3) Air conditioning
- 4) PCR work station
- 5) PCR thermocycler
- 6) Pipette (e.g. Pipetman set)
- 7) Pipette tips (e.g. Pipetman tips)
- 8) Refrigerator
- 9) PCR master mix reagent
- 10) *Glossina* ITS1 specific primers (*Glossina* ITS1_for GTGATCCACCGCTTAGAGTGA & *Glossina* ITS1_rev GCAAAAGTTGACCGAACTGA)

- 11) Agarose
- 12) Gel electrophoresis apparatus
- 13) Ethidium bromide
- 14) DNA ladder
- 15) Sterile distilled water
- 16) Gloves

4.1.4. Required skills

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

4.1.5. Procedure

Warning: The PCR detection method is very sensitive and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 uL of the DNA extracted from each sample in a 25 uL PCR reaction.
2. Conduct PCR using *Glossina* ITS1 specific primers with negative (no DNA template) and positive DNA samples (positive DNA sample of known tsetse species). Use the following PCR programme for *Glossina* ITS1 amplification: 5 min 95°C, 30 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 90 sec, then 72°C for 7 min as described by Dyer et al., 2008.
3. Separate the PCR products by electrophoresis on 2% agarose gel (**Figure 2**). Ethidium bromide can be used to stain the PCR product, although precautions should be taken to avoid contact with this chemical as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using a gel documentation system (**Figure 3**).
5. Analyse the PCR results of each sample with the positive control and record the band size/length of each sample using the DNA ladder (**Figure 4**).
6. Group all the samples with the same band size together and compare the band size/length of each group of samples with known species ITS1 size (see **Figure 4**).

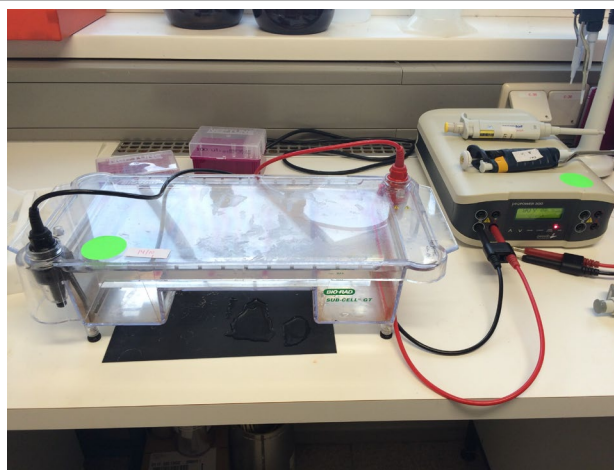


Figure 2. Gel electrophoresis system.

