Research Article

Molecular Characterization of Laboratory Reared *Glossina palpalis palpalis* and *Glossina morsitans submorsitans* Populations in Nigeria

Christopher U Orji[1]*, Ignatius O Onyeocha[2], Steven S Shaida[3], Peter M Dede[4], Bitrus Yakubu[5], Elijah E Ella[6] and Pam D Luka[5]

[1]Department of Agricultural Biotechnology, National Biotechnology Development Agency, Abuja, Nigeria; [2]Department of Biotechnology, Federal University of Technology, Owerri, Nigeria; [3]Vector and Parasitology Studies Department, Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Research (NITR), Kaduna, Nigeria; [4]Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC), Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Research (NITR), Kaduna, Nigeria; [5]Applied Molecular Biology Division, Nigerian Veterinary Research Institute (NVRI), Vom, Jos, Nigeria; [6]Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria.

Abstract | Tsetse fly (Diptera: *Glossinidae*) vector control measures can rely upon knowledge of genetic diversity between species' population. Two species were studied: *Glossina palpalis palpalis* and *Glossina morsitans submorsitans* using two mitochondrial DNA fragment primers- Cytochrome oxidase subunit II (COII) and Cytochrome b (CytB). Twelve samples of laboratory-reared population of each species were used for the study. Sequencing data were used to calculate haplotype, haplotype diversity and nucleotide diversity for the two species. Based on combined loci of cytochrome oxidase II (COII) and cytochrome b (CytB), twelve haplotypes were generated for *G. p. palpalis* and five for *G. m. submorsitans*. Similarly, for the combined loci, haplotype diversity (Hd) was higher for *G. p. palpalis* (0.90) than *G. m. submorsitans* (0.68). Nucleotide diversity was homogenous for *G. m. submorsitans* (0.24859) than *G. p. palpalis* (0.24093). A higher genetic diversity suggests a higher evolutionary rate. Haplotype diversity and Nucleotide diversity (genetic diversity) of a species population is often related to the ecological systems, evolutionary rate, adaptation of the species, gene flow, epidemiology and control strategies. Our study observed that the two laboratory grown species of tsetse flies may be genetically related.

Editor | Muhammad Abubakar, National Veterinary Laboratories, Park Road, Islamabad, Pakistan.
Received | June 06, 2016; **Accepted** | July 18, 2016; **Published** | July 22, 2016
***Correspondence** | Christopher U Orji, Department of Agricultural Biotechnology, National Biotechnology Development Agency, Abuja, Nigeria; **Email:** orjiuchristopher@yahoo.com
Citation | Orji, C.U., I.O. Onyeocha, S.S. Shaida, P.M. Dede, B. Yakubu, E.E. Ella and P.D. Luka. 2016. Molecular characterization of laboratory reared *Glossina palpalis palpalis* and *Glossina morsitans submorsitans* populations in Nigeria. *Veterinary Sciences: Research and Reviews*, 2(2): 31-41.
DOI | http://dx.doi.org/10.17582/journal.vsrr/2016.2.2.31.41

Introduction

Tsetse flies (Diptera: *Glossinidae*) found in sub-Saharan Africa are important vectors of trypanosomes causing Human African trypanosomiasis (HAT), or sleeping sickness and Animal African trypanosomiasis (AAT) across the West African region. Human African trypanosomiasis has impacted heavily on public health and human economic development of sub-saharan Africa with over 100,000 cases globally and 60 million people at risk of the disease of which 40% is from Africa (Cattand, 1995; Kioy et al., 2004). Animal trypanosomiasis and tsetse flies are widely distributed across five agro-ecological zones of Nigeria including areas that were initially thought to be free of the flies and disease possibly because of

the ability of the flies to disperse over a long distance (Kalu, 1996; Hargrove, 2003; Majekodunmi et al., 2013). Primarily, AAT is considered as an economic disease of cattle but losses also extend to pigs, camels, sheep and goats. Generally, trypanosomiasis caused by *Trypanosoma brucei rhodesiense* and *T. b. gambiense* are considered a zoonotic disease and deployment of appropriate control measures will affect both AAT and HAT.

Tsetse flies are vectors that belong to the genus *Glossina* and are subdivided into three subgenera or species groups: *palpalis, morsitans and fusca* made up of 33 species and subspecies (Gooding and Krafsur, 2005). The riverine *palpalis* and savannah *morsitans* species are the major vectors of human disease while the *fusca* group are responsible for AAT in animal (Aksoy et al, 2001).

Trypanosomiasis is caused by the flagellated protozoa, *Trypanosoma*. Of the known strains of trypanosomes, only *Trypanosoma brucei* species causes both HAT and AAT. Variability in vector competence of AAT or HAT depends on the species of trypanosomes as well as the species of *Glossina* (Geiger et al., 2005). The major human disease vectors belong to the *palpalis* and *morsitans* complex that inhabit the riverine and forest areas while the *fusca* group is found in forest areas where most of them are considered unlikely to feed on humans. Over the years as a result of human activities leading to reduced habitat, the distribution of the three major species: *G. pallidipes* and *G. brevipalpis* has reduced in population densities while that of *G. fuscipes* has continued to increase (representing the subgenera: *palpalis*, *morsitans* and *fusca*, respectively) across the East, Central and West Africa region. Sleeping sickness therefore exists in two forms, either the chronic form (West and Central African form) caused by *T. brucei gambiense* or the acute and more deadly form (East African form) resulting from infection with *T. brucei rhodesiense*. Uganda is the only country that has the two forms of the sleeping sickness parasites separated by a narrow 160 km belt (Abila et al., 2008).

