MOLECULAR CHARACTERIZATION OF SIMULIUM DAMNOSUM COMPLEX IN AN ENDEMIC FOCUS OF GURARA RIVER, KAGARKO LGA, KADUNA STATE, NIGERIA

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BY

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DEPARTMENT OF BIOCHEMISTRY
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AUGUST, 2018

DECLARATION

I declare that the work in the dissertation entitled "Molecular Characterization of *Simulium damnosum* complex in an Endemic Focus of Gurara River, Kagarko LGA, Kaduna State, Nigeria" has been performed by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Noah Oluwasegun, ASHADE _____ _____

Signature Date

CERTIFICATION

This dissertation entitled "MOLECULAR CHARACTERIZATION OF SIMULIUM DAMNOSUM COMPLEX IN AN ENDEMIC FOCUS OF GURARA RIVER, KAGARKO LGA, KADUNA STATE, NIGERIA" by Noah Oluwasegun ASHADE, meets the regulations governing the award of the degree of Master of Science (M.Sc.) of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

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ABSTRACT

The black flies (Diptera: Simuliidae), vectors of onchocerciasis are difficult to be distinguished morphologically. This study was carried out to characterize the residing *Simulium damnosum* complex collected along Gurara River focus, Kagarko LGA of Kaduna State, Nigeria. Morphological identity was determined using WHO/OCP colour classification criteria and transmission potential of the obtained adult female *S. damnosum* complex was done under stereo microscope as the first approach. Similarly, genomic DNA extractions and PCR were used to identify *S. damnosum* complex collected from morphologically verified populations based on ITS, ITS1, and ITS2 of nuclear ribosomal gene (NRG); and 16S rRNA of mitochondrial ribosomal gene (MRG). In addition, bioinformatics involving the use of BLAST and molecular evolutionary genetics analysis version six software (MEGA 6.06) were applied in the characterization. Results of morphological identification reflected the black flies to be *S. damnosum* sub-complex (i. e., savanna group) with zero transmission potential as the monthly transmission potential (MTP) was calculated to be zero. Whereas the PCR mediated assays yielded different amplicon sizes on agarose gel electrophoresis. The result of the ITS amplifications warrants further comments for heterozygous alleles – indicated by electrophoregram amplicon 2A and 2B of ITS/NRG. Also, the BLASTN results of ITS/NRG amplicon 2A and 3 nucleotide sequence suggested they might be new cryptic species as they did not have any corresponding sequences from the NCBI GenBank database. Meanwhile, the phylogenetic and molecular evolutionary analyses gave topology of trees that are quite similar for ITS, ITS1 and ITS2 of NRG, except that the tree topology for 16S rRNA of MRG are different from others. The different PCR amplicon sizes examined from the results was used to bypass the cytological examination of larvae for identification. However, *S. damnosum* clones (suspected to be *S. damnosum sensu stricto*), *S. sirbanum* (both savanna group); and *S. sanctipauli* (a forest group) were revealed as closely related sister members for ITS, ITS1 and ITS2 phylogenies. These results can also facilitate the understanding of the species groups of the black flies available at the study focus. At molecular level, the ITS marker was of the most identification utility among the markers of NRG, while by phylogenetic, the ITS, ITS1 and ITS2 markers gave phylogenies with better identification insight than the 16S rRNA marker from this study.

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LIST OF ABBREVIATIONS

A: Adenine

BLAST: Basic Local Alignment Search Tools

C: Cytosine

dam: *Simulium damnosum* sub-complex morphospecies Identification

ddH_2O: deionized distilled water

ddNTPs: dideoxyribonuleotide triphosphates

DNA: Deoxyribonucleic Acid

dNTPs: Deoxyribonucleotide triphosphates

DP: Dissection for Parousity of the Vector flies

DTCS: Dye Terminator Cycle Sequencing

EDTA: Ethylene diamine tetra-acetate

FCT: Federal Capital Territory

G: Guanine

gDNA: Genomic deoxyribonucleic acid

HB-A: Human Bait A

HB-B: Human Bait B

HB-C: Human Bait C

HB-D: Human Bait D

HCl: Hydrochloric Acid

ITS: Internal Transcribed Spacer

ITS-1: First Internal Transcribed Spacer

ITS-2: Second Internal Transcribed Spacer

LGA: Local Government Authority

MEGA: Molecular Evolutionary Genetics Analysis

ML: Maximum Likelihood Tree Method

MRG: Mitochondrial Ribosomal Gene

MSA: Multiple Sequence Alignment

MTP: Monthly Transmission Potential

NCBI: National Center for Biotechnology Information

NITR: Nigeria Institute of Trypanomiasis (and Onchocerciasis) Research

NP: Nulliparous vector flies

NRG: Nuclear Ribosomal Gene

n/a: Not available or not sequenced

OCP: Onchocerciasis Control Programme

PCR: Polymerase Chain Reaction

PM: Picomole

PR: Parous vector flies

RNA: Ribonucleic Acid

san: *S. sanctipauli* morphospecies Identification

s.a.n. w/a: Species available in Nigeria and other West Africa

squ: *S. squamosum* morphospecies Identification

T: Thymine

TAE: Tris-Acetate EDTA

TC: Total caught of adult female *S. damnosum* complex (vector flies)

TE: Tris-HCl EDTA

UV: Ultraviolet

WHO: World Health Organization

WHO-OCHRT: WHO-Onchocerciasis Control Human Resource Training

yah: *S. yahense* morphospecies Identification

16S rRNA: 16S ribosomal Ribonucleic Acid

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Onchocerciasis is a chronic parasitic and debilitating neglected tropical disease caused by the filarial nematode parasite – *Onchocerca volvulus* and it is transmitted by female black fly, *Simulium* (WHO, 1995). Onchocerciasis or river blindness is characterized by eye infection and skin lesion with severe troublesome itching. It is a chronic, communicable, slowly progressive, parasitic disease which has being rated as the second-leading infectious cause of blindness worldwide, after trachoma responsible for approximately 500,000 people blind annually (Mboera, 2010; WHO, 2011; Winthrop *et al.*, 2011).

In Nigeria, the disease is widespread and it is a cause of blindness in most rural communities of the northern part of the country. Of all the countries of the world, Nigeria has the largest number of persons with onchocerciasis, accounting for about a third of the global prevalence with about 7 million people infected, 1.5 million blinded and about 40 million at risk of infection (WHO, 2001). Onchocerciasis is therefore endemic to Nigeria (Evans *et al.*, 2011) with the highest burden and diversity of neglected tropical diseases (NTDs) in sub-Saharan Africa (Okorie *et al.*, 2014). The disease is transmitted to humans by the bite of infected adult female black flies of the genus *Simulium*, and is usually characterized by chronic skin disease, severe itching, and eye lesion that may progress to complete blindness (CDC, 2013), hence the disease has blindness in its commonly referred name – "river blindness".

The presence and abundance of the vector – *Simulium damnosum sensu lato* – has been observed to be directly related to the prevalence of human onchocerciasis (Crosskey, 1981). Onchocerciasis disease may be mild (dermatitis) or severe leading to visual impairment that can

progress into complete blindness which is caused by the human immune response to microfilaria released by female adult worms as they move across subcutaneous tissue and throughout the body (Gonzalez *et al.*, 2009) of the human host.

Black flies rank second in medical and veterinary importance among insects (Currie and Adler, 2008). And more than 25 of the world's 2142 species are vectors of the causal agent of human onchocerciasis (Adler and Crosskey, 2013).

There has been no sufficient works on molecular identifications of the cytospecies presumed to be vectors of *Onchocerca volvulus* in Nigeria as such. And it will be surprising that so little is known about the role and importance of the different cytospecies in the transmission of *O. volvulus* in Nigeria (Post *et al.*, 2011). While, most works pertaining to identification and/or characterization were more on the cytological identification of the larval developmental stage of these vector flies than on morphological identification of the adult vectors. Despite the fact that there has been research and publications on Onchocerciasis and its causative vectors – the *Simulium damnosum* complex, its life cycle and that of the parasite as well as the morphology, cytotaxonomy and infectivity of the vectors and its impacts; not much has been done in the area of molecular characterization of the vector of human onchocerciasis in Nigeria. The appropriate understanding of the medical, economic, and ecological importance of black flies relies on correct identification of species (Mafuyai *et. al.*, 1996; Ibeh *et. al.*, 2006; Ibeh *et. al.*, 2008; Adeleke *et. al.*, 2011; Post *et. al.*, 2011).

Meanwhile, the old traditional approach for taxonomy of black flies has been known to be impeded by a high degree of morphological uniformity, especially with the presence of cryptic biodiversity, otherwise recognized by details of chromosomal banding patterns that were not clearly possible with morphotaxonomy of the vector (Pramual *et al.*, 2011). Even at this, there is

still a limitation to the use of polytene chromosomes in the Simulidae, as it does not directly affect the culprit stage of the disease transmission – the adult female black fly – and also the need for the specialty of 'black flies taxonomists' that are scarcely available from my personal experience from this study.

However, other limitation to the use of polytene chromosomes is their under-replication in pupal and imago/adult tissues, making them less amenable to detailed band by band analysis. This limitation is problematic in linking the females of pests and vectors with the cytological segregates, based on larvae, at the breeding grounds (Adler *et al.*, 2010).

Hence, the epidemiological significance of many cytoforms of this insect-vector remains obscure, partly because identification of adult females is necessary for transmission studies, but this is rather possible only in few particular cases (Adler *et al.*, 2010).

To overcome these technical limitations, other approaches that have been used include: isozyme analysis (Meredith and Townson, 1981; Snyder, 1982; Snyder and Linton, 1983), DNA probes (Post and Crampton, 1988), random amplified polymorphic DNA (RAPD) (Duncan *et al.*, 2004) and inter simple sequence repeats (ISSRs) (Dusinsky *et al.*, 2006). Also, studies revealed that at least for some insects, DNA barcodes provide more information than do restriction fragment length polymorphism (RFLP) markers (Armstrong and Ball, 2005; Barr *et al.*, 2012).

Nevertheless, molecular methods were gradually coming in as an alternative remedy for the insufficiencies of the earlier traditional methods of vector identification. Hence, in 1993 a molecular method employed was able to detect at least two of the sibling species of the *S. damnosum s. l.* complex in Nigeria based on size polymorphism by PCR amplification of the entire internal transcribed spacer (ITS) region (Brockhouse *et al.*, 1993). So, advancements in

recombinant DNA technology can facilitate the development of simple and rapid molecular tools for the identification of sibling species (WHO, 2007), even in *Simulium damnosum* complex.

With reference to mosquitoes which have attracted more molecular studies than black flies, recent advances in DNA-based technology have made a wide range of molecular characteristics and markers available for taxonomic and systematics studies of mosquitoes where the ribosomal DNA is one of the regions that have received particular attention (Goswami *et al.*, 2005), of which the genus of *Simulium* – commonly called black flies may not be an exception.

Molecular methods, such as DNA barcoding, have been used to understand relationships between populations or between closely related species and also show utility for revealing cryptic diversity, which is one of the best methods for confirming taxonomic identification at molecular level (Sirin and Fidan, 2014).

The ITS is the spacer DNA situated between the small subunit ribosomal RNA and large subunit rRNA genes in the chromosome. However, ITS2 from the nuclear ribosomal DNA (rDNA) cluster, located on a single chromosome pair for most black fly species that were somehow earlier neglected (Rothfels, 1979), constituted one of the recent valuable additions to molecular research (Thanwisai *et al.*, 2005) that is still employed up to now. Also, there is a rather high degree of sequence similarity within copies of the rDNA unit among individuals in an interbreeding population or species, called homogenization or molecular drive (Linton *et al.*, 2002; Oshaghi *et al.*, 2008). The rDNA region consists of simultaneous repeated transcriptional units composed of structural genes (28S, 5.8S, and 18S), intergenic spacers (IGS) and two internal transcribed spacers, ITS1 and ITS2. Among these, ITS2 represents a specific interest because intra-specific sequence variation in this relatively quickly evolving region is considered to be than inter-specific variation. This makes ITS2 a good candidate for a DNA-based species

diagnostic assay (Goswami *et al.*, 2005; Oshaghi *et al.*, 2008). In fact in the studies of closely related anopheline mosquitoes, the ITS2 region has been successfully used by other researchers for identification purposes (Porter and Collins, 1991; Paskewitz *et al.*, 1993). Hence, the application can be extended to other insect vectors and in particular, black flies as well.

Despite the contributions of more than 50 years of chromosomal study of the Simuliidae, the current trend is a movement away from cytogenetic enquiry and toward molecular investigation (Adler *et al.*, 2010). And it is capable of probing deeper into the basis of genetic variability that cytogenetic and other earlier traditional methods of investigations in Simuliidae could not give.

1.2 STATEMENT OF RESEARCH PROBLEM

About 96% of the 120 million people globally at risk of Onchocerciasis are in Africa. A total of 18 million people are infected with the disease, of which 99% live in Africa. Of those infected with the disease, over 6.5 million suffer from severe itching or dermatitis and 270,000 from blindness (WHO, 2018; www.who.int/mediacentre/factsheets/fs095/en/).

Onchocerciasis has been a public health problem in Nigeria for over three decades leading to disabilities and loss of person-hours with its negative consequences on health and economy of the endemic area (Ogbonna, 2015). Kaduna State has been reported to be one of the states of the northern bio-geographical locations of Nigeria where the disease is severe, leading to visual impairments leading to blindness.

Black flies are a challenge for traditional taxonomy because of their small size and structural homogeneity (Adler *et al.*, 2004). It has been stated that the correct morphological identification of black fly species paramount for control and epidemiological studies is extremely difficult due to their morphological homogeneity (Hernandez-Triana *et al.*, 2014), meanwhile the banding

patterns of the polytene chromosomes from the black fly larval silk gland was been considered indispensable in addressing this challenge. But the further challenge was that the band by band patterns of the larvae' polytene chromosomes which are typically workable only for mid – to late instar larvae require skilled personnel for clarification. Hence, there will be need for other approaches that can give adequate clarity to this constant challenge encountered in identification of the vector of onchocerciasis.

1.3 JUSTIFICATION OF THE STUDY

One of the major obstacles for studies of the biology, ecology, and behavior of vector of human onchocerciasis has been lack of reliable and efficient means of identification of the many species or the sibling species of the *Simulium damnosum* complex at the adulthood stage. Although cytogenetic analyses of the immature stages of the vector (pupae and especially larvae) have been employed long ago; this approach is highly limited as it does not directly address the imago/adult stage of the vector – adult female black flies (*Simulium damnosum* complex) – responsible for transmission of human onchocerciasis. Hence, there is need for molecular analysis of the adult stage of the *S. damnosum* complex since the molecular characterization of *S. damnosum* complex is poorly studied in Nigeria.

Accurate identification of Simuliidae is becoming increasingly significant as more species become the target of pest and vector control operations, or the subject of epidemiological studies on the transmission of the black fly-borne parasites of man, *Onchocerca volvulus* and *Mansonella ozzardi* (Adler *et. al.,* 2004; Hernandez-Triana *et. al.,* 2014).

The ability to identify adult female *S. damnosum* complex, using molecular techniques as insect vector species is of obvious importance in human onchocerciasis, to understand their different

roles in pathogen transmission dynamics or to reduce consequently the incidence of human onchocerciasis through selective vector control.

Although, there were some reported studies on species composition on adult *S. damnosum* complex in some river systems in Nigeria (Mafuyai *et al.*, 1997; Ibeh *et al.*, 2008; Oluwole *et al.*, 2009; Adeleke *et al.*, 2011), the composition and molecular characterization of the biting adult female *S. damnosum* complex around Gurara River in Kaduna State have not been reported. Therefore, this study was carried out to fill that gap.

1.4 AIM AND OBJECTIVES OF THE STUDY

The aim of this study is to characterize and confirm the molecular and phylogenetic characteristics of residing *Simulium damnosum* complex in the endemic focus of Gurara River, Kagarko LGA of Kaduna State, Nigeria.

The specific objectives of the study were to:

1. Investigate the morphological identity and the transmission/infectivity potentials of the adult female *Simulium damnosum* complex obtained from the study focus.
2. Evaluate the utility of internal transcribed spacer (ITS) Nuclear Ribosomal Gene in discriminating amongst the collected adult female *S. damnosum* complex.
3. Examine the possibility of using 16SrRNA Mitochondrial Ribosomal Gene to facilitate adult female *S. damnosum* complex identification.
4. Determine the evolutionary relationships of the DNA sequence results through Phylogenetic and Molecular evolutionary analyses.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 ONCHOCERCIASIS: MEANING AND CAUSES

Human Onchocerciasis, commonly known as river blindness has *Onchocerca volvulus* (Filarioidea: Onchocercidae) as its causative agent which is transmitted by female black flies of the genus *Simulium* (Diptera: Simuliidae) (WHO, 1995). This disease is transmitted to humans by the bite of infected adult female black flies of the genus *Simulium*, and its resultant effect is usually characterized by chronic skin disease, severe itching, and eye lesions that can progress to complete blindness (CDC, 2013). The disease is called river blindness because the black flies that transmit the disease are found around riverside areas, where they breed in well oxygenated fast – flowing water, broken up by vegetation (WHO, 1995; Hougard *et. al.*, 1997).

Onchocerciasis disease may be mild (dermatitis) or severe leading to visual impairment and/or blindness which is caused by the human immune response to microfilaria released by female adult worms as they move across subcutaneous tissue and spread throughout the body (Gonzalez *et al.*, 2009).

The causative agent of human onchocerciasis is the nematode worm belonging to the genus Onchocerca. Amongst the 34 *Onchocerca* species known, only *Onchocerca volvulus* (Nematoda: Filarioidea) is anthropophilic, while others are zoophilic; hence, humans are the only known reservoirs. The occurrence of other filarial in sympatric with Onchocerciasis has been documented in the Americas and in Africa with Nigeria inclusive (Gonzalez *et al.*, 2009; Morales-Hojas, 2009; Tang *et al*., 2010).

Onchocerciasis is a chronic parasitic infection with a public health and socioeconomic problem of considerable magnitude in many Sub-Saharan African countries (WHO, 1997). The disease is

the fourth leading cause of preventable blindness and second cause of infectious blindness after trachoma (Hodgkin *et al*., 2007; Winthrop *et al*., 2011). With regards to Africa, onchocerciasis is seen as a major cause of about 60% of blindness in different parts of Africa where it affects the socio-economic development of people (Abiose, 1993; WHO, 1995).