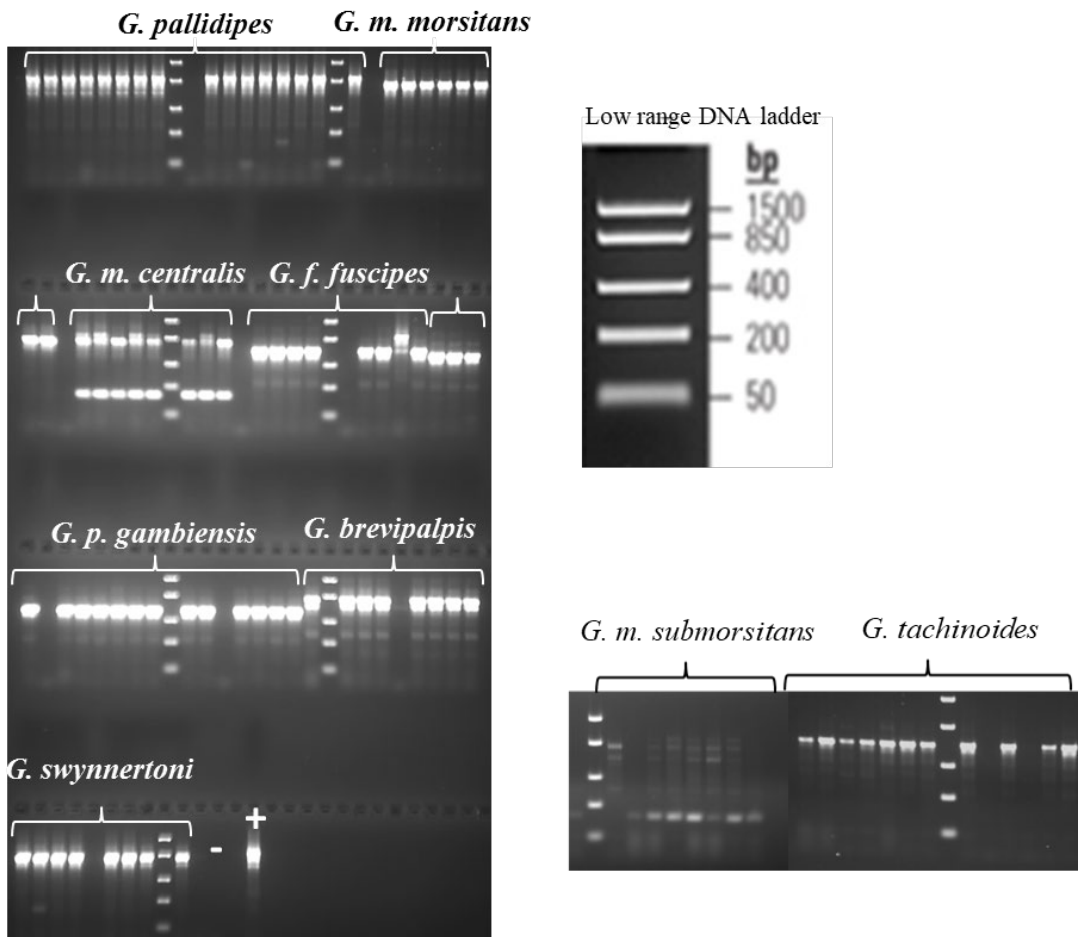
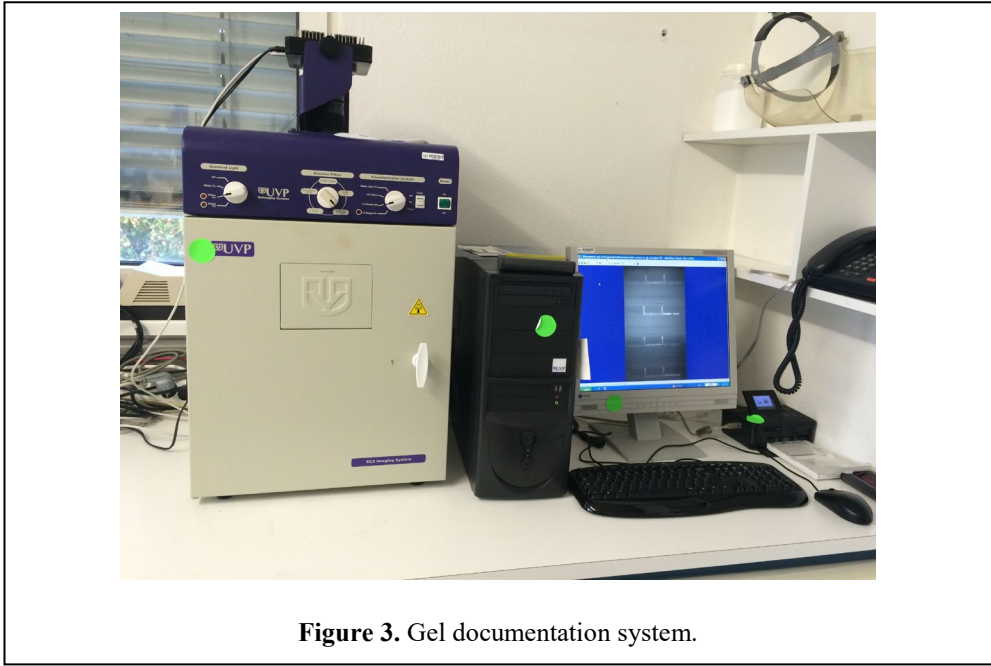


Figure 4. 2.5% agarose gel image showing different band sizes of ITS1 gene for tsetse species. Eight samples per species were analyzed. Low range DNA ladder was used to determine the size of the analyzed PCR products.

4.2. Identification of tsetse species using the microsatellite markers A10, GpB6b, GffA6, GpC10b, GffD6, Gmm14, Gff112 and GmcCA16c

4.2.1. Purpose

This method is used to identify specific tsetse species and subspecies.

4.2.2. Scope

This procedure is designed to correctly identify tsetse species from field collected and colonized flies.

4.2.3. Prerequisites

The following materials and equipment are needed:

- 1) DNA of the tsetse samples extracted in Section 3
- 2) Clean room
- 3) Air conditioning
- 4) PCR work station
- 5) PCR thermocycler
- 6) Pipette (e.g. Pipetman set)
- 7) Pipette tips (e.g. Pipetman tips)
- 8) Refrigerator
- 9) PCR master mix reagent
- 10) Primers for different microsatellite markers. A10_F & A10_R, GpB6b_F & GpB6b_R, GffA6_F & GffA6_R, GpC10b_F & GpC10b_R, GffD6_F & GffD6_R, Gmm14_F & Gmm14_R, Gff112_F & Gff112_R, GmcCA16c_F & GmcCA16c_R
- 11) Agarose
- 12) Gel electrophoresis apparatus
- 13) Ethidium bromide
- 14) DNA ladder
- 15) Sterile distilled water
- 16) Gloves

4.2.4. Required skills

Research or laboratory technician with the required skills for molecular biology work, including PCR technology and controlling PCR results on agarose gel.