HAT and AAT are endemic in some regions of sub-Saharan Africa, covering 37 countries. The number of reported cases of HAT dropped below 10,000 in 2009, for the first time in 50 years, and the downward trend was maintained in 2010 when 8,000 new cases were reported in 7 countries with a planned total eradication by 2031 (AU, 2010). The downward trend was attributed to some factors such as tsetse fly distribution, increasing human population and activity (Ford, 1963; Reid, 2000) leading to the destruction of some tsetse fly habitats. The other reasons for the reduction in sleeping sickness were the use of preventive measures resulting from avoidance of heavily infested areas, wearing of preventative clothing and the use of chemical sprays (CDC, 2012). An estimated total number of 30,000 people were infected as at 2012 (WHO, 2012) besides, the cases that go unreported.

Out of the 11 species of tsetse fly in Nigeria, four are of economic importance. *G. p. palpalis* and *G. tachinoides*, both of the *palpalis* species group are found in riverine and lakeshore habitats of both the northern and southern parts of the country. *G. m. submorsitans* and *G. longipalpis* are of the *morsitans* species group and found mainly in savannah areas (FAO, 1992; Leak, 1999). Tsetse flies have had a marked influence on cattle distribution and production systems in Nigeria and possibly across West Africa. Cattle keeping in Nigeria is practised mainly in the northern regions where grasses and shrubs of the savannah provide grazing areas. The southern parts of Nigeria and neighbouring countries are more forested and infested by the *palpalis* and *fusca* groups. The bulk of Nigeria's human trypanosomiasis and much of cattle trypanosomiasis are transmitted by the riverine species of tsetse fly (*palpalis* group). Human sleeping sickness in Nigeria was predominantly a disease of the drier northern savannahs and not of the southern forests where tsetse flies were more abundant (Leak, 1999). There is little information on the incidence of sleeping sickness in Nigeria although it appears to have decreased and appears in only small persistent foci (Leak, 1999). The Card Agglutination Test for Trypanosomiasis (CATT) used in an epidemiological survey of then Bendel State of Nigeria revealed that parts of the state had unreported endemic foci of sleeping sickness (Edeghere et al., 1989).

There are new reports indicative of changing ecology as regards localization of tsetse fly in Nigeria. Tsetse flies and cattle infections have been reported in previously known tsetse-free areas such as Jos, Mambilla and Obudu plateau (Dede et al., 2005), in Bassa Local Government Area of Plateau State (Qadeer et al., 2008) as well as Danja Local Government Area of Katsina State (Danbirni, 2008). This shows that Nigerian livestock population stand at greater risk of

infection. The need to accelerate effort in eradication of tsetse fly in view of the enormous losses associated with trypanosomiasis has prompted putting into consideration control measures that include genetic methods at national and international levels (Gooding and Krafsur, 2005). The genetic methods are expected to form part of an integrated control approach which is preferred to single strategies. A number of useful molecular genetics tools are becoming invaluable including molecular marker applications for pests and parasites species' characterization, population genetics and evolutionary studies. In addition to many applications of mitochondrial DNA, some recent *Glossina* phylogenetic studies have also used genetic markers such as microsatellites and internal transcribed spacer (ITS) 1 of rDNA (Abila et al., 2008; Dyer et al., 2008), while application of Single Nucleotide Polymorphism (SNP) is also advocated.

The knowledge of genetic diversity can help in determining the degree of structuring or genetic differentiation between populations of any species and establish if there is any gene flow between the populations. Similarly, a tsetse fly species' population structure (differentiated or undifferentiated) has implications for tsetse fly control because different genetic groups could display differences in infection rates and trypanosome identity (Bouyer et al., 2007; Ravel et al., 2007).

In this study, mitochondrial DNA (COII and CytB) data were used to determine the genetic relatedness between laboratory-reared populations of *G. p. palpalis* and *G. m. submorsitans*. This laboratory population study will be useful as a tool in initiating population genetic study of tsetse fly populations within Nigeria's ecological areas. The results from this study will provide useful information from a population genetics perspective to support an effective and sustainable tsetse fly control in Nigeria.

Materials and Methods

Laboratory Reared Tsetse Flies

The two tsetse fly species studied were *G. p.palpalis* and *G. m. submorsitans* which are two of the four species of economic importance in Nigeria. Representative flies from two laboratory colonies maintained at the Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Research (NITR), Kaduna, Nigeria were obtained. The increasing need of tsetse flies for research and biological control (Sterile Insect Control or SIT) has necessitated their laboratory rearing. At NITR the flies are reared on the blood of rabbit hence the tsetse flies are usually housed inside a production cage and made to suck blood through the ear of a restrained rabbit. Tsetse fly reproduction is viviparous as the female gives birth to live offspring. The larva is nourished within the mother and is larviposited at an advanced stage of development. The average female lifespan is 100-120 days with an average of about 10 pupae per female under laboratory conditions.

A total of 24 teneral tsetse flies were collected randomly from the laboratory, 12 flies for each species. Sample collection, laboratory analysis and sequencing were done between May and June 2012. Tsetse flies were preserved in 95% ethanol and stored in a 4°C 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 carried out at the Biotechnology Laboratory of Nigerian Veterinary Research Institute (NVRI), Vom, Jos, Plateau State, Nigeria. Each whole body of the 12 *G. p. palpalis* and 12 *G. m. submorsitans* were used. Homogenate was obtained by grinding each fly body in a mortar with a pestle, and 1000 µl of Phosphate Buffer Saline (PBS) was added to make a solution. Extraction of genomic DNA was performed using the ZR Tissue and Insect DNA MicroPrep™ (Zymo Research, CA, USA) based on manufacturer's instructions.