2.2 PREVALENCE AND GEOGRAPHICAL SPREAD OF ONCHOCERCIASIS

Prior to commencement of control strategies for onchocerciasis, an estimated 125 million people in 37 disease-endemic countries were at risk of infection and 96% of them are in thirty sub-Saharan Africa (Figure 2.1), one in Arabian Peninsula (Yemen) and six Latin American countries (Figure 2.2) (WHO, 1997; Hodgkin *et al*., 2007). About 99% of the 18 million infected people live in Africa where at least one million are either blind or severely visually impaired and 270,000 blind cases were recorded (WHO, 2018). Guatemala became the fourth country in the world to be verified and declared free of onchocerciasis by WHO in July 2016 after Mexico (third) in July 2015, Ecuador (second) in September 2014, and Colombia (first) in April 2013 after successfully implementing elimination activities for decades (Lawrence and Sodahlon, 2018).

Later on, the comprehensive researches supported the fact that onchocerciasis occurs mainly in tropical areas of the world, particularly with increase in African countries from 30 to 31 countries. So, about 99% of infected people live in 31 countries of Sub-Saharan Africa: Angola, Benin, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Republic of Congo, Côte d'Ivoire, Democratic Republic of the Congo, Equatorial Guinea, Ethiopia, Gabon, Ghana, Guinea, Guinea-Bissau, Kenya, Liberia, Malawi, Mali, Mozambique, Niger, Nigeria, Rwanda,

Senegal, Sierra Leone, South Sudan, Sudan, Togo, Uganda, Tanzania (WHO, 2014; Lawrence and Sodahlon, 2018, WHO, 2018).

Onchocerciasis is a chronic parasitic infection with public health and socioeconomic problem of considerable magnitude in many Sub-Saharan African countries (WHO, 1997). The disease is the fourth leading cause of preventable blindness and second cause of infectious blindness after trachoma (Hodgkin *et al*., 2007; Winthrop *et al*., 2011). Onchocerciasis is the cause of about 60% of blindness in different parts of Africa where it affects the socio-economic development of the people (Abiose, 1993; WHO, 1995).

In West Africa, Onchocerciasis is transmitted exclusively by sibling species of the *S. damnosum* Theobald complex (Diptera: Simuliidae) with blindness rates reaching as high as 15% in the savanna where up to 100% of adults may be infected. Infection rates can be similarly high in the forest bioclimatic zone and the ocular manifestation is usually very much less than in the savanna with blindness rates typically around 0.5%. Onchocercal skin disease can also be a significant socioeconomic problem (Duke, 1990; Remme, 1995).

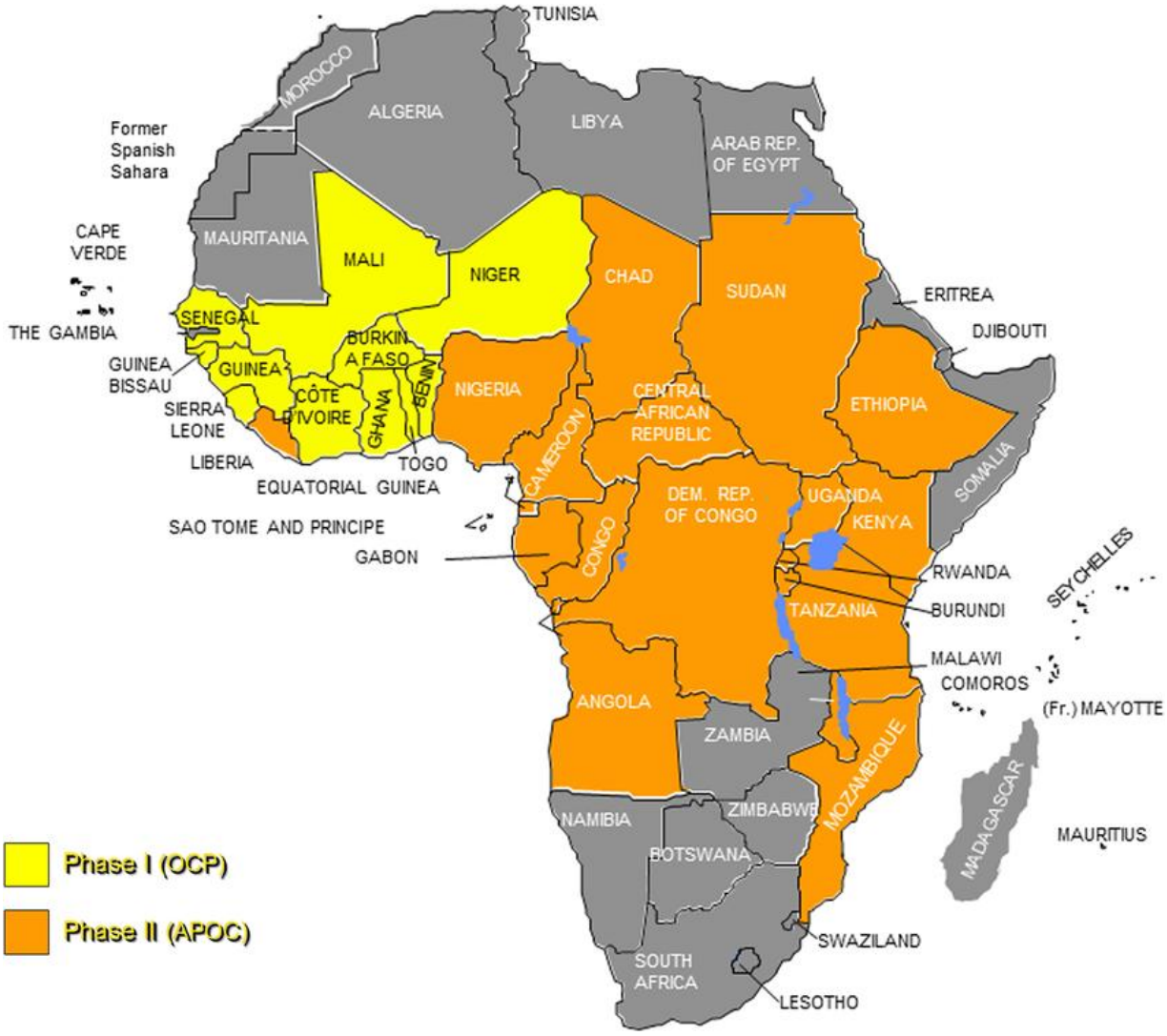


Figure 2.1: Onchocerciasis-endemic countries in Africa, showing countries covered by the Onchocerciasis Control Programme (OCP) and initially by Africa Programme for Onchocerciasis Control (APOC) Map from 2010. Note that South Sudan gained independence in 2011 after the information has been released, becoming the 20th APOC country.

Source: Tropical Disease Research, TDR news pre-view No. 79, December 2010, pp. 19. (www.who.int/tdr/publications/tdrnews/en/).

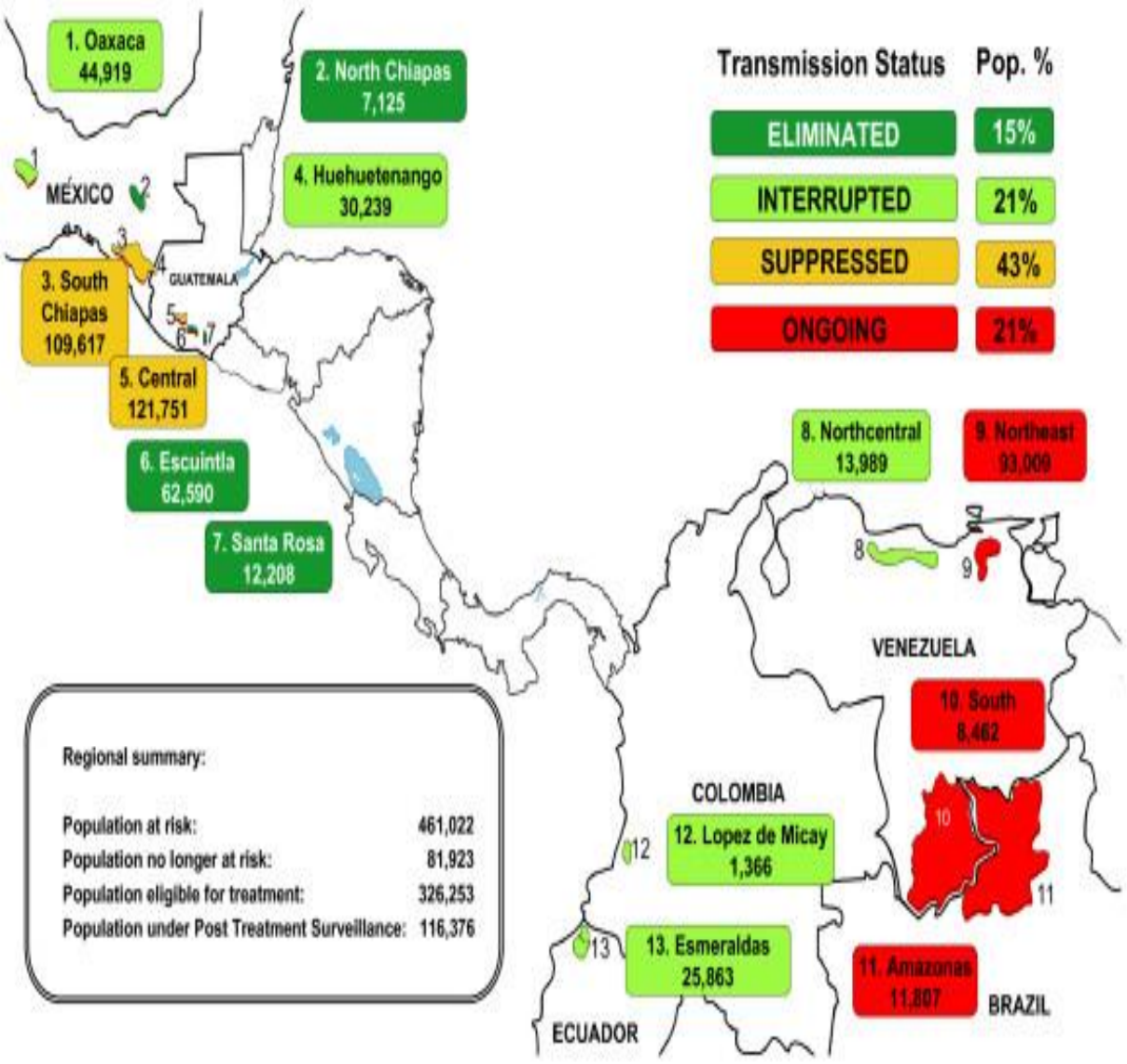


Figure 2.2: Distribution of onchocerciasis in Latin America in 2010. Biannual treatment was adopted in the Onchocerciasis Endemic Countries of Americas (OECA). In the Americas, onchocerciasis was endemic in six countries out of which some foci that include Colombia, Ecuador, and Mexico have been declared free of onchocerciasis. And WHO elimination efforts still continues in other foci countries such as Brazil and Venezuela.

Source: Guvstasen *et al.*, (2011).

2.2.1 Onchocerciasis in Nigeria

Onchocerciasis is endemic public health problem in Nigeria with a national spread (Edungbola *et al.*, 1987). Nigeria accounts for one third of the global estimates of onchocerciasis, with about 7 million infections, and endemicity involving about 32 states including the Federal Capital Territory, Abuja (FMoH, 2012). Researchers have however revealed that the worst cases of river blindness were found at the Southern part of Adamawa State around Katsina-Ala River Basin, down to Kogin Baba, near the Taraba River (Akogun, 1992).

Onchocerciasis is listed in the group of 10 neglected tropical diseases (NTDs) by the Nigerian Federal Ministry of Health. The strategic goal of Federal Ministry of Health in Nigeria is to progressively reduce morbidity, disability and mortality due to NTDs using integrated and cost-effective approaches with a view to eliminating them (FMoH, 2012). The map of Rapid Epidemiological Mapping of Onchocerciasis (REMO) (figure 2.3) showed the areas in Nigeria where Community Directed Treatment with Ivermectin (CDTI) was needed as at 2008, as released by Africa Programme on Onchocerciasis Control (APOC) management. The study of Okorie *et al.* (2014) as well revealed that Nigeria carries the highest burden and diversity of neglected tropical diseases (NTDs) in Sub-Saharan Africa.

Onchocerciasis is one of the five most prevalent neglected tropical diseases in the world, and each is frequently treated with mass drug administrations (Keenan *et al.*, 2013). The distribution of human onchocerciasis in Nigeria was mapped by Crosskey (1981) and subsequently between 1994 and 1996 the disease was re-surveyed by the National Onchocerciasis Control Programme in Nigeria (NOCP), using REMO, which is based on the prevalence of nodules in selected communities. This effort then yielded preliminary maps that were then refined and completed to

give a detailed working picture of onchocerciasis across Nigeria (Gemade *et al.*, 1998; Noma *et al.*, 2002; WHO-OCHRT, 2004; Basanez *et al.*, 2006).

Although approximately 40% of all the people blinded by *Onchocerca volvulus* are Nigerians, almost nothing was known about the various cytospecies of the black fly vectors present in Nigeria until the year 1981. The Nigerian cytospecies of the *S. damnosum* complex were first reviewed by Crosskey (1981). Gregory (1982), in his brief review of the vectors of *O. volvulus* in Nigeria, reported that six cytospecies had been identified in the country: *S. damnosum s. s.*, *S. sirbanum*, *S. sudanense*, *S. soubrense*, *S. sanctipauli* and *S. squamosum*. Four of these had already been reported by Crosskey (1981), but Gregory (1982) was the first to report *S. soubrense* and *S. sanctipauli* from Nigeria.

However, the study of Post *et al.*, (2011) had of recent recorded nine different cytoforms from Nigeria which were: *Simulium damnosum sensu stricto* Nile form, *S. damnosum s. s.* Volta form, *S. sirbanum* Sirba form, *S. sirbanum* Sudanense form, *S. soubrense* Beffa form, *S. squamosum* A, *S. squamosum* B, *S. squamosum* C and *S. yahense* typical form.

The cytospecies mentioned above are presumed to be vectors, although there has been almost no sufficient work on molecular identifications of the vectors of *O. volvulus* in Nigeria as such (Post *et al.*, 2011). Most works pertaining to identification and/or characterization were more on the cytological identifications of the larval developmental stage of these vector flies than morphological identification of the adult stage of the vector flies.

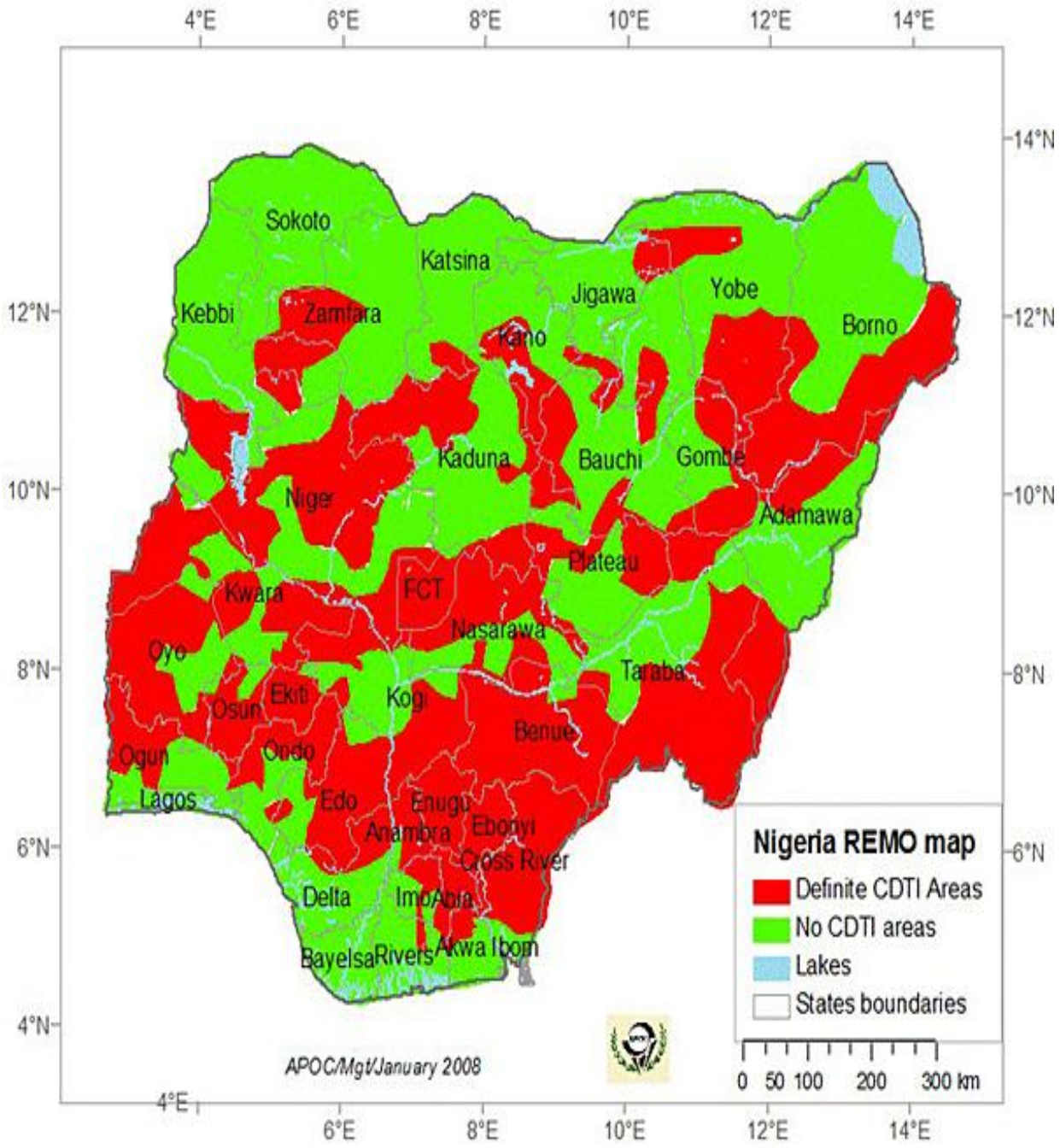


Figure 2.3: Rapid Epidemiological Mapping of Onchocerciasis (REMO) in Nigeria, showing areas (in red) where Community Directed Treatment with Ivermectin (CDTI) is needed as at 2008 when this information was released by APOC management.

Source: (**www.who.int/apoc/countries/nga/en).**

2.3 BLACK FLIES (DIPTERA: SIMULIIDAE): VECTOR OF CAUSATIVE AGENT OF ONCHOCERCIASIS

Black flies constitute a large and important family (Simuliidae) of Diptera (Adler, 2008) with about 2,219 species of black flies in the world as at year 2016, consisting of about 2,204 living as the total number of described species in the world and 15 extinct (Adler and Crosskey, 2016). This is representing a net increase of 27 living and 3 fossil species from the previous revision of the authors in 2015 (Adler and Crosskey, 2015; 2016).