4.2.5. Procedure

Warning: PCR detection method is very sensitive and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 μ L of the DNA extracted from each sample in a 25 μ L PCR reaction.
2. Conduct PCR using specific microsatellite primers with negative (no DNA template) and positive DNA samples (positive DNA sample of known tsetse species). Use the following PCR program for primers A10_F & A10_R: 3 min 95°C, 40 cycles of 95°C for 30 sec, 54.5°C for 30 sec and 72°C for 1 min, then 72°C for 5 min as described by Dyer et al., 2008. For the rest of the microsatellite marker primers use: 5 min 94°C, 40 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, then 72°C for 7 min.

3. Separate PCR products by electrophoresis on 1.5% agarose gel (**Figure 2**). Ethidium bromide can be used to stain the PCR product, although precautions should be taken to avoid any contact with this product as it is a powerful mutagenic.
4. Visualize the results of electrophoresis using gel documentation system (**Figure 3**).
5. Analyse the PCR results of each sample with the positive control and record the band size/length of each sample using the DNA ladder (**Figure 5**).
6. Group all the samples with positive signals and of the same band size together for each microsatellite marker as a single species. Use the known profile of each microsatellite marker to identify the correct species as shown in Figure 5 **Figure 6**.
7. Unlike all other analysed tsetse species, microsatellite GffD6 was not detected in *G. brevipalpis* and this microsatellite can be used to identify *G. brevipalpis*.

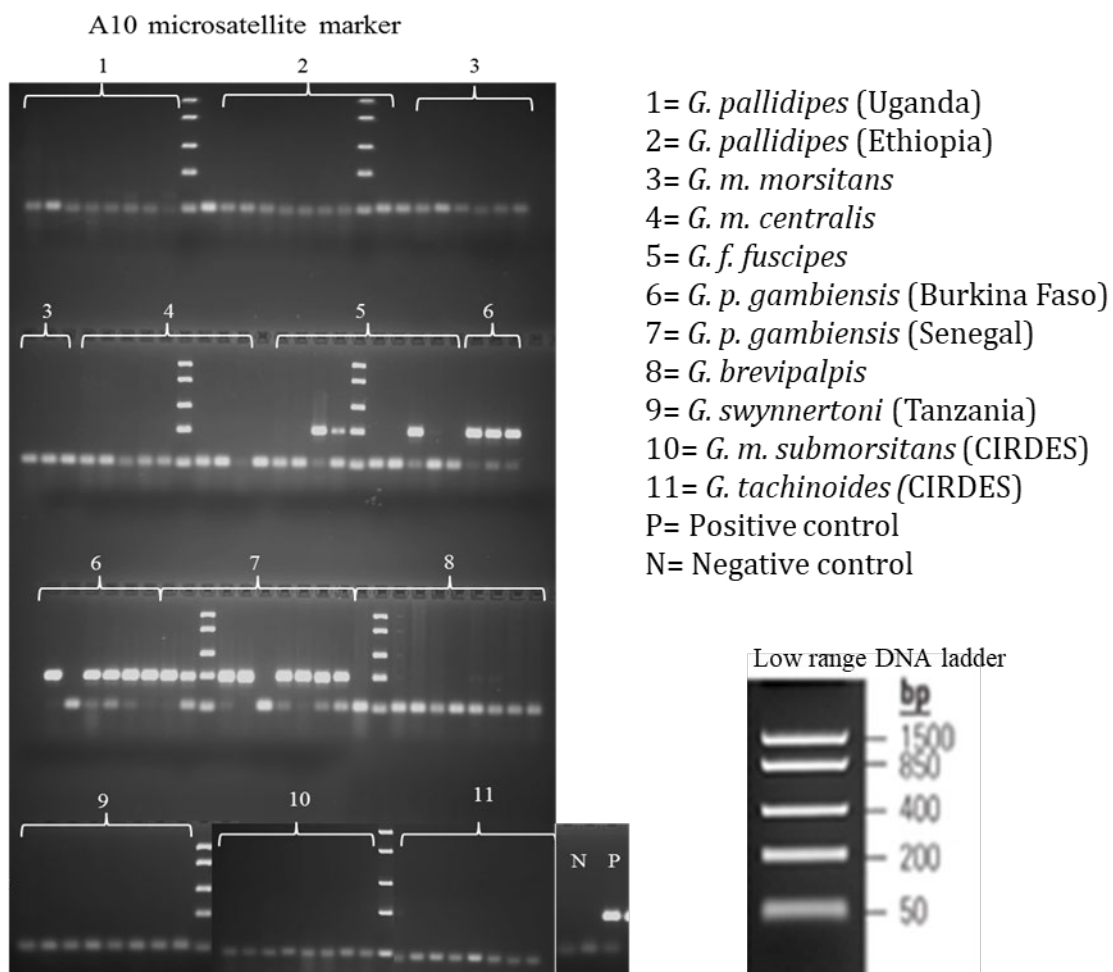


Figure 5. 2% agarose gel images showing example of microsatellite marker A10, and the species that it can identify. Eight samples per species were analyzed. A low range DNA ladder was used to determine the size of the analyzed PCR products.