Figure 1: *Amplification of a 400bp fragment of COII for 10 Glossina palpalis palpalis;* **Lane 1-8:** *amplification;* **Lane 9-10:** *no amplification;* **Lane M:** *100 bp ladder; Glossina morsitans submorsitans 400bp fragments of COII also amplified in a similar pattern*

Amplification was carried out for 400 bp region (Figure 1) of the mtDNA Cytochrome oxidase II (COII) and 500 bp region (Figure 2) of the Cytochrome b (CytB) gene using universal invertebrate primer pair's

Table 1: *The invertebrate universal primers for the amplification of the universal mitochondrial genome*

Name	Region in Genome	Sequence(5′ - 3′)
mtD-13	COII	COIIF – AATATGGCAGATTAGTGCA
mtD-15	COII	COIIR – TCATAAGTTCARTATCATTG
mtD-26	CytB2	CytB2F – TATGTACTACCATGAGGACAAATATC
mtD-28	CytB2	CytB2R - ATTACACCTCCTAATTTATTAGGAAT

mtD13/mtD15 and mtD26/mtD28, respectively (Simon et al., 1994) (Table 1). Polymerase chain reactions (PCR) were performed based on a 25 µl reaction mixtures containing 1 µl of template DNA, 2.5 µl 10X PCR buffer, 0.8 mM dNTP, 2mM $MgCl_2$, 0.4 µM of each primer, 1µl of BSA (Bovine Serum Albumin) and 1unit of AmpliTaq Gold (Applied Biosystems, Waltham USA). Gene Amp PCR® System 9700 (Applied Biosystems, Waltham, USA) was used for the amplification reactions. Thermal cycler conditions consisted of an initial 10 min denaturation step at 94° C, followed by 35 cycles of 1 min at 48°C and 1 min at 72°C. Reactions were terminated with a final extension time of 5 min at 72° C (Abila et al., 2008). PCR products were analyzed by electrophoresis on 2% agarose gel and visualized under ultra violet light using Gel Doc™ XR+ (BioRad, CA, USA) (Figure 1 and 2).

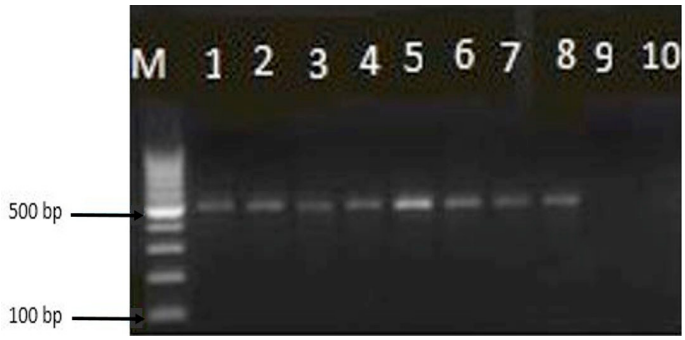

Figure 2: *Amplification of a 500 bp fragment of CytB gene for 10 Glossina palpalis palpalis;* **Lane 1-8:** *amplification;* **Lane 9-10:** *no amplification;* **Lane M:** *100 bp ladder; Glossina morsitans submorsitans 500bp fragments of CytB also amplified in a similar pattern*

A total of 44 amplicons were selected for nucleotide sequencing. For *G. p. palpalis,* they comprised 24 samples: 12 samples for COII and 12 samples for CytB while for *G. m. submorsitans* they comprised of 20 samples: 10 samples for COII and 10 samples for CytB that amplified. Sequencing of the 44 amplicons was done in the forward and reverse directions. Sequencing was performed by Macrogen USA, Rockville, Maryland using ABI 3730XL (Applied Biosystems) automated sequencer following standard manufacturer's protocols.

Phylogenetic Analysis of mtDNA Sequences for Genetic Diversity of the Species

BLAST (Basic Local Alignment Search Tool) of the sequences was done using BLASTIN 2.2.27 (Zang et al., 2000) and aligned by CLUSTAL W (Thompson et al., 1997) with default parameters and scrutinized and edited using MEGA 4 (Tamura et al., 2007).

The MEGA 4.1 data of the aligned sequences were exported to DnaSP v 5.0 (Librado and Rozas, 2009) and used to calculate DNA sequence polymorphism indices and generate the number of haplotypes, calculate haplotype diversity (Hd) (Nei, 1987) and nucleotide diversity (pi) (Nei, 1987; Nei and Miller, 1990). Haplotype is the genetic constitution of an individual at a set of linked genes. Nucleotide diversity is a concept in molecular genetics which is used to measure the degree of polymorphism within a population. This measure is defined as the average number of nucleotide differences per site between any two DNA sequences chosen randomly from the sample population, and is denoted by π (Nei and Li, 1979). Nucleotide diversity (Jukes and Cantor) is obtained using the Jukes and Cantor (1969) correction. The correction has been performed in each pairwise comparison; the Pi (p) estimates were obtained as the average of the values for all comparisons (Lynch and Crease, 1990). For each species in this study, haplotype diversity was estimated separately for each gene locus while the sequences for the two loci were later combined to obtain a combined value which is a better estimate than a single locus estimate. Nucleotide diversity estimate followed the same pattern.

Results

BLAST of the Sequences

Basic Local Alignment Search Tool (BLAST) of the database returned DNA sequences of related species for COII and CytB for the two species studied - *G. p. palpalis* and *G. m. submorsitans* with percentage identity ranging from 89-95%. No sequences of the two species for any of the two genes were found in the

GenBank implying that this is the first time that nucleotide database deposits was made for these sequences. Results of random BLAST of the sequences with one another for each gene revealed 91-99% identity.

Table 2: *Haplotype diversity for mtDNA for single and combined loci*

Species / mtDNA	No of Sequences (N)	No of Haplotypes (H)	Haplotype (gene) Diversity (Hd)
G. p. palpalis			
COII	10	5	0.88
CytB	9	8	0.972
CO II + CytB	19	12	0.901
G.m. submorsitans			
COII	8	3	0.464
Cyt B	9	3	0.417
CO II + CytB	17	5	0. 684

DNA Polymorphism based on Mitochondrial DNA Diversity Indices

DnaSP was used to compute the number of haplotypes and haplotype diversity, the results of which are presented in Table 2 (Column 3 and 4). The results for *G. p. palpalis* COII and CytB for single and combined loci for number of haplotypes and haplotype diversity are shown in the table. Similarly, the single and combined results for *G. m. submorsitans* for COII and CytB for the species are shown. A combined gene locus (COII and CytB) was used to generate 12 haplotypes for *G. p. palpalis* and 5 haplotypes for *G. m. submorsitans* (Table 2 column 3) Haplotype diversity when combined for the two genes was 0.901 for *G. p. palpalis* and 0.684 for *G. m. submorsitans* (Table 2 column 4).