The family Simuliidae is worldwide in distribution and is found only in most fast flowing streams and rivers, extending from the tropics to the Arctic Circle, even in desert ecosystems and in high polar latitudes, and coral islands (Doreen, 2006). The significance of these insects is manifested in both aquatic and terrestrial ecosystems and as economically important pests and vectors of disease agents to birds and mammals, including domestic animals and humans (Cherairiai *et al.*, 2014).

Black flies (Diptera: Simuliidae) represent 0.2% of all described species of insects and 1.3% of all described species of Diptera in the world. The species of black flies are distributed in the six zoogeographic regions of the world as follows: Afrotropical (11%), Australasian (12%), Nearctic (11%), Neotropical (16%), Oriental (17%), and Palearctic (33%). The subgenus *Simulium* consists of 24 species groups representing 439 (22%) of all black fly species worldwide, and includes most of the major pest (Adler, 2008). The black fly fauna of the Palearctic region is fairly well known, and that of the Afrotropical elements are poorly represented (Cherairiai *et al.*, 2014). However, the knowledge of Nigerian simuliids is not sufficient to accurately enumerate and identify species of black flies across the country covering both the vectors of savanna and forest strains of human onchocerciasis in the nation.

Afrotropical black flies of the *Simulium damnosum* Theobald complex include species responsible for the transmission of *Onchocerca volvulus* Leuckart (Nematoda: Onchocercidae), the filarial nematode that causes human onchocerciasis in Africa and Yemen. More than a dozen sibling species of the *S. damnosum* complex have been described and are still recognized on the basis of fixed inversion differences observed in polytene chromosomes of the larval silk glands. The challenge with adult individuals of these vector individuals is that they do not have easily workable polytene chromosomes and are difficult to identify to cytospecies level (Boakye, 1993; Morales-Hojas *et al.*, 2002).

The Simuliidae is a group that has important functions in the aquatic food chain and in the breakdown of organic substances, and is also a group of aggressive ecto-parasites. Females of many species are blood feeders and are responsible for various veterinary, medical and economic problems, especially in certain regions where mass emergences of biting adults take place (Crosskey, 1990).

2.3.1 Life cycle of Black fly (Diptera: Simuliidae)

Black flies undergo a type of development known as "complete metamorphosis" (Figure 2.4) in their general life cycle. After taking a blood meal, adult females develop a single batch of 200 – 500 eggs. Most species lay their eggs in or on flowing water, but some attach them to wet surfaces such as blades of aquatic grasses. The length of time it takes an egg to hatch varies greatly from species to species. Eggs of most species hatch in 4 – 30 days, but those of certain species may not hatch for a period of several months or longer (Hill *et al.*, 2010).

The number of larval stages ranges from 4 – 9, with 7 being the usual number. The duration of larval development ranges from 1 – 6 months, depending in part on water temperature and food supply. Larvae remains attached to stationary objects in flowing water, held on by silken threads

extruded from glands located at the end of the bulbous abdomen. Depending on species, mature larvae range from 5 – 15 mm in length and may be brown, green, gray, or nearly black in colour. They possess large head that bears two prominent structures known as "labral fans" that project forward. The labral fans are the primary feeding structures, filtering organic matter or small invertebrates out of the water current (Hill *et al.*, 2010).

The last larval stage molts into a non-feeding pupal stage which may be formed following spring or summer, typically in the same site as the last stage larva, but may develop downstream following larval drift with the current. Pupae remain attached to stationary objects in flowing water as well. They are typically orange and appear mummy-like because the developing wings and legs are tightly attached to the body. Pupae of many species produce a delicate, silken "cocoon" of varying density, weave, and size that partially or nearly entirely encloses them; other species produce hardly any cocoon at all (Hill *et al.*, 2010).

Adults emerge from the pupal stage 4 – 7 days after and live for a few weeks. The number of generations completed in one year varies among species, with some having only one generation, but most species that are major pests complete several generations per year. Black fly larvae and pupae develop in flowing water, typically non-polluted water with a high level of dissolved oxygen. Suitable aquatic habitats for black fly larval development vary greatly and include large rivers, icy mountain streams, trickling creeks, and waterfalls. Larvae of most species typically are found in only one of these habitats (Hill *et al.*, 2010).

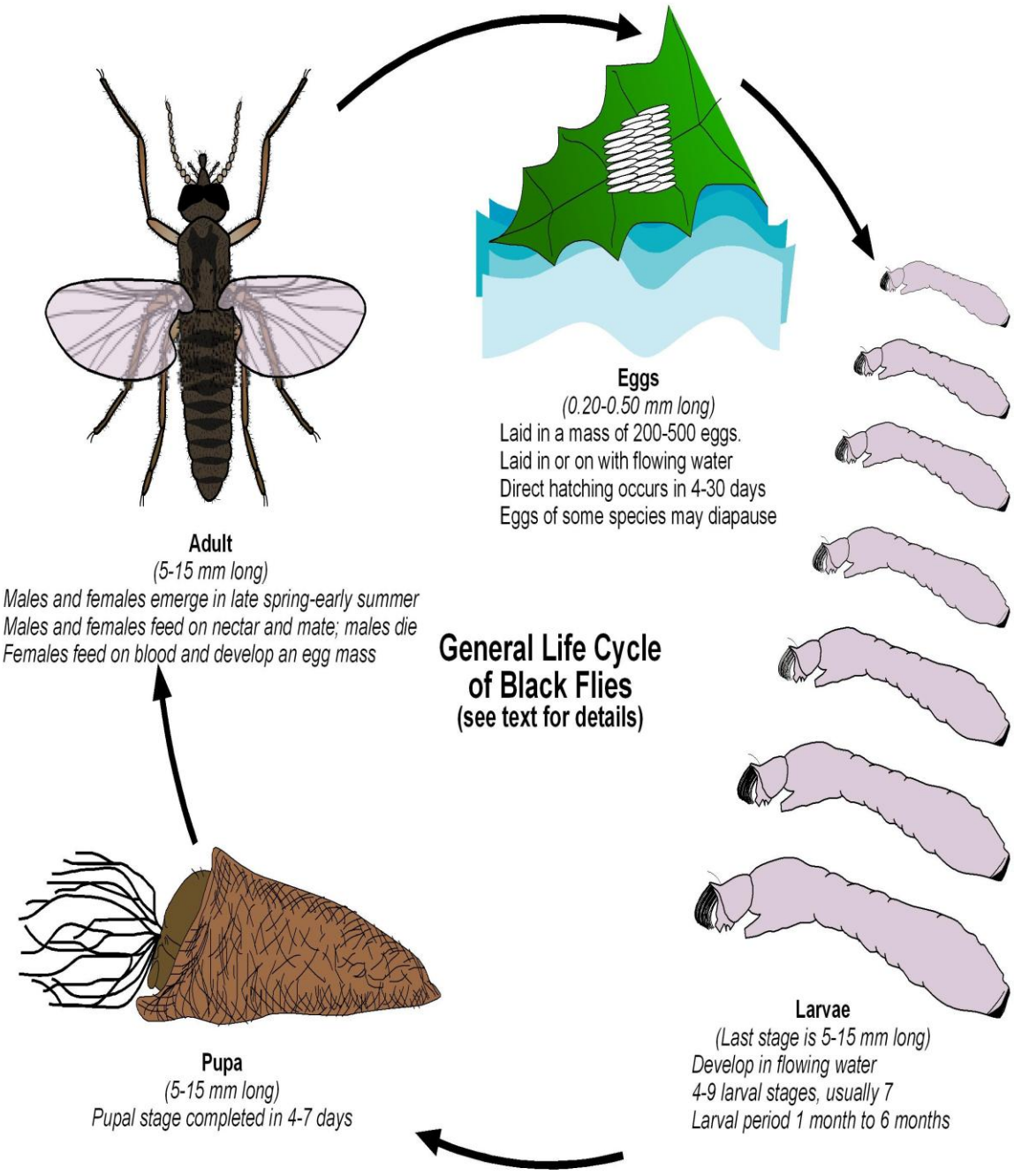


Figure 2.4: General Life Cycle of Black flies (from Purdue Extension Entomology, Purdue University)

Source: https://extension.entm.purdue.edu./publichealth/insects/blackfly.html.

2.3.2 Common Names for Black flies (Diptera: Simuliidae)

Black flies (Diptera: Simuliidae) have many common or vernacular names in different local languages in many parts of the world, especially where they are bloodsucking pests. Some of these vernacular names apply to Simuliids generally and others to a particular species (Davies, 2004). Amongst some Sub-ethnic local languages in Nigeria for instance, Yorubas call it "Amukuru", "Kpu kpu" in Igbo lands, "Kurukundu" or "Tsando" among the Hausas depending on their localities, while, Igalas call it "Ita dudu" (Personal Communications/findings). However, a more comprehensive documented vernacular/common names in some part of Nigeria is given in appendix III.

2.3.3 Classification of Black flies (Diptera: Simuliidae)

Black flies (Diptera: Simuliidae) are insects that form the family *Simuliidae*, for which the position in animal classification is Phylum: Arthropoda, Class: Insecta, Order: Diptera, Suborder: Nematocera, Family: Simuliidae and the Genus: *Simulium* with the common name – black flies. The *Simuliidae* are a cosmopolitan family with representatives in virtually every region of the world where sufficient running water allows the immature stages to develop (Crosskey, 1990).

There are four large genera of black fly which are of medical, veterinary and economic importance in many parts of the world. These are *Simulium*, *Austrosimulium*, *Prosimulium* and *Cnephia*. *Simulium* is the largest genus with 1,400 species arranged in 41 subgenera (Crosskey and Howard, 2004) and it has the most widespread distribution in all.

The history of Simuliids taxonomy is marked by an enormous amount of misidentification, not because of the incompetence of its practitioners but because of the extraordinary similarity of many species by the morphological appearance of the adult stages, sometimes especially when their early stages are still unknown and even when they are known. Some misidentifications,

however, have been serious and/or long persistent and still have some continuing potential for misunderstanding. As much of this was done long ago, some school of thought considered it would be unrealistic and pointless to attempt to capture every instance which bedevils Simuliids taxonomy literature (Adler and Crosskey, 2015).

According to Adler and Crosskey (2016), a total of 2,219 formally named species are listed as per the present knowledge which includes 2,204 living and 15 fossils representing a net increase of 27 living and 15 extinct species from 2015 revision.

2.3.4 Sibling Species/Cytospecies, Cytotypes and Cytoforms of Black flies (Diptera: Simuliidae)

Among many Diptera genera, such as *Drosophila, Simulium, Anopheles, Aedes, Sciara* and *Chironomus*, the populations within a morphologically defined species do not interbreed. These morphologically-similar, reproductively-isolated species within a taxon are known as cryptic, sibling or isomorphic species, and the taxon as a whole as a species complex. Sibling species are also found in other animal groups (WHO, 2007).

Sibling species complexes are reproductively distinct but cannot be distinguished by morphological features alone and require alternative methods for identification. It is important to distinguish these species in disease vector control programmes as the different species may exhibit differences in ecology, vectorial capacity and response to control measures (White, 1972).

Sibling species are pairs or groups of closely related species that are morphologically indistinguishable but reproductively isolated and live sympatrically. Berlocher (1978) called them cryptic or isomorphic species. And about 40 sibling species have been recognized within the *Simulium damnosum* complex alone (Sorungbe and Adegoke, 2012). Sibling species are real

species that may differ in aspects of their biology but usually cannot be distinguished by morphology. Such species in the *S. damnosum* complex are often called cytospecies because they have been defined on the basis of their chromosomal variation (Post *et al.*, 2007).

Simuliids are among a unique group of insects that have sibling species evolved in the genus with cytological evidence showing that they have an extraordinarily high rate of sibling speciation. A similar characteristic has been reported in other insects such as the mosquito genera *Anopheles, Culex* and many of the fruitflies of the family Tephritidae (Berlocher, 1978; Sorungbe and Adegoke, 2012).

Cytotypes, on the other hand, are chromosomally distinctive populations of unknown specific status, although further research will probably show that some of them are actually cytospecies. Cytospecies and cytotypes are known together as cytoforms. There are 55 named cytoforms within the *S. damnosum* complex (Post *et al.*, 2007), making it the largest known sibling species complex of any insect vector.

Black fly taxonomy is further complicated by sibling species complexes whose component species cannot be morphologically differentiated in adult life stages. Many sibling species are best characterized by larval salivary gland chromosome polymorphisms. Sibling species are inferred from lack of heterozygotes in areas of sympatry (Rothfels, 1979). Larvae of these sibling species are usually isolated temporally, spatially, or ecologically (Adler, 1987).

The black flies *Simulium damnosum s. l.* has been shown to be made up of a complex of sibling species on the basis of the banding sequences of the polytene chromosomes from the salivary glands of the larvae (Dunbar, 1966; Vajime and Dunbar, 1975).

Post *et al.*, (2011) later pointed out that the biogeographical distribution of the black flies cytoforms in Nigeria is broadly similar to that known of West Africa areas. Vajime and Dunbar

(1975) were the first to identify sibling species of the *S. damnosum* complex in West Africa. They recognized eight sibling species (*S. squamosum, S. yahense, S. sanctipauli, S. soubrense, S. damnosum s. s., S. sirbanum, S. sudanense* and *S. dieguerense*) and published an identification key based on chromosomal features. Although, Bedo (1977) criticized the earlier criteria given by Vajime and Dunbar (1975) for the identification of *S. sirbanum* and *S. sudanese*, arguing that the inversion 2L-7 was polymorphic within species and not diagnostic.

However, works after few decades pointed that *S. damnosum s. l.* (Diptera: Simuliidae) as the most important vector of onchocerciasis in sub-Saharan Africa comprises a complex of about forty to fifty sibling species that can be described on the basis of differences in their larval polytene chromosomes. And these cytospecies differ in their geographical distributions, ecologies and epidemiological roles (Ibeh *et al.*, 2006; Post *et al.*, 2013).

2.3.5 Black flies (Diptera: Simuliidae) Karyotyping/Cytogenetics

Cytogenetics – involving the karyotyping of polytene chromosomes – was pointed out as one of the earliest tools for the study of anopheline genetics (Lounibos and Conn, 2000), and other insect-vector genetics such as black flies, tsetse flies, etc. Karyotype is the chromosomal characteristics (number, size and shape) of the body cells of an individual species. Certain parameters are used to describe the karyotype of an organism. These include the diploid number (2n), the position of the primary constriction (the centromere) in relation to the total length of the chromosome, presence or absence of structural aberrations and the fundamental number (NF), which is the number of chromosomal arms possessed in the diploid state (Sorungbe and Adegoke, 2011).

Chromosome cytology is a useful tool in the identification of insect species and more so in the case of insect vectors of diseases, where it can assist in their control (Vajime, 1989). Mafuyai *et*

al., (1996) reported six sibling species of the *S. damnosum* complex in Nigeria, further strengthening the view that chromosome cytology can be exploited to the advantage of the control of the disease as positive identification of anthropophilic species aids in the design of disease control measures.

Among the disadvantages of this technique in Anopheles mosquitoes is that the polytene chromosome preparations must be made from ovarian tissue or fourth instar larvae. This limits the samples to either adult blood fed female mosquitoes or late instar larvae. In addition to the paucity of experienced personnel trained to read polytene chromosome preparations, markers are not abundant or particularly informative in some species (Lounibos and Conn, 2000). Despite the limitations, this method remains integral for much of the contemporary work (Norris, 2002).

2.3.6 Challenges of Black flies (Diptera: Simuliidae) Genetic Studies

The overwhelming constraint to classic genetic studies of Simuliids has been the difficulty of rearing black flies through multiple generations in the laboratory. Four challenges arise: (1) need for flowing water to complete larval development, required of all species, (2) obligatory diapause in about one-third of the world's species, (3) anautogeny in most species, and (4) aerial mating behavior in more than 95% of species. For the few species in which these limitations have been overcome, colony maintenance remains labor intensive (Edman and Simmons, 1985). The world's only continuous-generation colony is *S. vittatum* (Gray and Noblet, 1999).

The use of polytene chromosomes is ideal for cases where morphological characters are insufficient in discriminating species as in the case of sibling species, while mitotic chromosomes are sufficient enough to be used for identification to the species level (Sorungbe and Adegoke, 2012).

2.3.7 Black flies (Diptera: Simuliidae) Morphological Identifications in Nigeria

Researchers in Nigeria in the past have ascertained the prevalence of onchocerciasis by entomological evaluation and morphotaxonomy in the determination of the vector flies' parousity, infectivity rate and type (Ibeh *et al.*, 2005; Adeleke *et al.*, 2011; Sam-Wobo *et al.*, 2013). However, the problems inherent in morphological taxonomy are general enough to motivate the diffusion of molecular approach not to insect vectors alone, but to all life. In fact, there are a growing number of cases in which DNA-based identification systems have been applied to higher organisms (Bucklin *et al.*, 1999; Trewick, 2000; Vincent *et al.*, 2000).

2.4 MOLECULAR GENETICS

The evolutionary analysis of morphology is often difficult because its expression can be subject to environmental influence and may be determined by many genes having greater and lesser effects. However, morphological variation is important in its own right, because it can be directly adaptive. The shape of the tarsal claw, for example, seems to be an adaptation for clasping fur or feathers, and hence an adaptation to the preferred blood-host (mammal or bird). Mammalophilic species have simple claws, and ornithophilic species tend to have bifid claws. This relationship holds at different evolutionary scales. For example, sibling species of the *S. damnosum* complex show variation in claw morphology correlated with different degrees of blood-host preference (Krueger, 2006). Similar variation occurs in the *S. neavei* group and at a higher taxonomic level, all members of the subgenus *Pomeroyellum* are ornithophilic and have bifid claws (Crosskey, 1990).

Chromosomal variation is unlikely to be directly adaptive, but it can provide useful genetic markers. The range of cytogenetic variation, however, is limited and cannot reflect the full range

of genetic variation. A wealth of sibling species, therefore, might remain to be discovered, which is not correlated with chromosomal variation. Biochemical characters provide potentially more traits for analysis. They are traditionally divided into three groups: nucleic acids (usually DNA), proteins (enzymes and structural proteins), and secondary metabolites (e.g., lipids and carbohydrates). DNA variation is genetic and its expression is not subject to environmental influences. Protein variation is primarily the result of changes in amino acid sequences, which reflects the codon sequence of the relevant gene. However, it also can be subject to post-translational modification, and hence variation is not always genetically determined (Pasteur *et al.*, 1988).