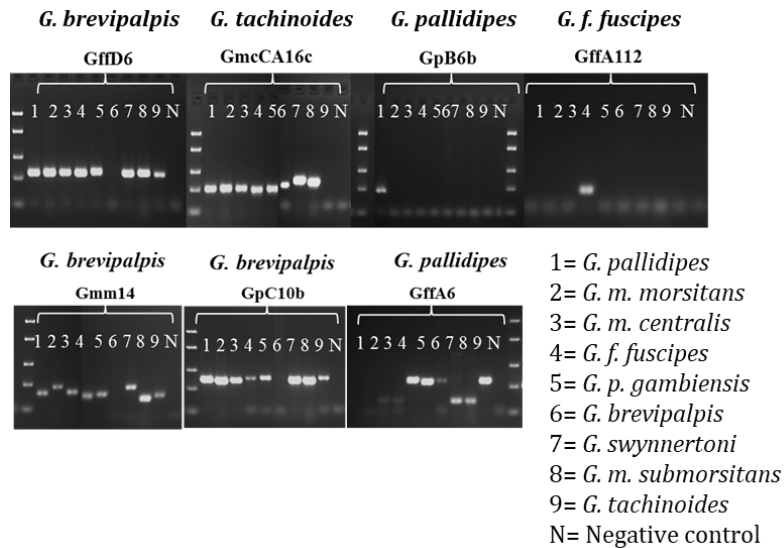


Figure 6. 2.0% agarose gel images showing examples of microsatellite markers and the species that they can identify. Each lane represents a single species. Nine tsetse species were used in the analysis with several microsatellite markers. A low range DNA ladder was used to determine the size of the analyzed PCR products.

4.3. Identification of tsetse species using mitochondrial markers (COI, 12S rRNA and 16S rRNA genes)

4.3.1. Purpose

This method is used to confirm identity of specific tsetse species and subspecies by PCR and sequencing.

4.3.2. Scope

This procedure is designed to correctly identify tsetse species from field collected and from colonized flies.

4.3.3. Prerequisites

The following materials and equipment are needed.

- 1) DNA of the tsetse samples extracted in Section 2
- 2) Clean room
- 3) Air conditioning
- 4) PCR work station
- 5) PCR thermocycler
- 6) Pipette (e.g. Pipetman set)
- 7) Pipette tips (e.g. Pipetman tips)
- 8) Refrigerator
- 9) PCR master Mix reagent
- 10) Primers for different mitochondrial markers. For COI: COI_F & CULR, for 12S rRNA: 12SCF & 12SCR and for 16S rRNA: NI-J-12585 & LR-N-12866.
- 11) Agarose
- 12) Gel electrophoresis apparatus
- 13) Ethidium bromide

- 14) DNA ladder
- 15) Sterile distilled water
- 16) PCR product purification kit. (High pure PCR product purification kit or QIAquick 96 PCR Purification Kit)
- 17) Microtubes
- 18) 96 well plates
- 19) 96 well plate strip caps
- 20) SeqMan Pro software
- 21) Gloves

4.3.4. Required Skills

Research or laboratory technician with the required skills for molecular biology work including PCR technology, controlling PCR results on agarose gel and analysis of sequencing results (bioinformatics).

4.3.5. Procedure

Warning: PCR detection method is very sensitive and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 ul of the DNA extracted from each sample in a 25 ul PCR reaction.
2. Conduct PCR using specific mitochondrial primers with negative (no DNA template) and positive DNA samples (DNA of known identify of tsetse species). Use the following PCR program for COI: 5 min 950C, 35 cycles of 930C for 1 min, 550C for 1 min and 720C for 2 min, then 720C for 7 min.
3. For 12S: 5 min 950C, 35 cycles of 950C for 45 sec, 550C for 45 sec and 720C for 30 sec, then 720C for 7 min.
4. For 16S: 5 min 950C, 30 cycles of 940C for 30 sec, 580C for 30 sec and 720C for 1 min, then 720C for 10 min as described by Dyer et al., 2008.
7. Separate PCR products by electrophoresis on 2% agarose gel (**Figure 2**). Ethidium bromide can be used to stain the PCR product, although precautions should be taken to avoid contact with this product as it is a powerful mutagenic.
8. Visualize the results of electrophoreses using Gel documentation system (**Figure 3**).
9. Analyse the PCR results of each sample with the positive control and record the band size/length of each sample using DNA ladder (**Figure 7**).
10. Select all the samples with positive signals. All the species present the same band size/length (see **Figure 7**).
11. Purify the remaining PCR products of each positive sample. For individual samples use High pure PCR product purification kit <https://pim-eservices.roche.com/LifeScience/Document/b73fbe5b-c5ed-e311-98a1-00215a9b0ba8> and for a full plate purification use QIAquick 96 PCR Purification Kit <https://www.qiagen.com/at/resources/resourcedetail?id=350f396d-b59f-480a-9f1b-6867a2e7e0a5&lang=en>.
12. Check the DNA concentration of the purified PCR products by Nano-drop spectrophotometer to ensure successful purification process or control by electrophoresis on 1.5% agarose gel (for more information see <http://www.nanodrop.com/nucleicacid.aspx>).
13. For sequencing, take 15 ul of the purified PCR product of each sample and add 2 ul of the forward primer and another 15 ul plus the reverse primer (Total 17 ul). For more information on preparation for sequencing samples check: https://www.eurofinngenomics.eu/media/892645/samplesubmissionguide_valuereadtube.pdf.