Table 3: *Nucleotide diversity for mtDNA for single and combined loci*

Species /mtDNA	No. of samples / sequences N	Nucleotide Diversity Pi(π)	Nucleotide Diversity Jukes and Cantor, Pi (π) (JC) (Corrected)
G. p. palpalis			
COII	10	0.02982	0.03135
CytB	9	0.03659	0.03829
COII + CytB	19	0.24093	0.34935
G.m. submorsitans			
COII	8	0.02918	0.03157
CytB	9	0.00299	0.00301
COII + Cyt B	17	0.24859	0.38109

Similarly, Nucleotide Diversity pi (π) and Nucleotide Diversity Jukes and Cantor, pi *(π)* (JC) (Table 3 columns 3 and 4) are shown for the two species as was done for haplotype diversity above. Nucleotide Diversity Pi (π) indices when combined for the two gene loci gave 0.2409 for *G. p. palpalis* and 0.2489 for *G. m. submorsitans*. Both the Haplotype Diversity and Nucleotide Diversity are often expressed in percentage by multiplying with 100.

Sequences were used for the computation of haplotypes, haplotype diversity and nucleotide diversity and also used to construct neighbour joining trees (Figure 3 and 4). The sequences were for *G. p. palpalis* COII (5)

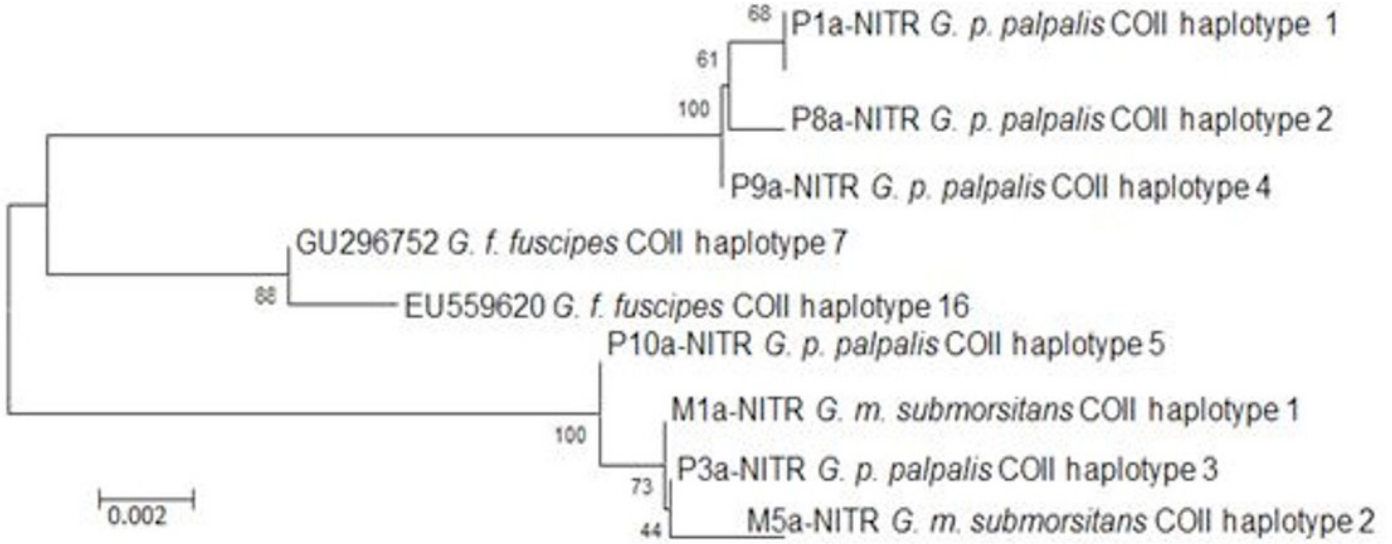

Figure 3: *Neighbour joining tree based on sequences of three species' COII. G. p. palpalis COII clustered differently from G. m. submorsitans COII and G. f. fuscipes. A few exceptions are P3a-NITR and P10a-NITR which are G. p. palpalis COII that clustered with G. m. submorsitans COII. Both G. p. palpalis and G. f. fuscipes COII (GU2967652 and EU559620) show a common ancestry as both are members of the morsitans species group. The scale bar indicates the number of nucleotide substitutions per site*