Secondary metabolites are produced by the action of enzymes and thus are one step further from a clear genetic basis. This straightforward genetic basis is one of the main reasons why DNA analysis came to dominate the nascent fields of ecology, systematics, and epidemiology. However, secondary metabolites remain the mainstay of biochemical entomology and have led to applications in black flies, such as estimation of the age of a fly through the accumulation of pteridines (Coyne, 1996).

The evolution of secondary metabolites and biochemical pathways can be adaptive, but secondary metabolites have hardly been used as genetic markers in the same way as protein and DNA variation. An exception is the assessment of cuticular hydrocarbon variation to provide markers for identification of adult sibling species in the *S. damnosum* complex (Coyne, 1996). Cuticular hydrocarbons were attractive because in some insects they function as contact pheromones in species-specific mate recognition systems, and variation can be under simple genetic control (Coyne, 1996).

2.5 GENOME OF THE PARASITE

When referring to the genome, one is usually indicating the nuclear genome. But most eukaryotic organisms contain a mitochondrial genome along with the nuclear genome, as *Onchocerca volvulus* does. Nevertheless, *O. volvulus* also contains a third genome - a bacterial endosymbiont of the genus *Wolbachia*, which plays a role in the parasite fecundity (Unnasch and Williams, 2000).

2.5.1 Nuclear Genome of Parasite and Insect Vector

The nuclear genome is predicted to be approximately 1.5 x 108bp and the genes seem to be very compact, averaging about 5kbp in size, distributed among four pairs of chromosomes. The nuclear genome of *O. volvulus* contains a large section of non-coding sequences, from which two families have been identified. These were discovered from genetic screens to identify DNA probes that would be useful to identify the parasite. One of the well-defined families in the nuclear genome is a tandemly repeated sequence with a length of 150bp, known as the O-150 repeat family. This represents about 1% of the total *O. volvulus* genome and is specific for parasites in the genus *Onchocerca* (Unnasch and Williams, 2000).

The intergenic spacer (IGS) and internal transcribed spacers (ITS1 and ITS2) within the nuclear ribosomal genome have become very popular targets for addressing taxonomic issues among insect vectors. So, researchers have discovered that the nucleotide sequence of these spacer regions are often much more polymorphic between species than within species of anophelines. This makes this region of the genome useful for delineating molecular differences between cryptic species by length or sequence polymorphisms (Fritz *et al.*, 1994; Marrelli *et al.*, 1999; Hackett *et al.*, 2000).

With reference to anopheline species that has attracted more molecular works than the black flies, sequence information is developed into PCR-based diagnostic tools that differentiate between cryptic taxa. Most of these molecular diagnostics fall into three categories. The first are based around sets of species-specific PCR primers that produce differential PCR products. This type of diagnostic has been extensively used for differentiation of species within the *A. gambiae* species complex (Scott *et al.,* 1993), the *A. funestus* group (Hackett *et al.,* 2000), *A. dirus* (Xu *et al.*, 1998), *A. fluviatilis* (Manonmani *et al.*, 2001), and the *A. quadrimaculatus* complex (Cornel *et al.*, 1996). More recently this method has been used for refinement of a RFLP-based diagnostic used for limited differentiation of chromosomal forms of *A. gambiae s. s.* (Favia *et al.*, 1997, 2001). The second type of rDNA diagnostics are based on unique RFLP patterns resulting from enzymatic digestion of a conserved PCR product. Molecular diagnostics of this type have been developed for *Anopheles maculatus s. l.* in Asia (Torres *et al.*, 2000). The third type of diagnostic usually centres on specific DNA hybridization to a species-specific sequence. Species-specific dot blots of this type have been constructed for taxa that are cryptic or difficult to differentiate from other vectors and non-vectors. An example of this utility includes a species-specific dot blot to differentiate *Anopheles aquasalis* from other members of the subgenus *Nyssorhynchus* (Perera *et al.*, 1998).

2.5.2 Mitochondrial Genome of Parasite and Insect Vector

The mitochondrial genome, present in almost all eukaryotic cells, contains genetic information that has greatly facilitated systematic and population genetic research over the past two decades. Characteristics such as a relatively rapid mutation rate, maternal inheritance, and a presumed lack of intermolecular recombination have resulted in its extensive use in investigations of population structure and phylogenetic relationships at different taxonomic levels (Avise, 1994;

2000). As at year 2000, approximately 460 eukaryote complete mitochondrial DNA sequences and corresponding gene orders have been determined, with approximately 75% representing vertebrates (Avise, 2000). The mitochondrial genome is very compact with just 13,747bp in size, making it the smallest metazoan mitochondrial genome (Unnasch and Williams, 2000).

Mitochondrial genome is frequently utilized in phylogenetic and population genetic studies. It was stated that the sequence and genome organization of the *A. gambiae* mitochondrial genome was published nearly some decades ago as at 1993 (Beard *et al.*, 1993). This information was quickly utilized in field and laboratory based studies where both coding (i.e. NADH dehydrogenase subunit 5 (ND5) and cytochrome oxidase subunits I and II (COI and COII)) and non-coding (16S and 12S rRNAs) regions are frequent targets for these analyses (Norris, 2002).

2.5.3 Bacterial Endosymbiont Genome of Parasite

The human onchocerciasis causative parasite – *Onchocerca volvulus* has been known to contain a third genome, which is a bacterial endosymbiont of the genus *Wolbachia*. It has been shown that this "bacterial endosymbiont genome" plays a role in the parasite fecundity (Unnasch and Williams, 2000) of human onchocerciasis.

2.6 DNA EXTRACTION

Deoxyribonucleic acid (DNA) extraction is the beginning of molecular markers analyses. Extraction and isolation of DNA (nuclear, mitochondrial, and/or chloroplast DNA) from sample to be studied is the first step for all molecular marker types. DNA can be extracted either from fresh, lyophilized, preserved or dried samples but fresh material is ideal for obtaining good quality DNA. There are many alternative protocols for DNA extraction and the choice of a protocol depends on the quality and quantity of DNA needed, nature of samples, and the

presence of natural substances that may interfere with the extraction and subsequent analysis. DNA extraction protocols vary from simple and quick ones (e.g., Clancy *et al.*, 1996; Dayteg *et al.*, 1998; von Post *et al.*, 2003) that yields low quality DNA but nevertheless good enough for routine analyses to the laborious and time-consuming standard methods (e.g. Murray and Thompson, 1980; Saghai-Maroof *et al.*, 1984) that usually produce high quality and quantity of DNA. Therefore, the researcher will need to test different protocols in order to find out the best one that works for the species of organism under investigation.

The most commonly used DNA extraction protocols involve breaking (through grinding) or digesting away cell walls and membranes in order to release the cellular constituents. Removal of membranes lipids is facilitated by using detergents such as sodium dodecyl sulphate (SDS), cetyl trimethyl ammonium bromide (CTAB) or mixed alkyl trimethyl-ammonium bromide (MTAB). The released DNA should be protected from endogenous nucleases and ethylene diamine tetra acetate (EDTA) is often included in the extraction buffer to chelate magnesium ions that is a necessary co-factor for nucleases. DNA extracts often contain a large amount of RNA, proteins, polysaccharides, (tannins and pigments, if from plant source), which may interfere with the extracted DNA. Most proteins are removed by adding a protein degrading enzyme (proteinase-K), denaturation at 65^0C and precipitation using chloroform and isoamyl alcohol. RNAs are normally removed using RNA degrading enzyme called RNase A. Polysaccharide-like contaminants are, however, more difficult to remove. NaCl, together with CTAB is known to remove polysaccharides (Murray and Thompson, 1980; Paterson *et al.*, 1993). Some protocols replace NaCl with KCl (Thompson and Henry, 1995).

As DNA will be released along with other compounds (lipids, proteins, carbohydrates, and/or phenols), it needs to be separated from others by centrifugation. The DNA in the aqueous phase

will then be transferred into new tubes and precipitated in salt solution (e.g. sodium acetate) or alcohol (100% isopropanol or ethanol) and re-dissolved in sterile water or buffer (Semagn *et al.*, 2006).

Finally, the concentration of the extracted DNA needs to be measured using either 1% agarose gel electrophoresis or spectrophotometer. Agarose gel is useful to check whether the DNA is degraded or not but estimating DNA concentration by visually comparing band intensities of the extracted DNA with a molecular ladder of known concentration is too subjective (Semagn *et al.*, 2006).

Spectrophotometer measures the intensity of absorbance of DNA solution at 260 nm wavelength. It indicates the presence of protein contaminants as well as hyperchromic effect upon DNA denaturation with increased absorbance.

There are three possible outcomes at the end of any DNA extraction: (a) there is no DNA, (b) the DNA appears as sheared (too fragmented), which is an indication of degradation for different reasons; and (c) DNA appears as whitish thin threads (good quality DNA) or brownish thread-DNA in the presence of oxidation from contaminants such as phenolic compounds (Semagn *et al.*, 2006).

2.7 MOLECULAR/GENETIC MARKERS

A molecular marker is a DNA sequence that is readily detected and whose inheritance can easily be monitored. The uses of molecular markers are based on the naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes. A marker must to be polymorphic i.e. it must exit in different forms so that chromosome carrying the mutant genes can be distinguished from the chromosomes with the normal gene by a marker

it also carries. Genetic polymorphism is considered as the simultaneous occurrence of a trait in the same population of two discontinuous variants or genotypes. Unlike protein markers, DNA markers do segregate as single genes and they are not affected by the environment (Kumar *et al.*, 2009).

Molecular markers are classified into two categories: type I are markers associated with genes of known function, while type II markers are associated with anonymous genomic segments (O'Brien, 1991). The usefulness of molecular markers can be measured based on their polymorphic information content (PIC). And PIC refers to the value of a marker for detecting polymorphism in a population (Liu and Cordes, 2004).

Molecular techniques are constantly developed, refined and made more cost effective, especially for population based studies that are revealing enormous amounts of insight into the population structure, dynamics and disease transmission by those important vectors of human disease (Norris, 2002).

All organisms are subject to mutations as a result of normal cellular operations or interactions with the environment, leading to genetic variation (polymorphism). In conjunction with selection and genetic drift, there arises genetic variation within and among individuals, species, and higher order taxonomic groups. For this variation to be useful to geneticists, it must be heritable and discernable to the researcher, whether as a recognizable phenotypic variation or as a genetic mutation distinguishable through molecular techniques. At the DNA level, types of genetic variation include: base substitutions, commonly referred to as single nucleotide polymorphisms (SNPs), insertions or deletions of nucleotide sequences (indels) within a locus, inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest (Liu and Cordes,2004).

Through long evolutionary accumulation, many different instances of each type of mutation should exist in any given species, and the number and degree of the various types of mutations define the genetic variation within a species. DNA marker technology can be applied to reveal these mutations. Large "indels" can cause shifts in the sizes of DNA fragments produced upon digestion by restriction enzymes, and are among the easiest type of mutations to detect, mainly by electrophoresis of the fragments on an agarose gel; smaller indels require DNA sequencing or more elaborate electrophoretic techniques to determine smaller changes in size. Inversions and rearrangements that involve restriction sites can be easy to detect because they disrupt the ability of a restriction enzyme to cut DNA at a given site and thus can produce relatively large changes in DNA fragment sizes. While point mutations are more difficult to detect because they do not cause changes in fragment sizes (Liu and Cordes, 2004).

Molecular methods play an important role in estimating the genetic diversity among individuals by comparing the genotypes at a number of polymorphic loci (Avise, 2004; Arif and Khan, 2009). Several types of molecular markers, including mitochondrial DNA (mtDNA) and nuclear DNA markers, are available but none of them can be regarded as optimal for all applications (Sunnucks, 2000). A large number of studies have utilized approaches with mtDNA sequencing; however, mtDNA only represents the genealogy of particular genes that are only inherited maternally (although, some exceptions do exist). Therefore, additional markers targeting nuclear DNA would be needed to be used for more accurate interpretation of population genetics and biodiversity (Arif and Khan, 2009).

For some decades now, the use of molecular markers in revealing polymorphism at the DNA level has been playing an increasing part in biotechnology and genetics studies. There are different types of markers viz. morphological, biochemical and DNA based molecular markers.

These DNA based markers are divided to two types: first is the non-PCR based marker and second is the PCR based markers. The microsatellite DNA marker has been the most widely used, due to its easy use by simple PCR, followed by a denaturing gel electrophoresis for allele size determination, and to the high degree of information provided by its large number of alleles per locus (Kumar *et al.*, 2009).

Molecular markers are indispensable tools for determining the genetic variation and biodiversity with high levels of accuracy and reproducibility. These markers are mainly classified into two types; mitochondrial and nuclear markers. The widely used mitochondrial DNA markers with decreasing order of conserved sequences are 12S rDNA > 16S rDNA > cytochrome b > control region (CR); thus the 12S rDNA is highly conserved and the CR is highly variable. The most commonly used nuclear markers for DNA fingerprinting include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites (Arif and Khan, 2009).

A genetic marker is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. It can be described as a variation, which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like mini & micro satellites. Recent years have witnessed a great interest towards molecular markers, revealing polymorphism at the DNA level, as they play an important role in animal genetic studies (Al-Samarai and Al-Kazaz, 2015).

A genetic marker is any visible character or otherwise assayable phenotype, for which alleles at individual loci segregate in a Mendelian manner. Genetic markers can be used to study the genetics of organisms, including trees, at the level of single genes. The development of the

discipline of genetics would not have been possible without genetic markers such as the visible characters in peas and *Drosophila* (Al-Samarai and Al-Kazaz, 2015).

Keys to the emergence of genomics were advances in DNA marker technology. These advances have resulted in a wealth of genetic markers including allozymes, mtDNA, RFLPs, RAPDs, AFLPs, microsatellites SNPs, and ESTs (Liu and Cordes, 2004). The genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate genetic marker depend on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and knowhow, time constraints and financial limitations (Kumar *et al.*, 2009).

2.7.1 DNA Barcoding Maker

A DNA barcode is a short DNA sequence from a standardized region of the genome used for identifying species. The essential aim of DNA barcoding is to use a large scale screening of one or more reference genes in order to assign unknown individuals to species, and to enhance discovery of new species. Biological taxonomists apply this principle to species classification. Tautz *et al.*, (2002) conducted the first application of using the DNA sequences in systematical biological taxonomy – also, called DNA taxonomy. And then, Herbert *et al.*, (2003) proposed the concept of DNA barcoding and suggested its use for a single mitochondrial DNA gene, mitochondrial cytochrome C oxidase 1, as a common sequence in animal DNA barcoding studies.

DNA barcoding was proposed as a solution to the limitations of traditional taxonomy (Hebert *et al.*, 2003). Its principal aim is to assign unknown specimens to known species, based on a short

DNA fragment. It also has been used to identify cryptic species (Witt *et al.*, 2006; Sheffield *et al.*, 2009), which then can be characterized morphologically, ecologically, and behaviorally (Hajibabaei *et al.*, 2007). Successful DNA barcoding depends on the distinction between intraspecific and interspecific genetic divergence. These values are influenced by two major factors: time since speciation and effective population size. If incipient species share a recent common ancestor, interspecific variation might not yet be sorted out, and the species could be genetically paraphyletic or polyphyletic (Avise, 2000). Species with large effective population sizes can have high intraspecific genetic diversity, which could overlap with interspecific divergence. DNA barcoding will be ambiguous in these situations. Imperfect taxonomy also could lead to erroneous identifications (Meyer and Paulay, 2005). Because different organisms have different population histories, the success of DNA barcoding will vary accordingly. Even within the same group of organisms (e.g., butterflies). Study revealed that the performance of DNA barcoding can vary among geographic regions and ecosystems (Elias *et al.*, 2007).

Previous studies of black flies have shown that DNA barcodes can be used to identify species. Nearly all of 24 new world morpho-species of the subgenus *Trichodagmia Enderlein* and 65 Nearctic morpho-species have been identified correctly by barcodes. So, DNA barcoding can distinguish morphologically distinct species of black flies (Rivera and Currie, 2009; Hernandez-Triana *et al.*, 2012).

One of the studies was of the opinion that DNA barcodes can also reveal cryptic biodiversity (Pramual *et al.*, 2011), but another was of the stand that it does not always distinguish morphologically uniform species (Ilmonen *et al.*, 2009). This approach of DNA barcoding has some disadvantages where representations of the genome fragments are very difficult to obtain, relatively conservative and have no enough variations. Therefore, some organisms cannot be

identified using mitochondrial cytochrome C oxidase 1 (mtCO1) because of low evolution rates of mtCO1 sequences in some species. Moreover, mtCO1 is a mitochondrial DNA sequence of maternal origin, which could be bias on accurate species diversity (Meyer and Paulay, 2005; Meier *et al.*, 2006).

However, it was given that DNA barcoding has a high accuracy of 97.9% in animal study (Thiel *et al.*, 2003), and as well provides a new, quick and convenient identification strategy for animal genetic diversity (Hajibabaei *et al.*, 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment

The list of equipment used in this research work includes: NanoDrop Spectrophotometer (GeneQuantpro RNA/DNA Calculator), centrifuge (Eppendorf centrifuge 5415C), refrigerated centrifuge (Eppendorf centrifuge 5402), micro-centrifuge (microcentrifuge model LX100), water baths/heat block pre-warmed at 65^0C (Multi-Block Heater LAB-LINE®), vortexer (Deluxe Mixer Cat. S8220), freezer, horizontal gel electrophoresis apparatus, rocking platform, PCR machine/Thermocycler (Programmable Thermal Controller, MJ Research, INC), UV transilluminator (GELDOC 2000 BIO-RAD), DNA Sequencer (CEQ 2000XL DNA Analysis System, BECKMAN COULTER), stereo microscope and dissecting implements, autoclave, microwave oven, and DC power supply.

3.2 METHODS

3.2.1 Study Area

The study was carried out around Gurara village along the Gurara River between $8^0$12'0" – $9^0$05'0"N and $6^0$42'0" – $7^0$30'0"E (www.iiste.org.>jees>article>download) within Kaduna State, located in the North Central geopolitical zone of Nigeria with a projection of 7.31 million of human populations (NPC, 2006). The state shares boundaries with Katsina and Kano States in the North, Plateau State to the East, Nasarawa State to the South east, Niger State to the West and Federal Capital Territory, Abuja (FCT) to the South. Some major rivers in the state include Kaduna, Galma and Gurara rivers. Vegetation is mainly guinea savanna grassland, with few

areas of forest mosaic. The climate consists of two distinct seasons: the dry season from November to March and wet season from April to October. The people are predominantly farmers, while some engage in petty trading with very few as fishermen (https://www.onlinenigeria.com/map.gif).