14. Once you receive the sequences of each sample sent, assemble the sequences using SegMan Pro software.

Group sample sequences with the same DNA sequence together and use the known mitochondrial sequences to identify the species of your unknown samples. (Figure 8).

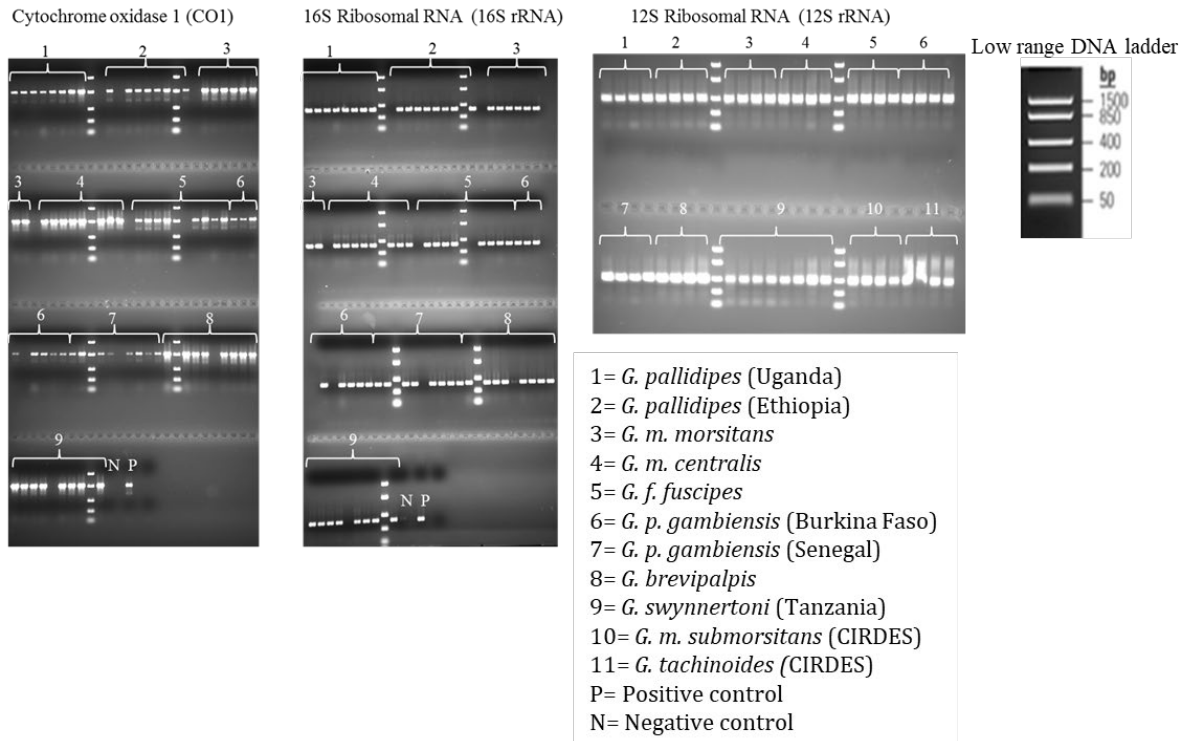


Figure 7. 2% agarose gel image for amplification of tsetse species mitochondrial markers (COI, 12S and 16S). All the genes present the same band size in all tsetse species. P is the positive control and N is the negative control. A low range DNA ladder was used to determine the size of the analyzed PCR products.