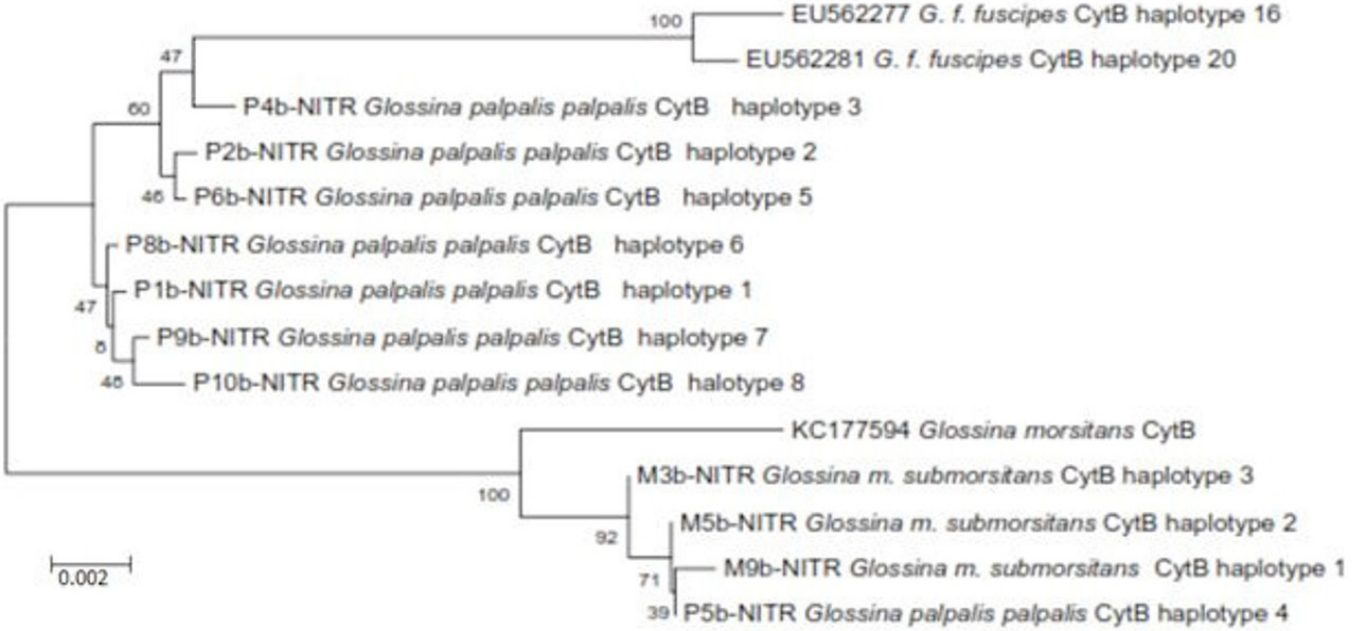

Figure 4: *Neighbour joining tree based on sequences of four species' CytB. G. p. palpalis CytB has a common ancestry with G. f. fuscipes CytB (EU562277 and EU562281) from GenBank as both are species of the palpalis species group. Members of each species also clustered more closely. G. morsitans (KC177594) from GenBank also showed a common ancestry with G. m. submorsitans which are species of the morsitans species group. The only exception was the P5b-NITR, a palpalis species member that clustered with the G. m. submorsitans. The scale bar indicates the number of nucleotide per site*

Table 4: *mtDNA sequences obtained from this study*

Isolate	Species	Gene	Haplotype	Accession No.
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

and *G. p. palpalis* CytB (8); *G. m. submorsitans* COII (3) and *G. m. submorsitans* CytB (3), giving altogether 19 sequences. The MEGA 4.1 Kimura's two-parameter model was used to calculate the genetic distances between pairs of sequences and constructed a Neighbour Joining trees (Saitou and Nei, 1987) using the Bootstrap Test of Phylogeny using other sequences from the GenBank. Neighbour joining tree was generated separately for the two genes (Figure 3 and 4). Though a bootstrap value of 70% is considered significant evidence for phylogenetic grouping (Hills and Bull, 1993), results of BLAST of the samples both with GenBank sequences and between the sample sequences to reveal their percentage identity was also considered in evaluating their level of relationship.

The mtDNA sequences of the flies were submitted at National Centre for Biotechnology Information (NCBI) (GenBank) database and Accession Numbers given to them as follows: *G. p. palpalis* COII KJ013516-20, *G. p. palpalis* CytB KJ013521-28 *G. m. submorsitans* COII KJ207383-5 and *G. m. submorsitans* CytB KJ207386-8 (Table 4).

Discussion

The BLAST results gave good values of percentage identity between the sampled species and the species found in the GenBank (89-95%). BLAST results between members of the sample for each species for each gene (COII and CytB) gave 91-99%. It can be seen that the percentage similarity observed between the samples and the other species from databases is less than the percentage similarity observed between the samples and other species from databases is less that the percentage similarity observed between members of the studied samples. Genetic diversity informa-

tion as seen in the haplotype and nucleotide diversity indices revealed whether a specie is differentiated (structured) or undifferentiated (unstructured) within a population.

Haplotype diversity and nucleotide diversity are important population genetics and evolutionary studies parameters. Number of haplotypes obtained based on single locus of COII and CytB as well as for the combined loci were higher for in *G. p. palpalis* compared to *G. m. submorsitans* (Table 2). The haplotypes and nucleotide diversity indices generated were useful in further identifying and characterizing the species. The differences obtained for the two gene loci when used in combination and individually showed that a better resolution was obtained when the two genes were combined. A table of haplotype diversity and nucleotide diversity (of different mtDNA markers) that includes the two species in this study and other published *Glossina* taxa (Table 5) further support the result and showed they compare with similar studies and thus help to show their relevance. For instance, haplotype diversity of 0.901 for lab-reared *G. p. palpalis* obtained in this work compared well with 0.95 for lab-reared *G. f. fuscipes* obtained by Krafsur et al. (2008) for the different mtDNA markers used in both studies. Both species are members of the *palpalis* species group. Similarly, nucleotide diversity of 0.24093 was obtained for lab-reared *G. p. palpalis* in our study while 0.012 was obtained for lab-reared *G. f. fuscipes* by Krafsur et al. (2008). In addition to specie differences, differences between the mtDNA markers employed for natural and laboratory environments may explain the differences observed between the species. However, our findings are in agreement with that of Krafsur et al. (2008) between wild and captive species of *Glossina*.

A strong relationship exists between nucleotide diversity and evolutionary rate that is useful in *Glossina* epidemiology and eradication. The higher the nucleotide diversity the higher the evolutionary rate (Lenski, 2001). Evolutionary rate is a measurement of the rate of genotype change of species and organisms over a period of time. The changes of interest may be in the genome itself or in the phenotypic expression of underlying genetic events. Mutation and natural selection are factors that account for differences in evolutionary rate. Evolutionary rate is a factor in an individual's adaptation to its environment (Lenski, 2001). Evolutionary concepts have also been influencing the thinking in the health sciences (Ewald, 1983; Stearns, 2008) leading to the field evolutionary epidemiology (Galvani, 2003). Examples of evolution in action in a medical setting are development of resistance following the use of drugs against pathogens (such as antimalarial) or resistance following the use of insecticides against disease vectors such as mosquitoes (Coleman and Hemingway, 2007).