3.2.2 Adult Female Vector Flies Collection

Both adult female vector flies collection and morphological identifications was done with the aid and participation of Onchocerciasis entomological team of Nigeria Institute of Trypanosomiasis (and Onchocerciasis) Research, Kaduna ("NITOR, Kaduna"). The entomological sampling of adult female *Simulium damnosum* complex was carried out along Gurara River (figure 3.1) near Gurara village, Kagarko Local Government Area (LGA), Kaduna State in the month of June and July 2016. Prior to the commencement of the study, prospection of the river (appendix II) was done earlier in May 2016.

The adult female *S. damnosum* complex collection took place at four "marked fly catching points" with four human baits (Walsh *et al.*, 1978; Taye *et al.*, 2000) serving as the attractants for collection. Engorged flies were discarded immediately, as only those vector flies that have not ingested blood were selected. The human baits are situated at these "four marked fly catching points" with the lower part of their legs exposed for biting. So, adult female *S. damnosum* complex landing on their exposed lower part of the legs for blood meal were caught by inverted tube (figure 3.2). The four volunteered human baits were from the Gurara community participating in the on-going ivermectin (Mectizan®) treatment which served as a preventive measure to them. The caught samples of adult female *S. damnosum* complex were preserved alive and transported to the laboratory in cold chain for further analyses. The samples for morphological identifications were preserved in 80% isopropanol after assessment and

forwarded for molecular analyses where they were kept at -20^0C, while the remaining were dissected to determine their transmission/infectivity potentials.

The larvae specimens were picked with fine forceps on the trailing substrates floating on the water surface and immediately preserved in Carnoy's solutions (ethanol: acetic acid – 3:1) (Brockhouse and Tanguay, 1996). Thereafter, these were transported in cold chain to the laboratory for preservation at -20^0C and any further molecular analyses.

3.2.3 Morphological Identifications of Adult Female Vector Flies

Twenty selected adult female *S. damnosum* complex were examined under stereo microscope. External morphology such as criteria for wing turfs, arculus and antenna classification according to "WHO/OCP Entomological surveillance network criteria keys for the morphological identification of adult female *Simulium damnosum sensu lato*" (Wilson *et al.*, 1993; 1994; WHO-OCHRT, 2004) were used.

3.2.4 Transmission/Infectivity Potentials of caught Adult Female Vector Flies

The parousity of the remaining 502 flies was determined before assessment of transmission potential. The ovaries of the vector flies were stretched and classified as parous or nulliparous after observing other character like absence or presence of fat bodies and colour of malphigian tubes (Garms *et. al.*, 2015).

The observed parous adult female flies will be subsequently dissected with micro-dissection instruments under stereo microscope for the presence or absence of parasite (microfilaria worms' i. e., *Onchocerca volvulus*) L3 stage in the head part to determine their transmission potential/ competence. While, both *O. volvulus* L2 and L1 stages will be observe in the thorax and abdomen respectively for confirmation of their infectivity potential.



Figure 3.1: Gurara River near Gurara village within Kagarko Local Government Area of Kaduna State showing rapid moving/flowing water which is a good breeding site for *Simulium damnosum* complex.



Figure 3.2: Capture of adult female *Simulium damnosum* complex landing on the exposed lower part of the leg of one of the consented human baits.

3.3 GENOMIC DNA EXTRACTIONS

Genomic DNA extractions from collected adult female *S. damnosum* complex specimens were performed using the phenol-chloroform DNA extraction method of Moreira *et al.,* (2013) with little modification as follows: the selected frozen adult female *S. damnosum* complex from the preserved samples (in 80% isopropanol at -20^0C) were rinsed in deionized distilled water after de-freezing/thawing and the rinsed samples were allowed to air dried. Genomic DNA (gDNA) was extracted from rinsed dried samples of morphologically identified adult female *S. damnosum* complex either as a single specimen or two pooled specimen. Each of the sample specimens (single or pooled) was grounded in sterile eppendorf tube using sterile micro plastic pestle. Then, 400µl of lysis buffer was added, followed by 10µl of proteinase K, and mixture was transferred to the heating block already set at 65^0C for 2hours with intermittent vortexing every 20minutes. To the lysate, 400µl phenol chloroform (1:1) was added and vortexed for 1minute. The lysate mixture was spinned at 14,000g for 10 minutes to separate the phases. The supernatant was carefully pipetted (without pipetting the white interphase) and transferred to a fresh/new labeled sterile eppendorf tube. To the recovered supernatant/clear upper layer, 400µl chloroform was added, and gently agitated for 10 seconds and then spinned at 14,000g for 5minutes. The supernatant was carefully pipetted (without pipetting the white interphase) and transferred to a fresh/new sterile eppendorf tube. Equal volume of 100% cold ethanol (i. e., 1000µl) was added alongside with 40µl of 3M sodium acetate; and it was mixed by gently inverting the tubes carefully several times. Then, it was incubated at -20^0C overnight.

The overnight incubated sample was centrifuged at 14,000g for 10 minutes at 4^0C in refrigerated centrifuge (Eppendorf Centrifuge 5402). The supernatant layer (ethanol phase) was carefully

pipetted out and discarded. After which, 400µl of 70% cold ethanol was added and spinned at 14,000g for 5 minutes at 4^0C in refrigerated centrifuge. Again the supernatant (ethanol phase) was carefully pipetted out and thereafter short spinned at 14,000g for about 30 seconds long. The supernatant (i.e., ethanol phase) was then carefully pipetted out leaving behind the pellets phase. Pellets were allowed to air dried by keeping the pellets at 37^0C as the eppendorf tubes were opened for 3 – 10 minutes. The air dried pellets were re-suspended in 100µl of nuclease free water. Thereafter, 3µl of the eluted DNA sample was quickly subjected to agarose gel electrophoresis to estimate its quality, as well as DNA concentration to estimate its quantity, purity and integrity; and all the remaining extracted gDNA samples were thereafter stored at – 20^0C for downstream applications.

3.3.1 DNA Concentration and Purity Determination of extracted gDNA Samples

The concentration and the purity of the extracted gDNA were determined by measuring the absorption of ultraviolet light at wavelengths 230, 260 and 280nm using the NanoDrop Spectrophotometer (Gene Quantpro RNA/DNA Calculator). Nuclease free water was used as blank and 2µl gDNA sample was used for determining both concentration and purity of the DNA using A_{260}/A_{280} ratio. The DNA sample (1µl) was diluted with the elution solvent by a dilution factor of 1:100 (i. e., 1µl DNA sample was diluted into 99µl elution solvent). After which the optical density was taken at $A_{230,}$ A_{260} and A_{280} to calculate the amount of DNA concentration and purity of the extracted gDNA.

3.3.2 Preparation of 1% Agarose Gel for extracted gDNA Samples

Qualitative analysis of the extracted gDNA was carried out to visualize the DNA bands after electrophoresis using agarose gel. Agarose (0.5g) was weighed and dissolved in 50 ml of 1xTAE buffer in a 250 ml conical flask. The solution was then heated in an oven to boiling until a clear

solution was obtained. The boiled solution was allowed to cool; 2µl EtBr solution was added and mixed by gentle swirling; and was thereafter, poured into a pre-set gel tray. The poured gel solution in the gel tray was allowed to cool and harden to form gel with appropriate thickness as the comb was gently removed.

The prepared gel was removed and carefully transferred to the electrophoresis tank already filled with 1xTAE buffer. Then, 8µl of DNA samples and 2µl of 5x gel loading dye were prepared, and loaded into the wells appropriately. Electrodes were connected appropriately to the power pack/source and ran at 60 – 100V for 20 minutes. The progress of the gel was monitored with bromophenol blue as tracking dye and the run was stopped when the mark was at three-quarter (i. e., $3/4^{th}$) of the gel. The electrophoresed gel was placed on the UV trans-illuminator and check for the orange-purple colour bands.

3.4 POLYMERASE CHAIN REACTION TECHNIQUES

Nuclease free water (µl) was used for reconstituting/re-suspending the primer (nano mole) according to individual label on them in ratio 10:1 to make up 100 pico mole of the primer which was vortexed, spinned briefly and later stored in the cold pack rack. Then, 1 µl of each forward primer and reverse primer made from the working 10 micro molar primer was used for the PCR reaction. The components of "master-mix" were prepared in the reagents hood, with exception of DNA samples (appendix IV). The master-mix was divided into five epicup tubes with each containing 18µl of the mixtures and the sixth part was there to be discarded after composing the five reactions needed for the amplification. Then, 2µl of the corresponding DNA samples were added on the bench to each labeled epicup tube to make up a final volume of 20µl each. The caps of the epicup tubes were close firmly and placed in the thermocycler (PCR Programmable

Thermal Controller, MJ Research PTC-100 Thermal Cycler). The thermocycler was set on a required programme and conditions for 33 cycles to amplify the specific gene fragments of the extracted gDNA samples. The cycling conditions for the PCR amplifications were below: The initial denaturation step was for 94^0C for 5 minutes, followed by 33 cycles of 94^0C for 1 minute, 60^0C for 1 minute, 72^0C for 1 minute and a final extension step at 72^0C for 3 minutes 30 seconds. When the cycling was completed, the epicup tubes were placed at 4^0C for further downstream applications of gel electrophoresis and DNA nucleotide sequencing. And the epicup tubes with the remaining PCR products not needed for immediate further downstream applications were later kept in the cold park rack for storage at -20^0C.

Table 3.1: Primers used for PCR Amplifications

Maker	Gene	Forward Sequence 5' - 3'	Reverse Sequence 5' - 3'	Reference
ITS	NRG	GTTGGTTTCTTTTCCTC	TCGTAACAAGGTTTCCG	Brockhouse *et al.* (1993)
ITS1	NRG	TTACTATCTTATTTCCACAA	CCCCTGTCTAGATGTTAT	Tang *et al.* (1996)
ITS2	NRG	GCGCCGCGGTGTGAACTGCAGGACACATG	GCGGGTACCATGCTTAAATTTAGGGGGTA	Thanwisai *et al.* (2005)
16SrRNA	MRG	CGCCTGTTTATCAAAAACAT	CTCCGGTTTGAACTCAGATC	Shouche and Patole (2000)

ITS = Internal Transcribed Spacer
ITS1 = Internal Transcribed Spacer 1
ITS2 = Internal Transcribed Spacer 2
16SrRNA = 16S ribosomal RNA
NRG = Nuclear Ribosomal Gene
MRG = Mitochondrial Ribosomal Gene

3.4.1 Gel Electrophoresis of PCR Product

The 1.5% w/v gel (3g of agarose powder was added to 100 ml 1xTAE buffer) was dissolved in a microwave oven. It was allowed to cool to 60^0C, then, 10 µl EtBr was added and swirled gently with magnetic stirrer. The mixture liquid was poured into electrophoresis tank that was already having the "comb" in place with appropriate tapping of the tank and it was ensured that no air bubble was formed. It was allowed to solidify for about 20 – 25 minutes to obtain 4 – 5 mm thick gel, as the comb was carefully removed. The tray was placed in the electrophoresis tank as TAE buffer was poured into the tank to completely cover the gel surface. Using 20µl pipette tip, 5µl sample mixed with 2µl loading dye were carefully loaded into the wells created by comb. And 5µl PCR products were loaded accordingly with appropriate molecular weight maker (1000bp ladder) to determine the expected size of base pairs of bands of amplicon of the amplified PCR products relative to the marker. The negative control was also loaded accordingly. Electrodes were connected to the appropriate terminal and electrophoresed at 60 – 100V till migration of dye reached three-quarter of gel. Then, current was turned off as the electrodes were disconnected. To obtain gel electrophoregram and view the PCR amplicon, the gel was observed under UV Trans-illuminator and the molecular weight bands were documented in gel Documentation Imaging System with camera linked to the laboratory computer.

3.5 DNA SEQUENCING

Samples were prepared for sequencing reactions, loaded and sequenced on a DNA sequencer (CEQ 2000XL DNA Analysis system, BECKMAN COULTER). The visualized bands of amplicon were sliced out and extracted and/or purified by ethanol precipitation.

Fresh stop solution/glycogen mixture (2µl of 3M Sodium acetate, 2µl of 100mM Na_2-EDTA and 1µl of 20mg/ml of glycogen or as provided in the kit) was prepared per sequencing reaction. To each of the labeled tubes 5µl of the stop solution/glycogen mixture was added. The sequencing reaction was transferred to each of the appropriately labeled tube and mixed thoroughly. Thereafter, 60µl cold 95% (v/v) ethanol from -20^0C freezer was added and mixed thoroughly. It was immediately centrifuged at 14,000g at 4^0C for 15 minutes. And supernatant was carefully removed with a micropipette (the pellet should be visible). The pellet was rinsed with 200µl 70% (v/v) ethanol from -20^0C freezer, centrifuged at 14,000g at 4^0C for a minimum of 2 minutes. And the supernatant was carefully removed with a micropipette and the other left over in the tube was vacuum dry for 10 minutes or until noticed dry. The sample was re-suspended in 40µl Sample loading solution provided in the kit.

The re-suspended samples were transferred to the appropriate wells of the sample plate (PN 609801). Each of the re-suspended samples was overlay with one drop of mineral oil from the kit. The sample plates were loaded into the instrument to start the desired method.

3.5.1 Dye Terminator Cycle Sequencing with Quick Start kit for Sequencing Reaction

All reagents were kept on ice while preparing the sequencing reactions and were added in the listed order as follows: ddH_2O (0 – 9.5µl), DNA template (0.5 – 10.0µl), Forward primer (2.0µl) and DTCS-dye terminator cycle sequencing – Quick start master mix (8.0µl). The sequencing reaction was set up in the Sequencing machine with thermal cycling program: 96^0C for 20 seconds, 50^0C for 20 seconds x 30 cycles, and 60^0C for 4 minutes. The protocols for purifying PCR fragments, preparing extension products, spin column purification and sequencing of single strand DNA (ssDNA) were strictly according to the manufacturer's instruction.

3.6 MULTIPLE SEQUENCE ALIGNMENT

The multiple sequence alignment (MSA) of all the acquired DNA nucleotide sequences from the BLASTN result was carried out using molecular evolutionary genetics analysis software version 6 (MEGA 6.06) before conducting the phylogenetic analyses. MSA was created from selected nucleotide sequences from the data of BLASTN that were imported into the alignment editor/explorer of MEGA 6.06. The alignment explorer was launched by selecting align/edit/build alignment on the launch bar of the MEGA 6.06 main window. Create new alignment was selected and the button labeled DNA was clicked for OK subsequently in answer to the question "are you building a DNA or protein sequence alignment". The MSA was created in MEGA using Clustal W algorithm by clicking "OK" button to accept the default settings (Tamura *et al.*, 2013).

3.7 PHYLOGENETIC ANALYSES

Molecular Evolutionary Genetic Analysis (MEGA) program was used among the several other programs for the phylogenetic analyses. The phylogenetic and molecular evolutionary analyses of the generated alignments of all the DNA nucleotide sequences from the MSA were conducted using MEGA 6.06 by Maximum Likelihood Tree Method – ML (Tamura *et al.*, 2013).

CHAPTER FOUR

4.0 RESULTS

4.1 ADULT FEMALE VECTOR FLIES COLLECTION

Human bait technique was used to catch 522 adult female *S. damnosum* complex at "four marked catching points" along Gurara River in the months of June and July (table 4.1).

4.1.1 Adult Female Vector Flies Morphological Identifications

Twenty out of the total adult female *S. damnosum* complex were identified morphologically under stereo microscope as *Simulium damnosum* sub-complex (plate 4.1) using the criteria for colour classification of wing turfs, arculus, and the antennae amongst other morphological identification criteria keys developed for the OCP area of West Africa (Wilson *et al.*, 1993; 1994; WHO-OCHRT, 2004). All the morphologically identified *S. damnosum* sub complex flies (i. e., commonly called savanna group) completely have pale wing tufts, arculus and antennae; and they were kept for further molecular analyses on them.

4.1.2 Adult Female Vector Flies Transmission/Infectivity Potentials

Higher percentage of the remaining 502 vector flies was revealed to be 68.92% nulliparous with a lesser percentage of 31.08% parous (Table 4.1). Meanwhile, absence of L3 *O. volvulus* larvae was viewed in the 156 parous vector flies with "zero transmission potential" as their monthly transmission potential (MTP) was calculated to be zero. Likewisely, zero infectivity potential was observed for both L2 and L1 *O. volvulus* larvae in both thorax and abdomen respectively.