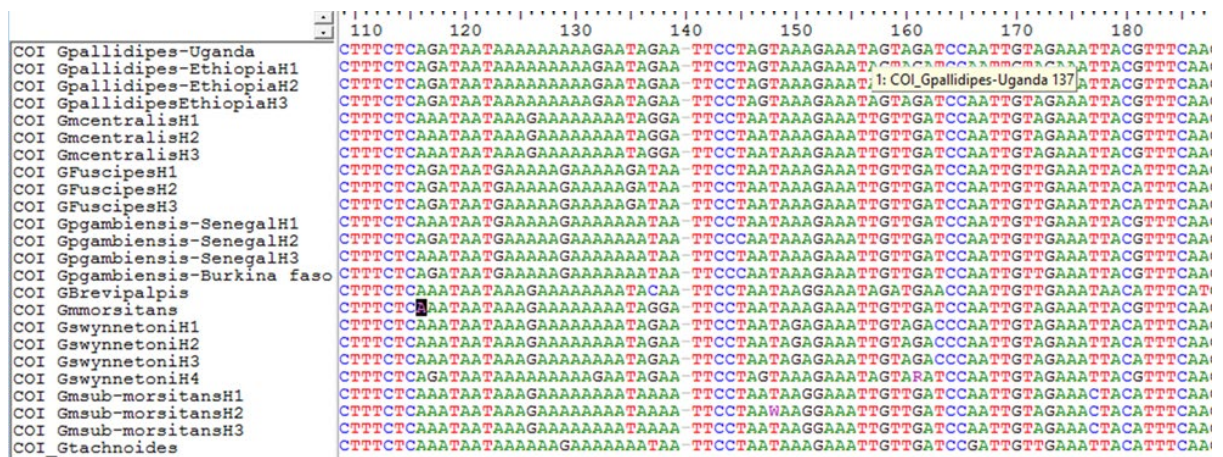


Figure 8. A screen shot of the aligned COI sequences of tsetse species. Each tsetse species presents a distinctive COI sequence. Some tsetse species have more than 2 haplotypes according to the COI sequence.

4.4. Identification of tsetse species using *Wolbachia*-based markers

4.4.1. Purpose

This method is used to confirm identify of specific tsetse species and subspecies using *Wolbachia*-based markers.

4.4.2. Scope

This procedure is designed to correctly identify tsetse species in field collected or in colonized tsetse fly species based on the fact that some tsetse species are *Wolbachia*-infected while others are not.

4.4.3. Prerequisites

The following materials and equipment are needed.

- 1) DNA of the tsetse samples extracted in Section 2
- 2) Clean room
- 3) Air conditioning
- 4) PCR work station
- 5) PCR thermocycler
- 6) Pipette (e.g. Pipetman set)
- 7) Pipette tips (e.g. Pipetman tips)
- 8) Refrigerator
- 9) PCR master Mix reagent
- 10) Primers for *Wolbachia* detection (WspecF & WspecR)
- 11) Agarose
- 12) Gel electrophoresis apparatus
- 13) Ethidium bromide
- 14) DNA ladder
- 15) Sterile distilled water
- 16) Gloves

4.4.4. Required Skills

Research or laboratory technician with the required skills for molecular biology work including PCR technology and controlling PCR results on agarose gel.

4.4.5. Procedure

Warning: PCR detection method is very sensitive and precautions should be taken to avoid cross contamination between samples (from instruments, equipment etc.).

1. Use 1.5 ul of the DNA extracted from each sample in a 25 ul PCR reaction.
2. Conduct PCR using *Wolbachia* detection primers (WspecF & WspecR) with negative control (no DNA template) and positive DNA samples (DNA of known *Wolbachia*-infected tsetse species). Use the following PCR program: 5 min 940C, 35 cycles of 940C for 45 sec, 550C for 45 sec and 720C for 30 sec, then 720C for 7 min as described by Doudoumis et al., 2012.
3. Separate PCR products by electrophoresis on 2% agarose gel (**Figure 2**). Ethidium bromide can be used to stain the PCR product, although precautions should be taken to avoid contact with the chemical as it is a powerful mutagenic.
4. Visualize the results of electrophoreses using Gel documentation system (**Figure 3**).

5. Analyse the PCR results of each sample with the positive control and record the band size/length of each sample using DNA ladder (**Figure 9**).
6. Select all the samples with positive signals (*Wolbachia*-infected). See **Figure 9** for tsetse species with presence/absence of *Wolbachia* infection. Note that in *G. m. morsitans*, 2 bands are presented due to the integration of a 16S RNA *Wolbachia* gene in the Gmm genome with an internal deletion.