Phylogenetically, distinct species clustered differently for each of the species however, with a few exceptions suggesting its utility in differentiation. The three species (*palpalis, morsitans, and fusca*) clustered separately i.e. for the COII gene, *G. p. palpalis* clustered more closely with *G. f. fuscipes* both of which belong to the *palpalis* species group while *G. m. submorsitans* which belong to the *morsitans* species group clustered separately (Figure 3). Similarly, utilizing the CytB gene, *G. p. palpalis* clustered more closely with *G. f. fuscipes* both of which are also members of the *palplis* species group while *G. morsitans* and *G. m. submorsitans* clustered more closely as both are members of the *morsitans* species group (Figure 4).

Table 5: *Comparison of the mtDNA diversity indices (haplotype diversity and nucleotide diversity) with other published Glossina taxa*

Taxon	No of Populations	Hd (mean pops Hd)	Nucleotide Diversity, pi (mean pops)	mtDNA markers involved	References
G. f. fuscipes (Lab reared)	1	0.95	0.012	COI, 16S2	(Krafsur et al, 2008)
G. f. fuscipes	3	0.84	0.009	COI, 16S2	(Krafsur et al, 2008)
G. pallidipes	20	0.63	-	COII, 16S2,	(Krafsur & Wohlford, 1999)
G.m. submorsitans	7	0.89	-	12S, 16S2	(Krafsur et al, 2000)
G. f. fuscipes	10	0.74	0.0130	COII, CytB	(Abila et al, 2008)
G. p. palpalis (Lab reared)	1	0.901	0.24093	COII, CytB	This study
G.m. submorsitans (Lab reared)	1	0.684	0.24859	COII, CytB	This study

Comparatively, gene sequence (and wing morphometry) have been used to demonstrate tsetse fly species groupings (Patterson and Schofield, 2004). However, the utility of gene sequences for tsetse fly species groupings and identification will continue to increase with increasing deposits in the in the GenBank. We therefore recommend that phylogenetic analysis be used to support conventional morphological technique in tsetse identification (Buxton, 1955; Pollock, 1992).

The importance of these mitochondrial DNA diversity indices are better appreciated in a study of the genetic variation of a tsetse fly species involving different populations in different localities. It makes it possible to compare the structure of the populations and to evaluate if there is gene flow between the populations. It has been observed that the different populations of savannah species and forest species such as *G. morsitans* tend to be substantially distinct in structure apparently due to barriers brought by thick vegetation which isolates the populations (Ouma et al., 2006; Ouma et al., 2007). This suggests that there is wide genetic diversity between different populations for that species. On the other hand, the riverine species *G. palpalis gambiensis* found in West Africa are lesser structured thus showing lower levels of genetic differentiation between populations (Solano et al., 2000; Bouyer et al., 2007). Together with *G. p. palpalis* these species live in the humid savannah vegetation and can easily disperse along the river banks through the savannah and forest vegetation. However, although a member of the *G. palpalis* species group which are riverine in nature, both microsatellite and mtDNA data analyses revealed high levels of differentiation between the studied *G. f. fuscipes* populations in Uganda. This is because almost all pair-wise comparisons of *Fst* values were significant indicating some restriction in gene flow between populations. In this regard, the riverine *G. f. fuscipes* is more similar to the savannah species *G. morsitans* and *G. pallidipes, by having patchy or isolated populations*, than it is to the other riverine species *G. p. gambiensis* (Abila et al., 2008; Krafsur et al., 2008).

A differentiated population based on a significant pair-wise comparison of Fst values between different field populations of any species would mean that there is restriction in gene flow between the populations of that species. Gene flow refers to the movement of individuals among populations. It is a collective term that includes all mechanisms resulting in the movement of genes from one population to another. Gene flow generally occurs within species and can be due to migration of individuals or as a result of movement of extra-nuclear segments of DNA such as mitochondria, plasmids and viruses (Slatkin, 1985). A review of the breeding structures of the principal trypanosome vectors - *G. morsitans G. pallidipes*, *G. palpalis* and *G. f. fuscipes* revealed that all showed highly structured populations among which there is little detectable gene flow (Krafsur, 2009). Tsetse fly population structure has implications for tsetse fly control because different genetic groups could display differences in infection rates and trypanosome identity. The population indices are thus related to the tsetse fly species evolution, their adaptation to their environment and epidemiology of the diseases. In conclusion, the two species of tsetse fly from Nigeria are diverse and this diversity is more in *morsitans* compared to *palpalis* and can be utilized in specie differentiation.

Acknowledgements

We thank Bamidele Solomon and Lucy Jumeyi Ogbadu, the former and the present Directors General, National Biotechnology Development Agency and Mohammed Mamman Director General, Nigeria Institute for Trypanosomiasis Research (NITR) for their support for this research.

Authors' Contribution

CUO, IOO, EE, SSS and PMD conceived and designed the experiment, SSS and PMD reared the laboratory tsetse flies, CUO, EE, BY and PDL did laboratory and bioinformatics analysis, and CUO, IOO and PDL wrote the manuscript and all read approved it for submission.