Table 4.1: Number of Adult Female *Simulium damnosum* complex showing Morphospecies Identifications and Parousity

Week	TC	DP	NP	PR	squ	san	yah	dam
Week 1	98	88	80	8	-	-	-	10
Week 2	145	135	109	26	-	-	-	10
Week 4	147	147	83	64	-	-	-	-
Week 6	132	132	74	58				
Total	522	502	346	156	-	-	-	20

TC = Total caught of adult female *S. damnosum* complex
DP = Dissection for parousity of the vector flies
NP = Nulliparous vector flies
PR = Parous vector flies which were further dissected for infectivity and transmission potentials
squ = *S. squamosum* morphospecies identification
san = *S. sanctipauli* morphospecies identification
yah = *S. yahense* morphospecies identification
dam = *S. damnosum* sub-complex morphospecies identification

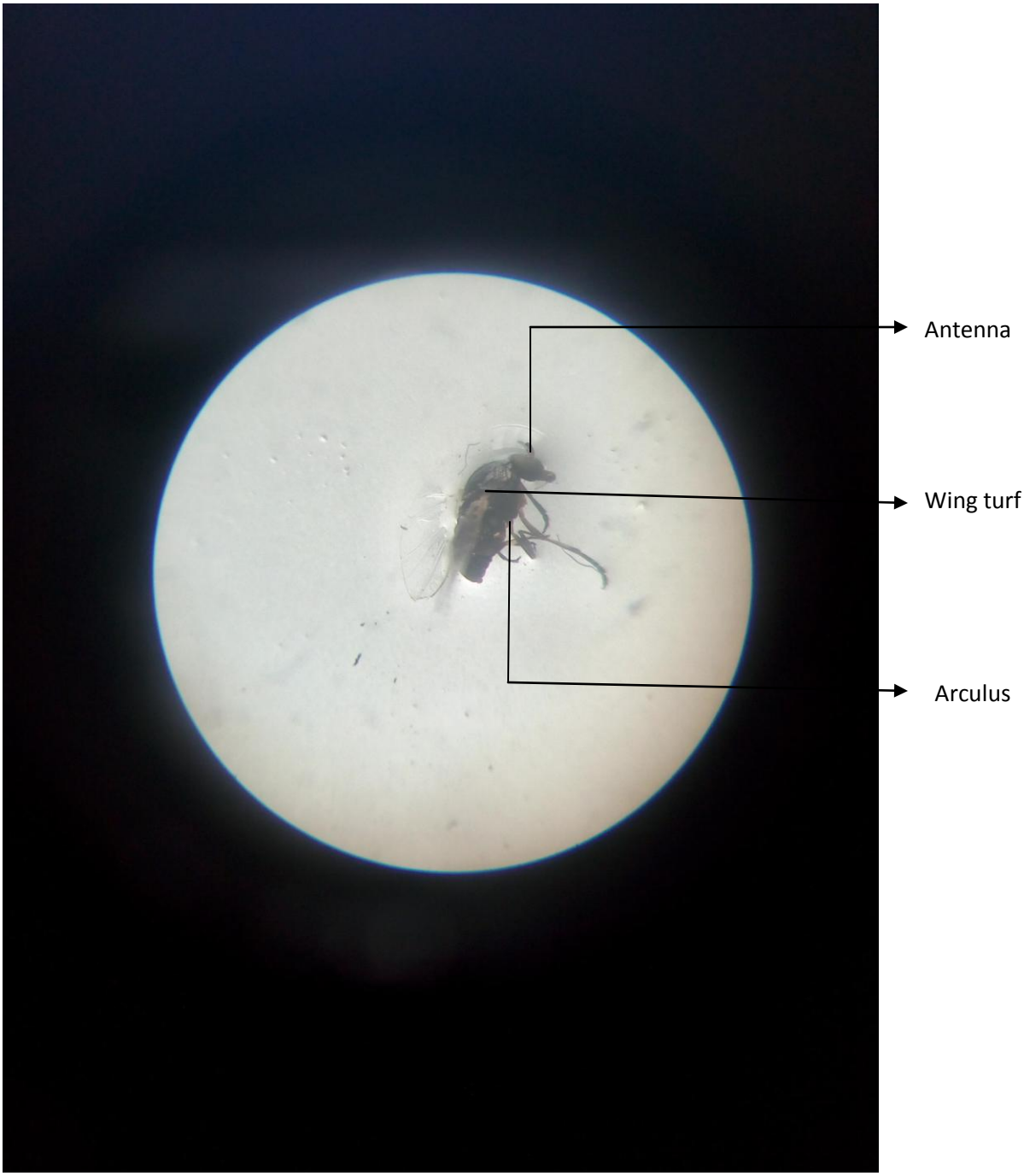


Plate 4.1: Morphological Identification of an Adult female *S. damnosum* complex collected from Gurara River Study Focus.

The criteria for colour classification of Wing turfs, Arculus and Antennae were considered amongst other criteria of WHO-OCHRT (2004) for morphological identification.



Plate 4.1.1a: Separated Head, Thorax and Abdomen parts an Adult female *S. damnosum* complex collected from Gurara River study focus.

The morphologically identified adult female *S. damnosum* sub-complex (savanna group) was further separated into head, thorax and abdomen parts to enhance further investigation of the transmission/infectivity potentials.

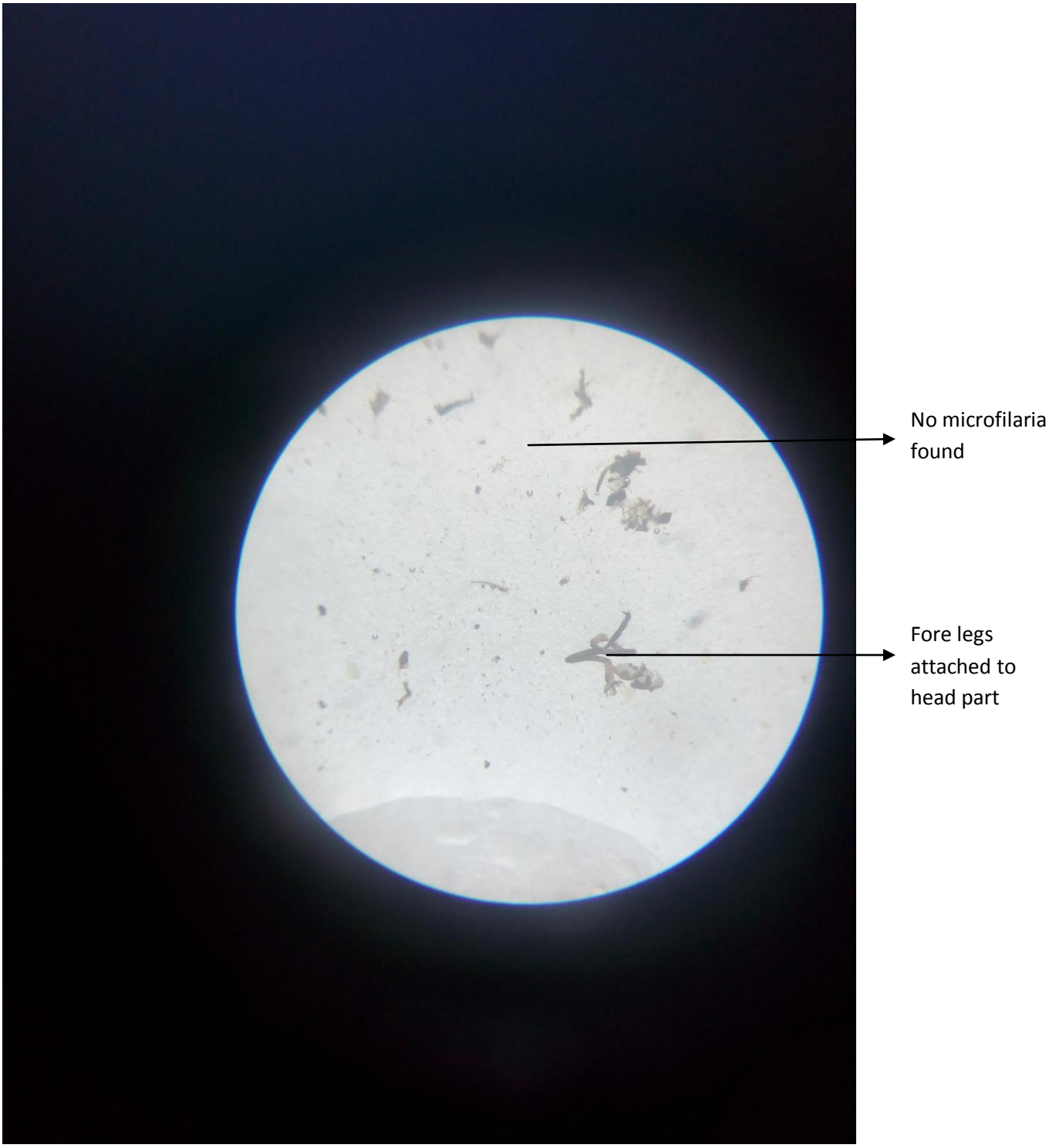


Plate 4.1.1b: Dissected head part of an adult female *S. damnosum* complex collected from Gurara River study focus with the absence of the L3 *O. volvulus* stage (micro filarial parasite of human onchocerciasis).

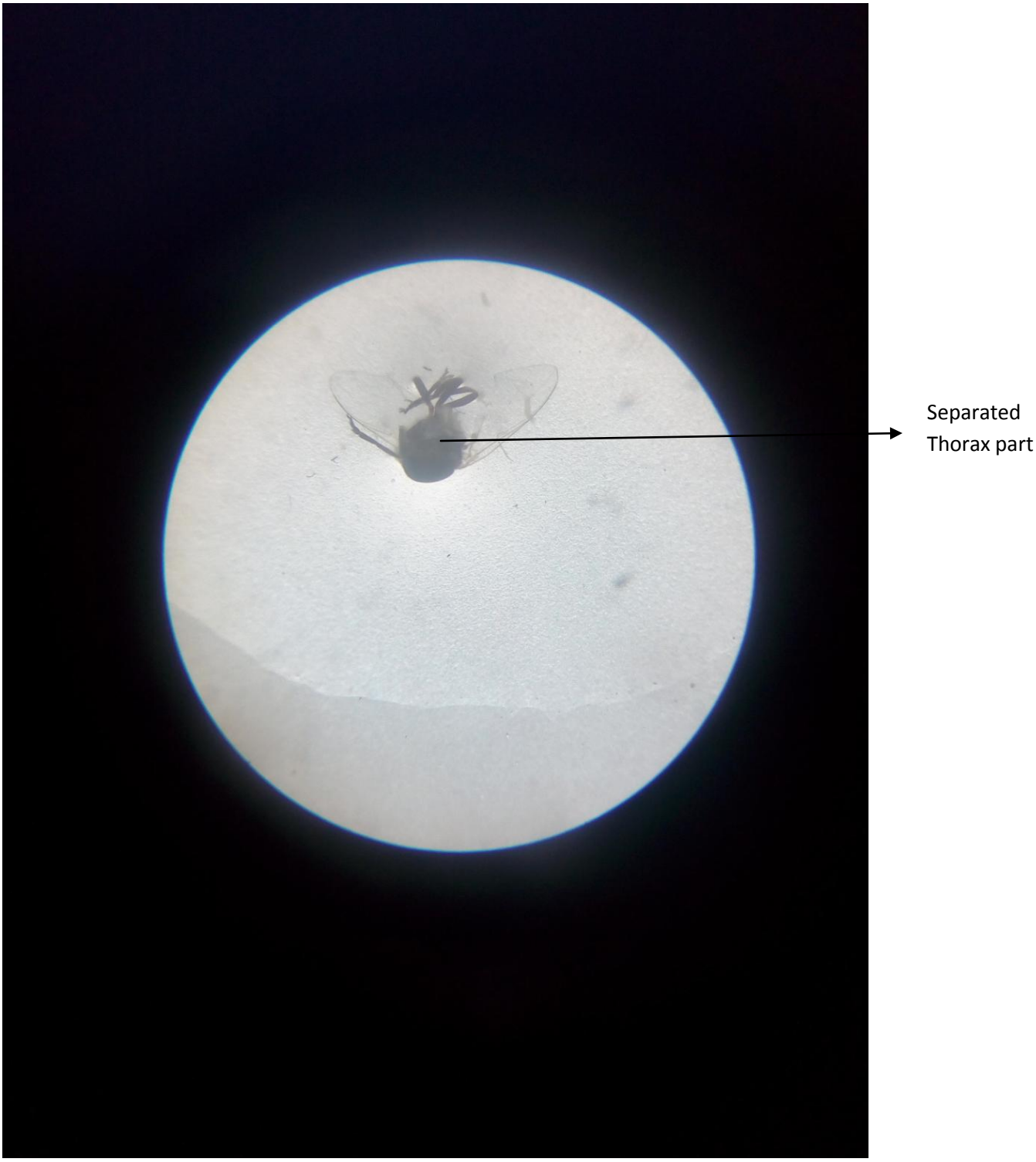


Plate 4.1.2a: Thorax part of an adult female *S. damnosum* complex collected from Gurara River Study Focus.



Plate 4.1.2b: Dissected Thorax part of an Adult female *S. damnosum* complex collected from Gurara River Study Focus with the absence of L2 *O. volvulus* stage (micro filarial parasite of human onchocerciasis).

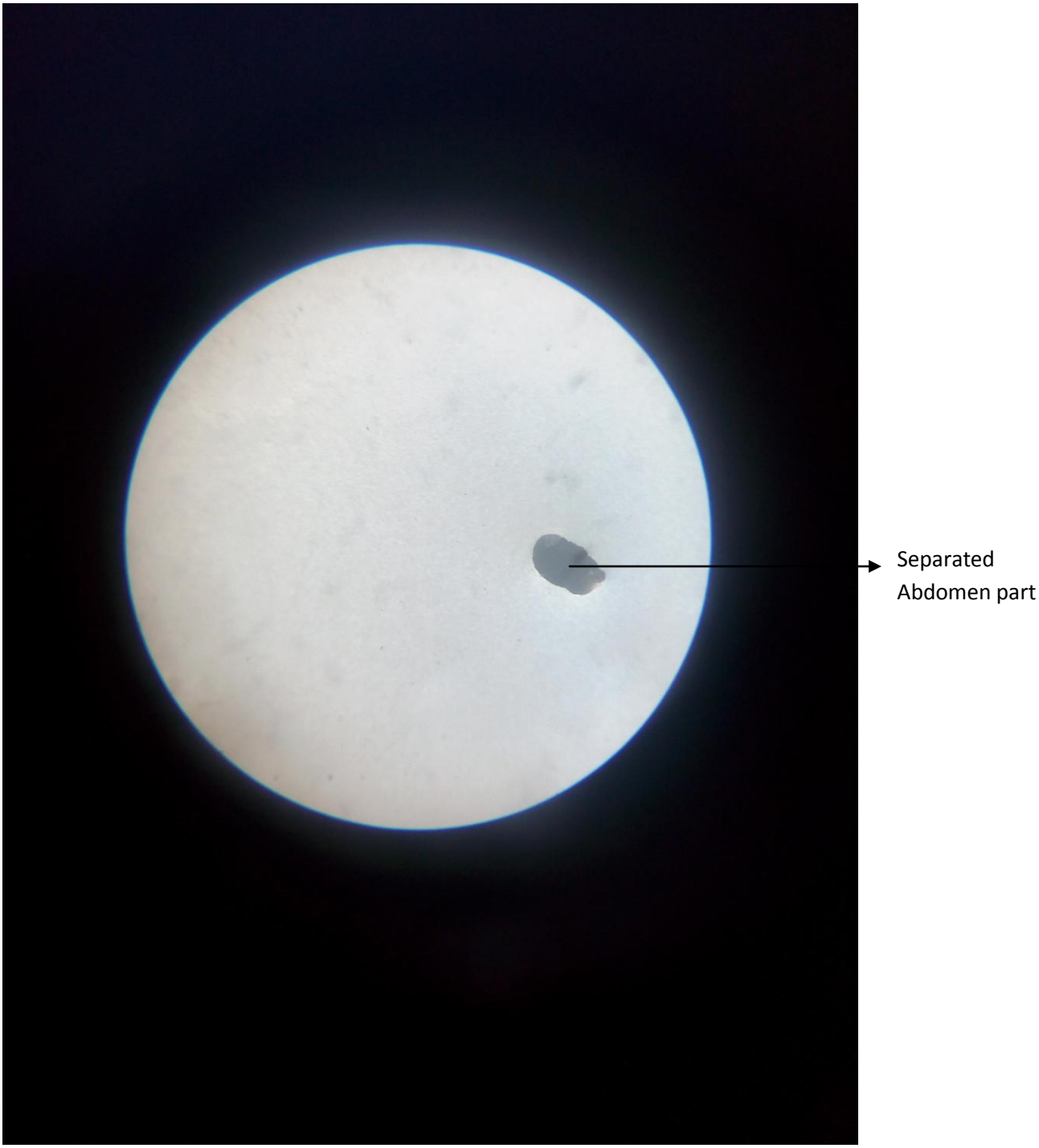


Plate 4.1.3a: Abdomen part of an Adult female *S. damnosum* complex collected from Gurara River Study Focus.

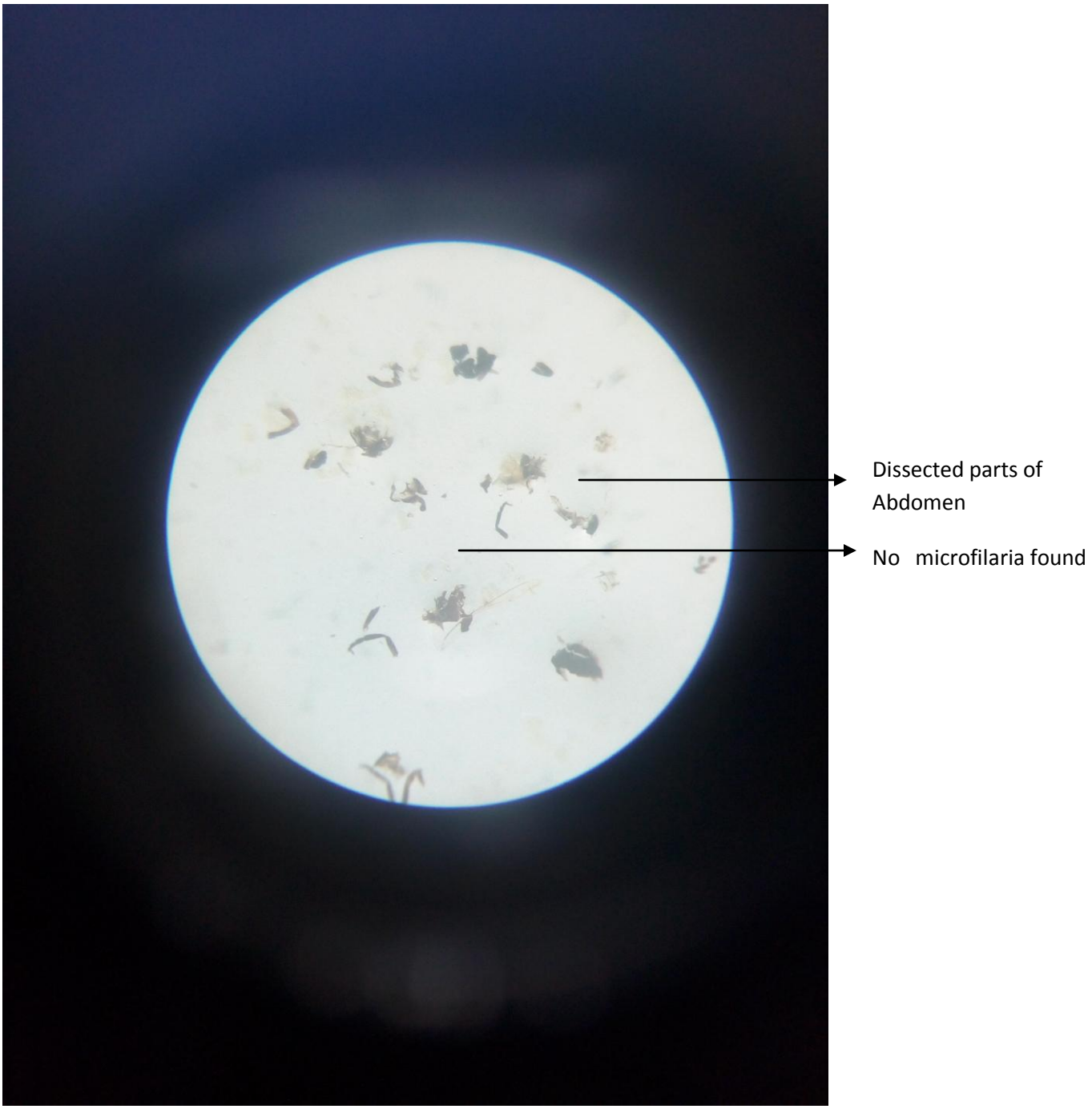


Plate 4.1.3b: Dissected Abdomen part of an Adult female *S. damnosum* complex collected from Gurara River Study Focus with the absence of the L1 *O. volvulus* stage (micro filarial parasite of human onchocerciasis).