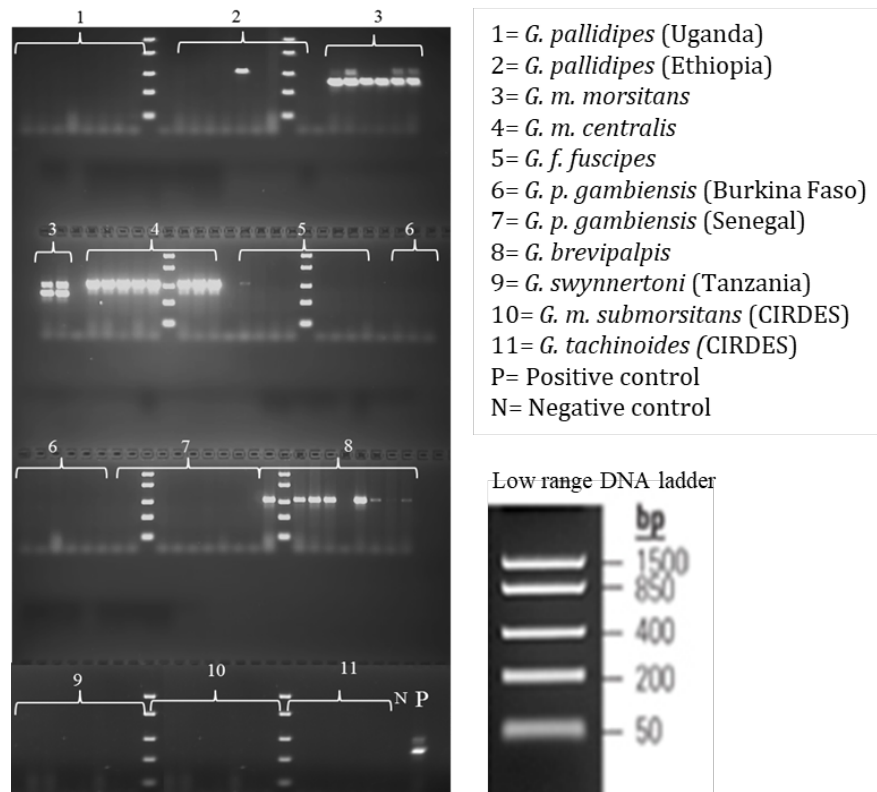


Figure 9. 2% agarose gel image showing the *Wolbachia* infection status in different tsetse species. A positive signal indicates *Wolbachia* presence. P is the positive control and N is the negative control. Eight samples per species were analyzed. A low range DNA ladder was used to determine the size of the analyzed PCR products.

4.5. Conclusion

The following scheme (**Figure 10**) can be used as guidance for the steps required identifying or distinguishing tsetse species.

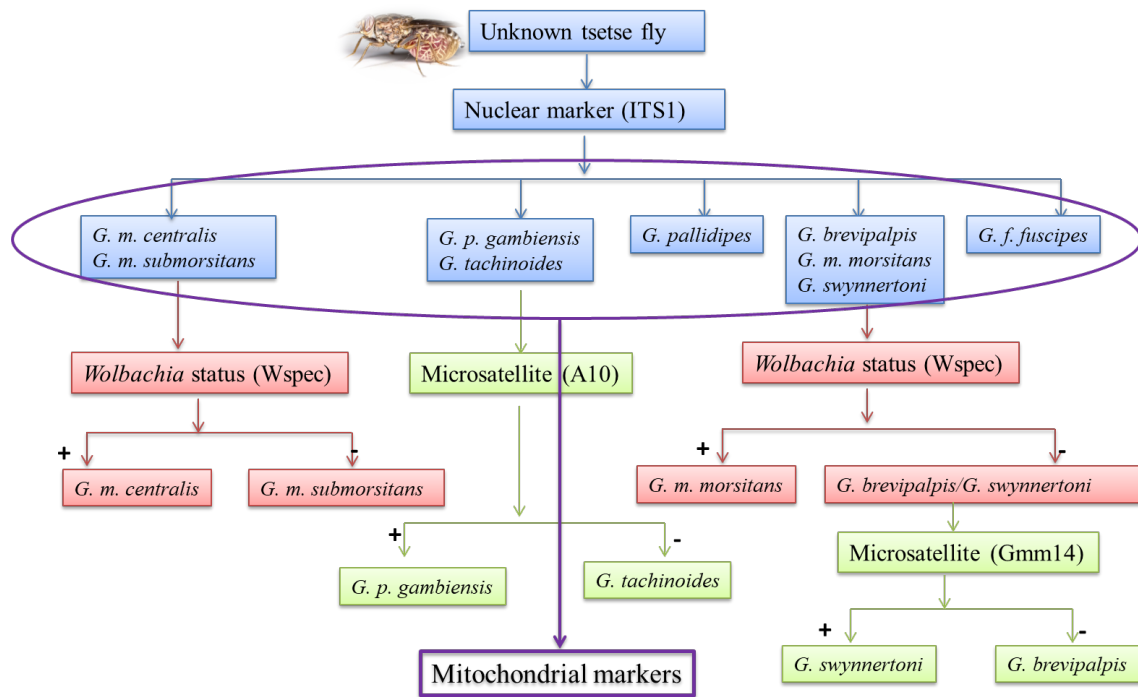


Figure 10. Schematic representation of step by step application of molecular tools for tsetse species identification.