References

- Abila, P.P., Slotman, M.A., Parmakelis, A., Dion, K.B., Robinson, A.S., Muwanika, V.B., Enyaru, J.C.K., Okedi, L.M.A., Aksoy, S., Caccone, A. High levels of Genetic differentiation between Ugandan *Glossina fuscipes fuscipes* populations separated by Lake Kyoga. Neglected Tropical Diseases, 2008; 2(6): e242.
- Aksoy, S., Maudlin, I., Dale, C., Robinson, A.S., O'neill, S.L. Prospects for control of African trypanosomiasis by tsetse vector manipulation. Trends

in Parasitology, 2001; 17: 29-35. http://dx.doi.org/10.1016/S1471-4922(00)01850-X
- AU. Pan African Tsetse and Trypanosomiasis Eradication Campaign – PATTEC, 2010. Available at: http://rea.au.int/en/RO/PATTEC. (Accessed 2 Sept 2014).
- Bouyer, J., Ravel, S., Dujardin, J.P., De Meeüs, T., Vial, L., Thévenon, S., Guerrini, L., Sidibé, I., Solano, P. Population structuring of *Glossina palpalis gambiensis (*Diptera*: Glossinidae)* according to landscape fragmentation in the Mouhoun river, Burkina Faso. Journal of Medical Entomology, 2007; 44(5): 788-95. http://dx.doi.org/10.1603/0022-2585(2007)44[788:PSOGPG]2.0.CO;2
- Buxton, P.A. 1955. The natural history of tsetse flies, Memoirs of the London School of Hygiene and Tropical Medicine. 10, 816 pp.
- Cattand, P. The scourge of human African trypanosomiasis. African Health, 1995; 7: 9–11.
- Centers for Disease Control and Prevention (CDC). Parasites – African Trypanosomiasis, 2012. Available at: http://www.cdc.gov/parasites/sleepingsickness/ (Accessed 12 March 2013).
- Coleman, M., Hemingway, J. Insecticide resistance monitoring and evaluation in disease transmitting mosquitoes. Journal of Pesticide Science, 2007; 32(2): 69-76. http://dx.doi.org/10.1584/jpestics.R07-01
- Danbirni, S., Sackey, A.K.B., Fadason, S.T., Bello, A.A. *Trypanosoma brucei* infection in a herd of sedentary cattle in Danja Local Government Area, Katsina State, Northern Nigeria- A possible resurgence of tsetse flies in the previous tsetse-free area of Nigeria. Nigerian Veterinary Journal, 2010; 32(1): 87-89.
- Dede, P.M., Omoogun, G.A., Uzoigwe, N.R., Njoku, C.I., Daniel, A.D., Dadah, A.J. Current tsetse and trypanosomiasis situation on the Plateau, Nigeria: Epidemiological factors that may enhance disease acquisition and spread. Revue d'elevageet de medicine veterinaire des pays tropicaux, 2005; 58: 25-32.
- 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., Donelly, M.J. 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, 2008; 49 (1): 227–239. http://dx.doi.org/10.1016/j.ympev.2008.07.011
- Edeghere, H., Olise, P.O., Olatunde, D.S. Human African trypanosomiasis (sleeping sickness): new endemic foci in Bendel State of Nigeria. Tropical Medicine and Parasitology, 1989; 40: 16-20.
- Ewald, P.W. Host-parasite relations, vectors, and the evolution of disease severity. Annual Review of Ecology and Systematics, 1983; 14: 465-485. http://dx.doi.org/10.1146/annurev.es.14.110183.002341
- FAO, 1992. Tsetse biology, systematics and distribution techniques. Training Manual for Tsetse fly Control Personnel Vol. 1, Pollock, J.N. (edn). FAO, Rome. Available at: http://www.fao.org/docrep/009/p5178e/P5178E00.htm#TOC (Accessed 12 Sept 2014).
- Ford, J. The distribution of the vectors of African pathogenic trypanosomes. Bulletin of World Health Organization, 1963; 28(5-6): 653–669.
- Galvani, A.P. Epidemiology meets evolutionary ecology. Trends in Ecology & Evolution, 2003; 18(3): 132-139. http://dx.doi.org/10.1016/S0169-5347(02)00050-2
- Geiger, A., Cuny G., Frutos, R. Two tsetse fly species, *Glossina palpalis gambiensis* and *Glossina morsitans morsitans*, carry genetically distinct populations of the secondary symbiont *Sodalis glossinidius*. Applied and Environmental Microbiology, 2005; 71: 12. http://dx.doi.org/10.1128/AEM.71.12.8941-8943.2005
- Hargrove, J.W. 2003. Tsetse eradications: sufficiency, necessity and desirability. Research report, DFID Animal health programme, Centre for Tropical Veterinary Medicine, University of Edinburgh.
- Hills, D.M, Bull, J.J. 1993. ´An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis´, Systematic Biology, 42: 182–192.
- Gooding, R.H., Krafsur, E.S. Tsetse genetics: Contributions to biology, systematics, and control of tsetse flies. Annual Review in Entomology, 2005; 50: 101–123. http://dx.doi.org/10.1146/annurev.ento.50.071803.130443
- Jukes, T.H., Cantor, C.R. 1969. Evolution of protein molecules. In: Munro, HN. (edn.), pp. 21-132. Mammalian protein metabolism. Academic Press, New York, NY. http://dx.doi.org/10.1016/b978-1-4832-3211-9.50009-7
- Kalu, A.U. Current status of tsetse fly and animal trypanosomosis on the Jos Plateau, Nige-

ria. Preventive Veterinary Medicine, 1996; 27 (3–4): 107-113. http://dx.doi.org/10.1016/0167-5877(96)01014-8