4.2 DETERMINATION OF CONCENTRATION AND PURITY OF GENOMIC DNA

Qualitative analysis of the extracted gDNA was carried out to visualize the DNA bands by agarose gel electrophoresis (data not shown). The presences of DNAs were indicated by all samples as the electrophoregram/chromatogram had varying band intensities. Also, the gel electrophoresis was employed to further confirm the purity of the DNA samples.

The results of the concentration and purity of the gDNAs using NanoDrop Spectrophotometer (GeneQuantpro RNA/DNA Calculator) are shown in Table 4.2. The mean concentration of gDNAs was 91.91ng.μl^{-1} with ranges of 54.9 to 171.0 respectively. And the purity of the gDNAs given as ratio of the absorbance at 260/280nm gave a mean of 1.8 with ranges of 1.4 to 2.3 as a result of some contaminations but they were still good for the PCR amplification.

Table 4.2: Concentration and Purity of gDNA of Adult female *S. damnosum* complex collected along Gurara River Study Focus

Sample	G01*	G02*	G03	G04	G05	G06	G07	G08	G09	G10	Mean
DNA Concentration (ng/µl)	168.0	171.0	91.7	79.5	59.1	54.9	74.7	74.2	86.5	59.5	91.91
RNA/Protein Contamination (A_{260}/A_{280})	2.3	1.6	2.0	2.2	1.5	1.6	1.4	1.7	1.7	1.6	1.8

Key: * = gDNA extraction from two morphologically identified adult female *S. damnosum* complex' pooled sample.

4.3 PCR AMPLIFICATIONS

Plate 4.2a showed the PCR amplification of ITS region of the nuclear ribosomal gene (NRG) loci from the extracted gDNAs from adult female *S. damnosum* complex. Double bands of amplicon were observed with lane 2 (containing two morphologically identified pooled adult female *S. damnosum* complex); and one band of amplicon with lane 3 (containing single morphologically identified adult female *S. damnosum* complex). While lane 1 (containing empty sample) and lane 4 (containing single morphologically identified adult female *S. damnosum* complex) indicated no band of amplicon as well as the negative control of lane 5. The observed molecular weights or band sizes of the double bands of lane 2 for ITS sequence of NRG were approximately 450bp and 250bp while that of the single band of amplicon of lane 3 for ITS sequence of NRG was approximately 550bp.

Plate 4.2b showed the PCR amplification of ITS-1 region of the nuclear ribosomal gene loci from the extracted gDNAs from adult female *S. damnosum* complex. All the amplified lanes 1 to 4 for ITS1 sequence of NRG were with uniform single band size of amplicon of approximately 350bp each, except lane 4 that had faint band of amplicon.

Plate 4.2c showed the PCR amplification of ITS-2 region of the nuclear ribosomal gene loci from the extracted gDNAs from adult female *S. damnosum* complex. All the amplified lanes 1 to 4 for ITS2 sequence of NRG gave uniform single band size of approximately 450bp each.

Plate 4.3 showed the PCR amplification of 16S rRNA region of the mitochondrial ribosomal gene from the extracted gDNAs from adult female *S. damnosum* complex. Lanes 1 to 4 gave uniform single band of amplicon of approximately 500bp with lanes 2 and 3 sharper than others.

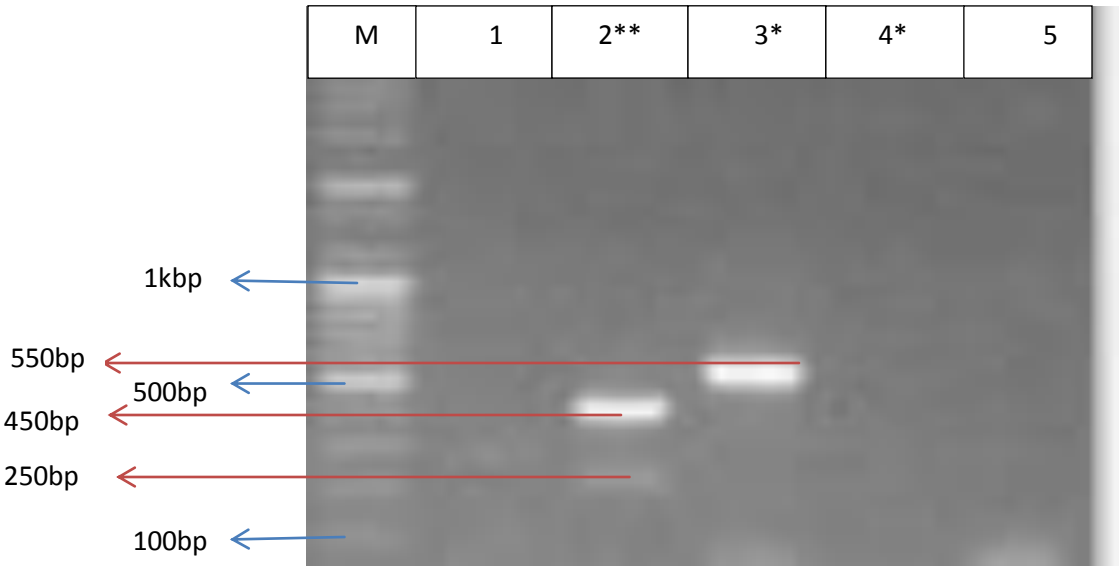


Plate 4.2a: Electrophoregram for PCR amplification of ITS region of nuclear ribosomal gene (NRG) of extracted gDNA from Adult female *S. damnosum* complex vector flies.

Lane 1 = No sample at all.
Lane 2** = Two morphologically identified adult female *S. damnosum* complex pooled sample.
Lanes 3* = Single morphologically identified adult female *S. damnosum* complex sample each.
Lanes 4* = Single morphologically identified adult female *S. damnosum* complex sample each.
Lane 5 = Negative control.
Lane M = Molecular ladder.

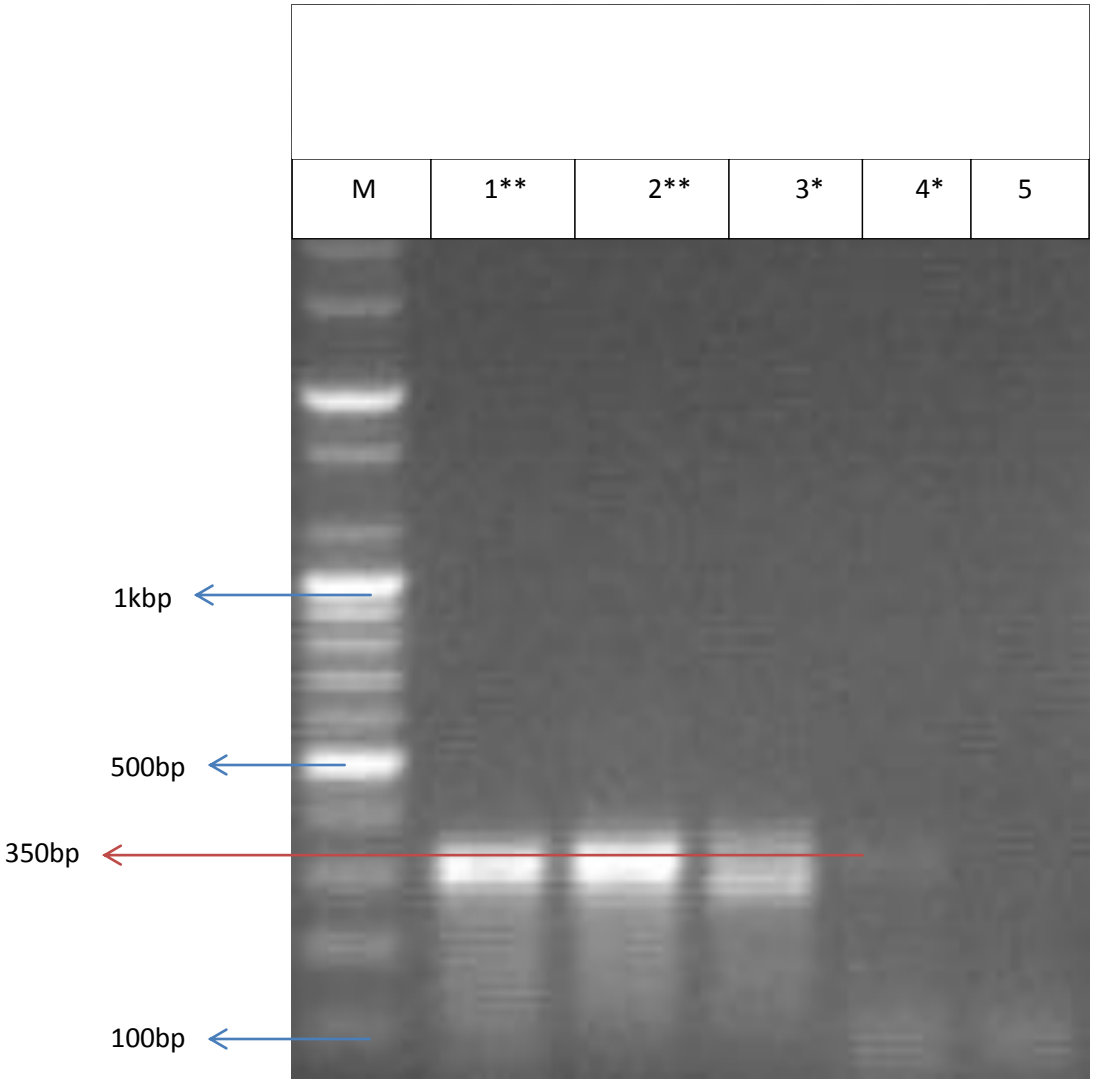


Plate 4.2b: Electrophoregram for PCR amplification of ITS-1 region of nuclear ribosomal gene (NRG) of extracted gDNA from Adult female *S. damnosum* complex vector flies.

Lanes 1** & 2** = Two morphologically identified adult female *S. damnosum* complex pooled samples each.
Lanes 3* & 4* = Single morphologically identified adult female *S. damnosum* complex sample each.
Lane 5 = Negative control.
Lane M = Molecular ladder.

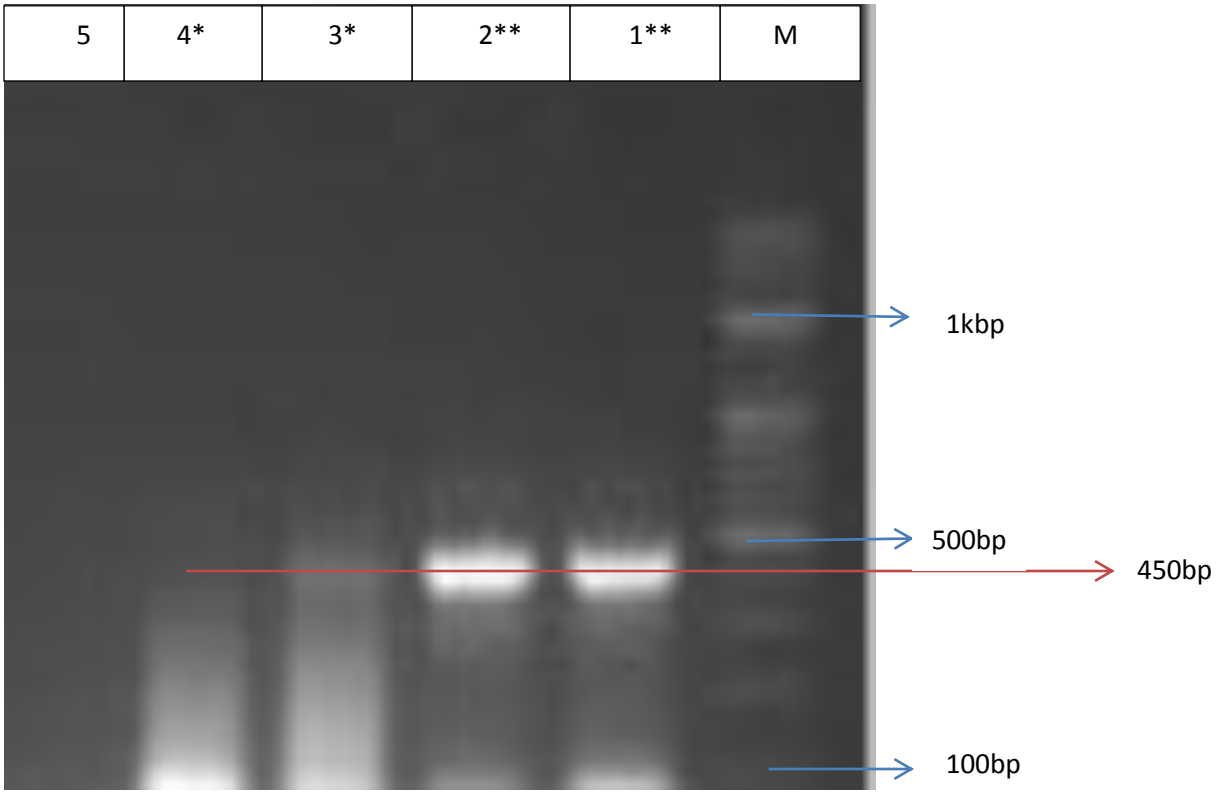


Plate 4.2c: Electrophoregram for PCR amplification of ITS-2 region of nuclear ribosomal gene (NRG) of extracted gDNA from Adult female *S. damnosum* complex vector flies.

Lanes 1** & 2** = Two morphologically identified adult female *S. damnosum* complex pooled samples each.
Lanes 3* & 4* = Single morphologically identified adult female *S. damnosum* complex sample each.
Lane 5 = Negative control.
Lane M = Molecular ladder.

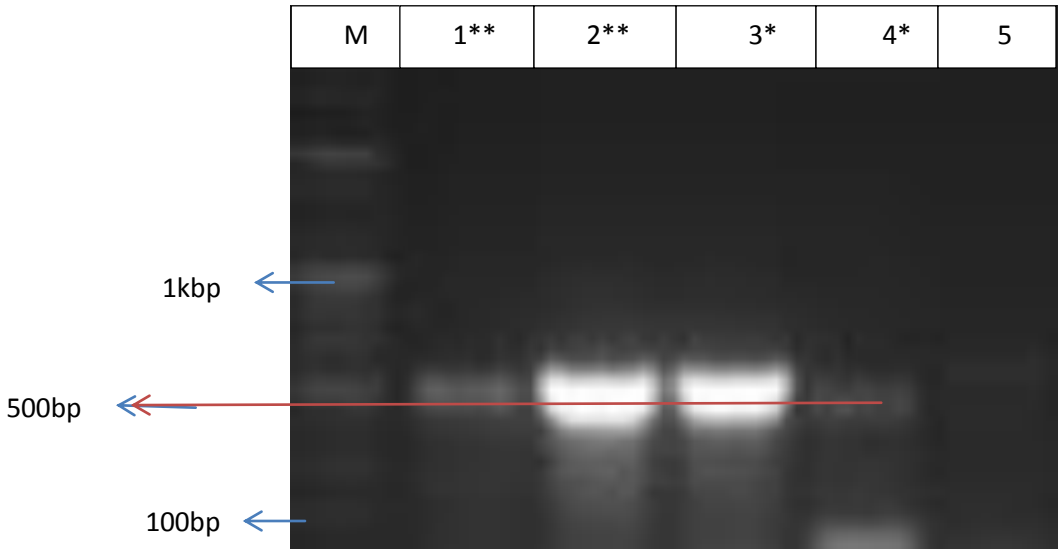


Plate 4.3: Electrophoregram for PCR amplification of 16S rRNA region of mitochondrial ribosomal gene (MRG) of extracted gDNA from Adult female *S. damnosum* complex vector flies.

Lanes 1** & 2** = Two morphologically identified adult female *S. damnosum* complex pooled samples each.
Lanes 3* & 4* = Single morphologically identified adult female *S. damnosum* complex sample each.
Lane 5 = Negative control.
Lane M = Molecular ladder.

4.4 DNA NUCLEOTIDE SEQUENCES

The PCR amplicon gel purified (double bands and single band for ITS gene, and two selected each from the single band of amplicon for ITS1, ITS2 and 16SrRNA genes) was successful for DNA sequencing of eight PCR amplicon (Table 4.4).

Table 4.3: Nucleotide Compositions of DNA Sequence Results

Lane	Maker/ Gene	Length (bases)	A%	T%	C%	G%	AT%	CG%
2A	ITS/NRG	160	30.0	28.1	14.4	27.5	58.1	41.9
2B	ITS/NRG	344	30.5	30.5	20.9	17.7	61.0	38.7
3	ITS/NRG	489	23.9	31.6	22.4	21.8	55.6	44.4
7	ITS1/NRG	219	22.4	42.9	17.4	17.4	65.3	34.7
8	ITS1/NRG	n/a	n/a	n/a	n/a	n/a	n/a	n/a
12	ITS2/NRG	314	34.4	32.5	18.5	14.6	66.9	33.1
13	ITS2/NRG	275	35.6	33.8	16.4	13.5	69.5	29.8
22	16S/MRG	423	33.1	36.4	18.4	11.3	69.5	29.8
23	16S/MRG	402	24.6	36.6	24.9	13.9	61.2	38.8

A = adenine, G = guanine, T = thymine, C = cytosine.

n/a = not available or not sequenced.

ITS = internal transcribed spacer.

ITS-1 = first internal transcribed spacer.

ITS-2 = second internal transcribed spacer.

16S rRNA = 16S ribosomal RNA.

NRG = nuclear ribosomal gene.

MRG = mitochondrial ribosomal gene.

4.5 MULTIPLE SEQUENCE ALIGNMENT OF DNA NUCLEOTIDE SEQUENCES SELECTED FROM BLASTN SEARCHES OF DNA SEQUENCING PRODUCTS

The DNA nucleotide sequence of double bands of amplicon of lane 2A for ITS gene did not show any significant alignment while that of lane 2B for ITS gene showed alignment (table 4.4) for the BLASTN searches (using the Somewhat Similar Sequence program) from the database of NCBI Genbank. The remaining DNA nucleotide sequences for ITS1, ITS2 and 16SrRNA genes also showed significant alignment with the BLASTN searches in the NCBI GenBank Database. Multiple Sequence Alignment (MSA) of some selected DNA nucleotide sequences from the BLASTN searches using DNA sequence result of ITS gene of Amplicon 2B (figure 4.5a) were carried out using Clustal W method (Tamura *et al.*, 2013).