5. REFERENCES

1. Augustinos, A., Meki, I.K., Demirbas-Uzel, G., Ouedraogo, G.M.S., Saridaki, A., Tsiamis, G., Parker, A.G., Abd-Alla, A.M.M., Bourtzis, K., (2018). Nuclear and *Wolbachia*-based multimarker approach for the rapid and accurate. *BMC Microbiol.* <https://doi.org/10.1186/s12866-018-1295-4>. (in press)
2. Doudoumis, V., Tsiamis, G., Wamwiri, F., Brelsfoard, C., Alam, U., Aksoy, E., Dalaperas, S., Abd-Alla, A., Ouma, J., Takac, P., Aksoy, S., Bourtzis, K. (2012). Detection and characterization of *Wolbachia* infections in laboratory and natural populations of different species of tsetse flies (genus *Glossina*). *BMC Microbiology*, 12(Suppl 1), S3-S3.
3. Dyer, N.A., Lawton, S.P., Ravel, S., Choi, K.S., Lehane, M.J., Robinson, A.S., Okedi, L.M., Hall, M.J.R., Solano, P., Donnelly, M.J., (2008). Molecular phylogenetics of tsetse flies (Diptera: Glossinidae) based on mitochondrial (COI, 16S, ND2) and nuclear ribosomal DNA sequences, with an emphasis on the palpalis group. *Molecular Phylogenetics and Evolution*, 49(1), 227-239.

6. DEFINITIONS

Nano-drop technology: A technology developed for micro-volume quantitation and analysis.

Gel documentation system: A gel image system or gel imager, is equipment used in molecular biology for the imaging and documentation of nucleic acid and protein suspended within polyacrylamide or agarose gels.

DNeasy®: This is the registered trade mark for a kit used for DNA extraction

7. APPENDIX 1: Special equipment and materials with specifications.

Item	Specification	Example of supplier and/or distributor
1. Sterile micropestles	Hand-operated or motor-driven grinders for disrupting tissue in microcentrifuge tubes. Pestle ends should be specially designed to mate with 0.5 ml or 1.5 ml microtubes.	Labsco or Sigma
2. Autoclave	An autoclave is a pressure chamber used to sterilize equipment and supplies by subjecting them to high pressure and saturated steam at 121 °C for around 15–20 minutes depending on the size of the load and the contents.	Labsco or Sigma
3. PCR work station	A self-contained work area that will help protect PCR runs against contamination. It should have HEPA H14 filter, stainless steel work surface, and a UV light with a timer. Dual UV bulbs irradiate the work area prior to use, reducing the possibility that any contaminating DNA will be amplified. Containment features reduce the chance of airborne contamination.	Labsco, Erlab or Sigma
4. PCR thermocycler	A device to conduct DNA amplification; it should be a compact 96-well thermal cycler with thermal gradient optimization and reliable performance.	Bio-Rad, Eppendorf, Labsco or VWR
5. Pipette (e.g. Pipetman set)	A set of pipette includes 6 adjustable-volume pipettes (0.1–2.5 µl, 0.5–10 µl, 2–20 µl, 10–100 µl, 20–200 µl, 100–1,000 µl).	Eppendorf or Gilson

6. Pipette tips (e.g. Pipetman tips)	Sterile tips with reload or filtered, which fit the different pipettes size.	Labsco or VWR
7. Water bath	Water bath with microprocessor controller for temperature selection, rapid heat-up and excellent stability. It should be equipped with an adjustable over-temperature safety cutout, which sounds an audible alarm and shuts the heater off to protect samples, and recessed control panel to protect against spills. Also, it should have a seamless passivated tank to prevent against rust and corrosion.	Labsco or VWR
8. Microcentrifuge. i.e. Centrifuge 5424 / 5424 R, Eppendorf.	Micro-centrifuge with the following specification as example: max. rcf: 20,238 x g, max. speed: 14,680 rpm, max. rotor capacity: 24x 1.5/2.0 ml, no. of rotors: 4, acceleration time to max. speed: 16 s, braking time from max. speed: 18 s, SOFT ramp: adjustable, Noise level with rotor FA-45-24-11: <51 dB(A), dimensions in cm (W x D x H): 24 x 32 x 23, weight without rotor: 13.4 kg, power supply: 230 V/50–60Hz, power requirement max.: 250 W	Eppendorf, Labsco or VWR
9. DNeasy kit	Kit for DNA extraction	Qiagen or Labsco
10. PCR master Mix reagent	Ready-made mix for PCR reaction; it contains all the required components, including MgCl ₂ , buffer, dNTPs, DNA polymerase for PCR reaction, except primers and DNA template	Qiagen or Labsco

11. Gel electrophoresis apparatus	A horizontal electrophoresis chambers with a wide (15 cm) platform to provide higher capacity. It should include a buffer tank, safety lid with cables, and leveling bubble.	Bio-Rad, Or Labsco
12. DNA ladder	A mixture of DNA fragments with known length, which helps to determine the size of unknown DNA fragments using gel electrophoresis.	Qiagen or Invitrogen
13. Nano-drop spectrophotometer	A spectrophotometer that measures 1 μ l nucleic acid samples with high accuracy and reproducibility. It should have a full spectrum (220nm-750nm) spectrophotometer. It requires a PC or laptop with suitable software to analyze and calculate the nucleic acid concentration.	Thermo Scientific or Labsco