- Kioy, D., Jannin, J., Mattock, N. Human African trypanosomiasis. Nature Review in Microbiology, 2004; 2: 186–187. http://dx.doi.org/10.1038/nrmicro848
- Krafsur, E.S. Tsetse flies: Genetics, evolution, and role as vectors. Infection Genetics and Evolution, 2009; 9(1): 124–141. http://dx.doi.org/10.1016/j.meegid.2008.09.010
- Krafsur, E.S., Madsen M., Wohlford D.L., Mihok S., Griffiths, N.T. Population genetics of *Glossina morsitans submorsitans* (Diptera: Glossinidae). Bulletin of Entomology Research, 2000; 90: 329–335. http://dx.doi.org/10.1017/s0007485300000456
- Krafsur, E.S., Wohlford, D.L. Breeding structure of *Glossina pallidipes* populations evaluated by mitochondrial variation. Journal of Heredity, 1999; 90: 635-642. http://dx.doi.org/10.1093/jhered/90.6.635
- Krafsur, E.S., Marquez, J.G., Ouma, J.O. Structure of some East African *Glossina fuscipes fuscipes* populations. Medical and Veterinary Entomology, 2008; 22(3): 222-227. http://dx.doi.org/10.1111/j.1365-2915.2008.00739.x
- Leak, S.G.A. 1999. Tsetse biology and ecology: Their role in the epidemiology and control of trypanosomiasis, pp. 50-291, CABI Publishing, New York. http://books.google.com.ng/books.
- Lenski, R.E. 2001. Evolutionary Rate. Academic Press. Available at: myxo.css.msu.edu/lenski/pdf/2001, EncyGen, Lenski.pdf. (Accessed 24 Feb 2015.
- Librado, P., Rozas J. DnaSP v. 5.0: software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 2009; 25: 1451-1452. http://dx.doi.org/10.1093/bioinformatics/btp187
- Lynch, M., Crease, T.J. The analysis of population survey data on DNA sequence variation. Molecular Biology and Evolution, 1990; 7: 377-394.
- Majekodunmi, A.O., Fajinmi, A., Dongkum, C., Picozzi, K., Thrusfield, M.V., Welburn, S.C. A longitudinal survey of African animal trypanosomiasis in domestic cattle on the Jos Plateau, Nigeria: prevalence, distribution and risk factors. Parasites & Vectors, 2013; 6: 239. http://dx.doi.org/10.1186/1756-3305-6-239
- Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, NY.
- Nei, M. & Li, W.H. Mathematical Model for Studying Genetic Variation in Terms of Restriction Endonucleases". Proceedings of the National Academy of Sciences, 1979; 76 (10): 5269–73. http://dx.doi.org/10.1073/pnas.76.10.5269
- Nei, M., Miller J.C.A simple method for estimating average number of nucleotide substitutions within and between populations from restriction data. Genetics, 1990; 125:873-879.
- Ouma, J.O., Marquez J.G., Krafsur, E.S. Microgeographical breeding structure of the tsetse fly, *Glossina pallidipes* in south-western Kenya. Medical and Veterinary Entomology, 2006; 20: 138–149. http://dx.doi.org/10.1111/j.1365-2915.2006.00609.x
- Ouma, J.O., Marquez, J.G., Krafsur, E.S. Patterns of genetic diversity and differentiation in the tsetse fly *Glossina morsitans morsitans* Westwood populations in east and southern Africa. Genetics, 2007; 130:139–151. http://dx.doi.org/10.1007/s10709-006-9001-0
- Patterson, J. S, Schofield, C. J. 2004. Preliminary study of tsetse fly wing morphometrics: a potential tool for vector surveillance. *IX European Multicolloquium of Parasitology*, Valencia, Spain.
- Pollock, J.N. 1992. Training manual for tsetse control personnel. Vol 1. FAO, Rome.
- Qadeer, M.A., Danbirni, S., Usman, M., Akogun, O. B., Gundiri, M.A., Bobbo A.G. Prevalence of bovine trypanosomiasis in Bassa Local Government Area, Plateau State, Nigeria. Nigerian Journal of Parasitology, 2008; 29 (2): 136-139. http://dx.doi.org/10.4314/njpar.v29i2.37919
- Ravel, S., DeMeeus, T., Dujardin, J.P., Ze´ze´, D.G., Gooding, R.H., Dusfour, I., Sane´, B, Cuny, G., & Solano, P. The tsetse fly *Glossina palpalis palpalis* is composed of several genetically differentiated small populations in the sleeping sickness focus of populations in east and southern Africa. Infection, Genetics and Evolution, 2007; 7(1): 116–125. http://dx.doi.org/10.1016/j.meegid.2006.07.002
- Reid S.R., Kruska, L.R., Deichman, U., Thornton, K.P., Leak, S.G. Human population growth and the extinction of the tsetse fly. Agriculture, Ecosystem and Environment, 2000; 77: 227-236. http://dx.doi.org/10.1016/S0167-8809(99)00103-6
- Saitou, N, Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

- Schauff, M. E. (ed.) 1986. Collecting and preserving insects and mites: Techniques and tools. National Museum of Natural History, Washington, D.C. USA.
- Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America, 1994; 87: 651– 701. http://dx.doi.org/10.1093/aesa/87.6.651
- Slatkin, M. Gene flow in natural populations. Annual Review of Ecology and Systematics, 1985; 16: 393-430. http://dx.doi.org/10.1146/annurev.es.16.110185.002141
- Solano, P., De La Rocque, S., De Meeus, T., Cuny, G., Duvallet, G., Cuisance, D.Microsatellite DNA markers reveal genetic differentiation among populations of *Glossina palpalis gambiensis* collected in the agro-pastoral zone of Sideradougou, Burkina Faso. Insect Molecular Biology, 2000; 9: 433–439. http://dx.doi.org/10.1046/j.1365-2583.2000.00205.x
- Stearns, S.C., Koella, J. C. 2008. Evolution in health and disease. New York: Oxford University Press.
- Tamura, K., Dudley, J., Nei, M., Kumar, S. MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 2007; 4: 1596-1599. http://dx.doi.org/10.1093/molbev/msm092
- Thompson, J.D., Gison, T.J., Plewniak, F., Jeanmough, F., Higgins, D.G. The Clustal X. windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 1997; 25: 4876-4882. http://dx.doi.org/10.1093/nar/25.24.4876
- WHO Media Centre, (2012) Fact sheet No. 259: Trypanosomiasis, Human African (sleeping sickness).
- Zhang, Z., Schwartz, S., Wagner, L., Miller, W. "A greedy algorithm for aligning DNA sequences". Journal of Computational Biology, 2000; 7(1-2): 203-14. http://dx.doi.org/10.1089/10665270050081478