Table 4.4: List of Selected Simulium species from BLASTN to build MSA and Phylogeny of Amplicon 2B of ITS/NRG

S/N	Accession	Species or Group Name	Length (bases)	Max Score	Total Score	Query Cover (%)	Expect Value	Identities (%)
01	KF057785.1	*S. gravely*	1337	116	148	40	2e-26	83
02	HQ864622.1	*S. sp6* BR-2011 strain9A	439	53.6	53.6	15	2e-07	81
03	EU429888.1	*S. intermedium*	666	50.0	50.0	28	2e-06	72
04	U36210.1	*S. sirbanum*	855	75.2	75.2	15	5e-14	91
05	U36206.1	*S. sanctipauli*	810	75.2	75.2	15	5e-14	91
06	U36205.1	*S. damnosum*	856	75.2	75.2	15	5e-14	91
07	EF535155.1	*S. limbatum*	682	64.4	64.4	12	9e-11	93
08	EF535152.1	*S. quianense*	653	64.4	64.4	12	9e-11	93
09	AF427961.1	*S. roraimense*	686	64.4	64.4	12	9e-11	93

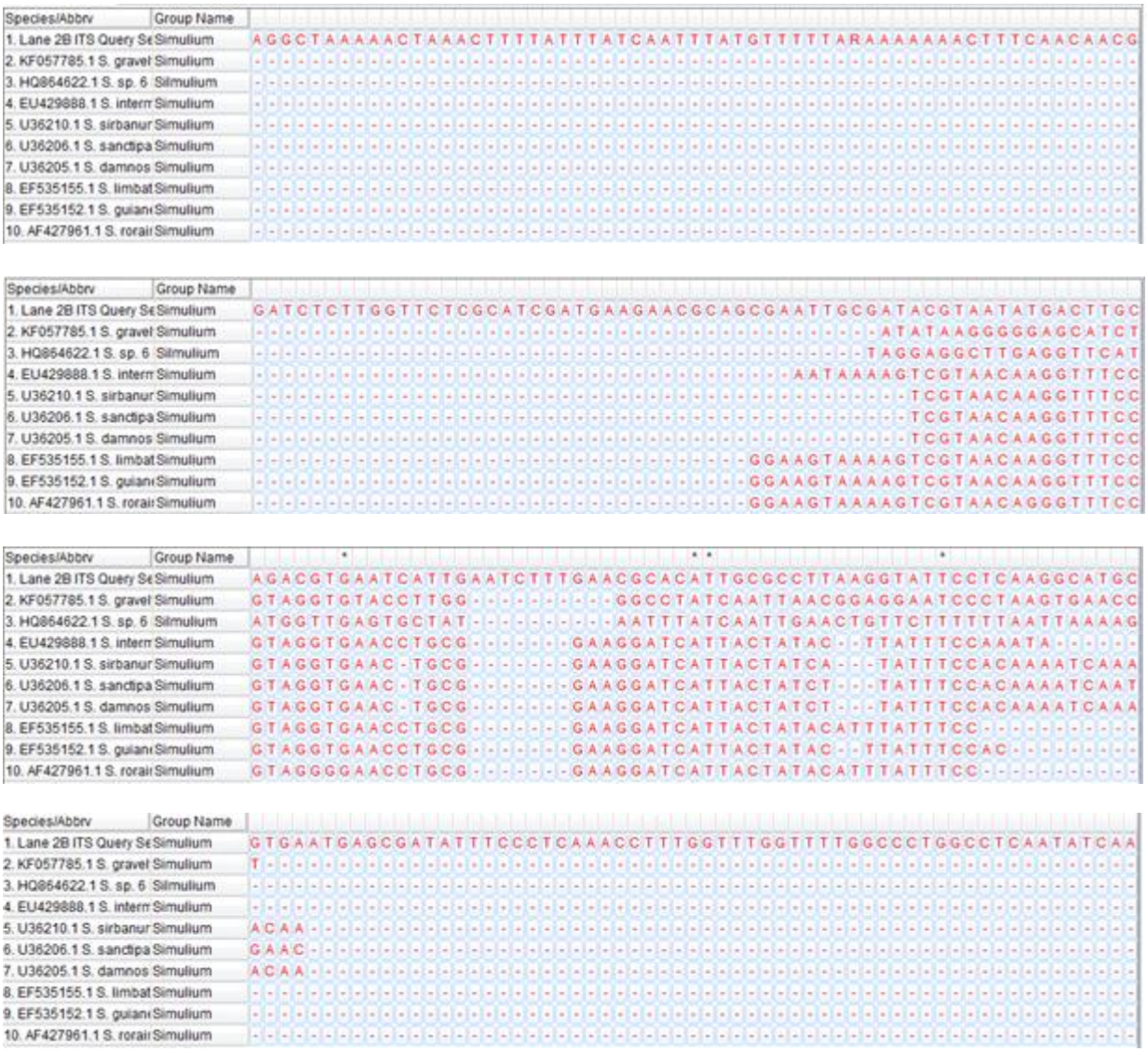


Figure 4.1a: Amplicon 2B ITS/NRG MSA required/needed to build Phylogeny.

An asterisk represents agreement with the consensus at that position. And a dot within the alignments represents disagreement with the consensus at that position.

4.6 PHYLOGENETIC ANALYSES

The phylogenetic and molecular evolutionary analyses of the generated alignments of all the DNA nucleotide sequences were conducted from the MSA using Clustal W tool following default parameters of MEGA 6.06 by Maximum Likelihood Tree Methods – ML (Tamura *et al.*, 2013).

The evolutionary history for amplicon 2B ITS/NRG sample (Figure 4.1a) was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-254.8772) was shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 65 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.06 (Tamura *et al.*, 2013).

Table 4.5a showed the estimates of evolutionary divergence between selected sequences from amplicon 2B ITS/NRG query sequence BLASTN result by pairwise distances. The numbers of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al.*, 2004). The analysis involved 10 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 52 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.06 (Tamura *et al.*, 2013).

The phylogenetic and molecular evolutionary analyses (Figures 4.2b – 4.2d) and evolutionary divergence estimates (Tables 4.5b – 4.5d) between the selected sequences from the MSA was also conducted for ITS1, ITS2 and 16S rRNA respectively using MEGA 6.06.

Table 4.6 gave the summary of the order of the implicated Simulium species from both the molecular and phylogenetic analyses of ITS/NRG to be *S. damnosum* clone (savanna group with the possible occurrence tally of 5), followed by *S. sirbanum* clone (savanna group with possible occurrence tally of 2), and followed by *S. sanctipauli* (forest group with lowest possible occurrence tally of 1).

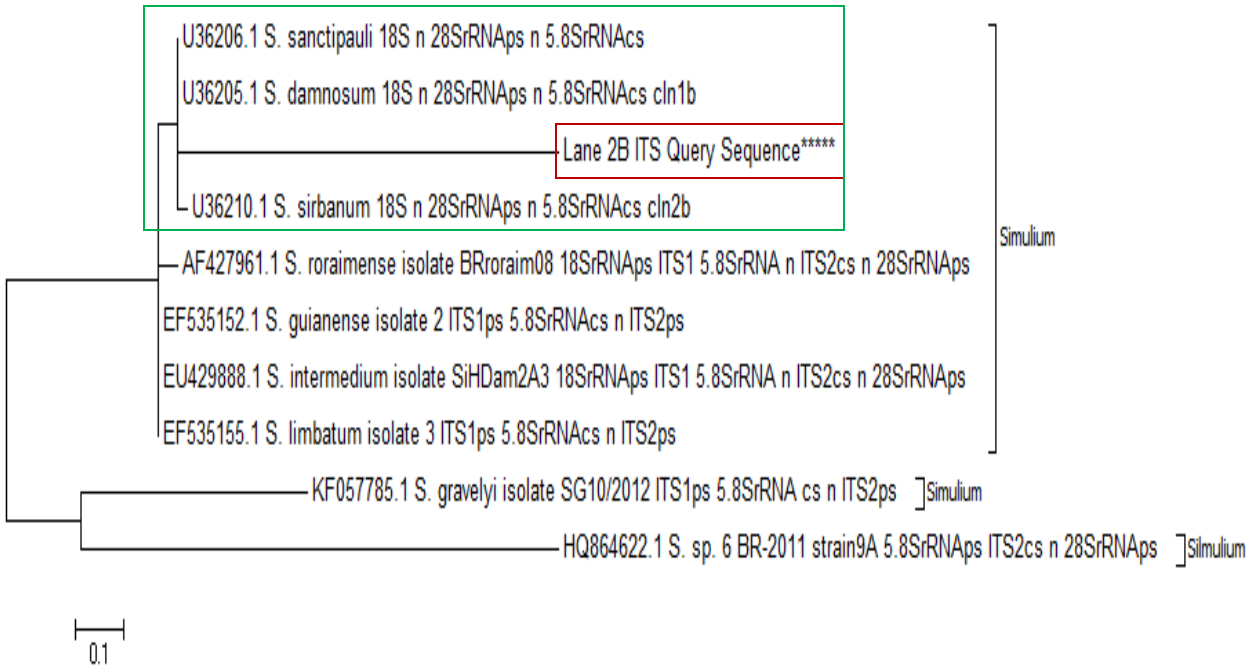


Figure 4.2a: Phylogenetic Tree based on Amplicon 2B ITS/NRG generated by MLTree Method (MEGA 6.06).

Red square = Nucleotide sequence from the query gene
Green square = clades of evolutionary relationship that the queried gene belong
ITS1 = first internal transcribed spacer
ITS1ps = first internal transcribed spacer partial sequence
5.8SrRNAcs = 5.8S ribosomal RNA complete sequence
ITS2cs = second internal transcribed spacer complete sequence
n = and
28SrRNAps = 28S ribosomal RNA partial sequence
18SrRNAps = 18S ribosomal RNA partial sequence
cln2b = clone 2b
cln1b= clone 1b

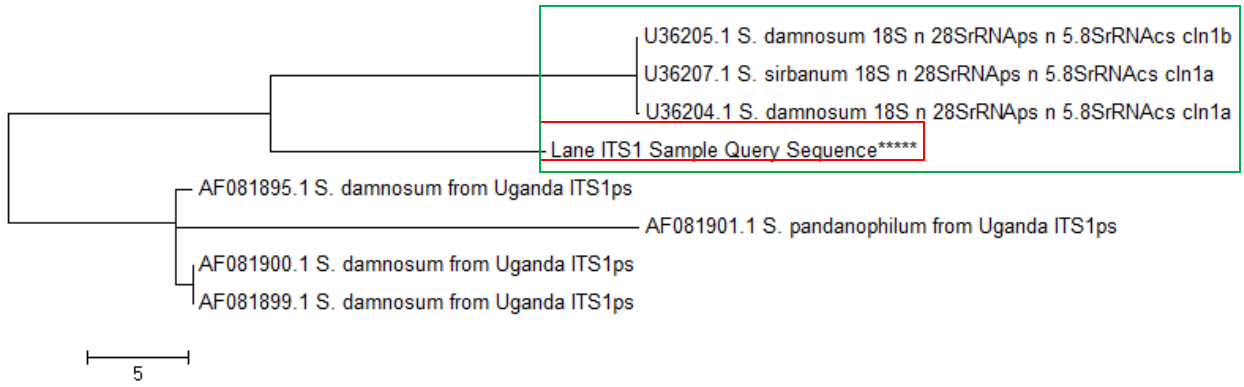


Figure 4.2b: Phylogenetic Tree based on Amplicon 2 ITS1/NRG generated by ML Tree Method (MEGA 6.06).

Red square = Nucleotide sequence from the query gene
Green square = clades of evolutionary relationship that the queried gene belong
ITS1 = first internal transcribed spacer
ITS1ps = first internal transcribed spacer partial sequence
5.8SrRNAcs = 5.8S ribosomal RNA complete sequence
n = and
28SrRNAps = 28S ribosomal RNA partial sequence
cln1a = clone 1a
cln1b= clone 1b

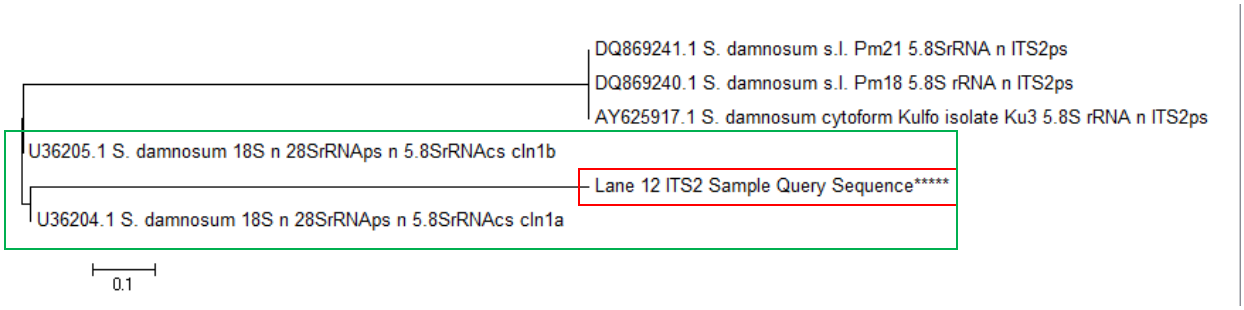


Figure 4.2c: Phylogenetic Tree based on Amplicon 2 ITS2/NRG generated by ML Tree Method (MEGA 6.06).

Red square = Nucleotide sequence from the query gene
Green square = clades of evolutionary relationship that the queried gene belong
5.8SrRNA = 5.8S ribosomal RNA
5.8SrRNAcs = 5.8S ribosomal RNA complete sequence
ITS2cs = second internal transcribed spacer complete sequence
n = and
28SrRNAps = 28S ribosomal RNA partial sequence
cln1a = clone 1a
cln1b= clone 1b

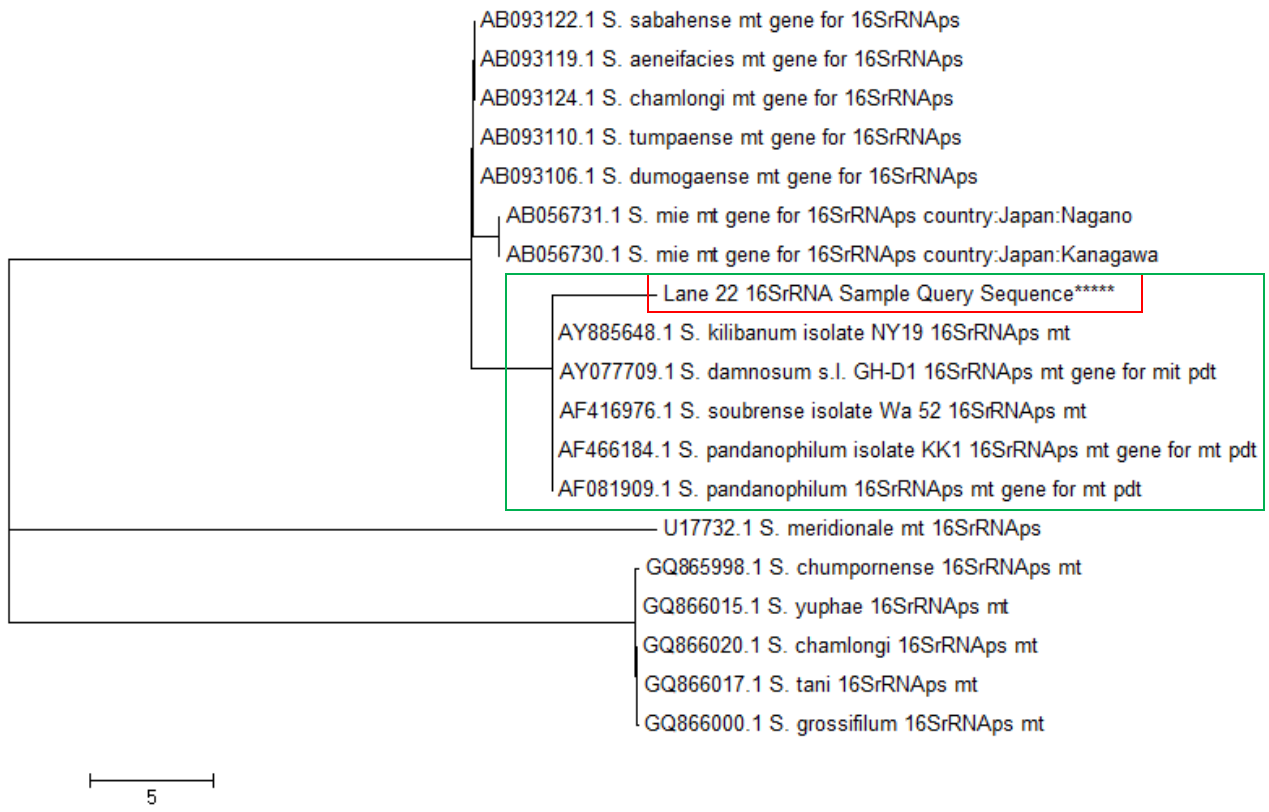


Figure 4.2d: Phylogenetic Tree based on Amplicon 2 16SrRNA/MRG generated by ML Tree Method (MEGA 6.06).

Red square = Nucleotide sequence from the query gene
Green square = clades of evolutionary relationship that the queried gene belong
16SrRNAps = 16S ribosomal RNA partial sequence
16SrRNAps mt = mitochondrial 16S ribosomal RNA partial sequence
mt pdt = mitochondrial product

Table 4.5a: Estimates of Evolutionary Divergence between selected sequences from Amplicon 2B ITS Query Sequence BLASTN Result by Pairwise Distances

S/N	Accession	Name	01	02	03	04	05	06	07	08	09	10
01	New Sample	Lane 2B ITS Query Sequence*****										
02	KF57785.1	*S. gravelyi* isolate SG10/2012 ITS1ps	1.46									
03	HQ864622.1	*S. sp.6* BR-2011 strain9A 5.8SrRNAps ITS2cs n 28SrRNAps	2.02	1.38								
04	EU429888.1	*S. intermedium* isolate SiHDam2A3 18SrRNAps ITS1 5.8SrRNA n ITS2cs n 28SrRNAps	0.84	0.92	1.27							
05	U36210.1	*S. sirbanum* 18S n 28SrRNAps n 5.8SrRNAcs cln2b	0.84	1.08	1.27	0.04						
06	U36206.1	*S. sanctipauli* 18S n 28SrRNAps n 5.8rRNAcs	0.77	1.05	1.39	0.04	0.02					
07	U36205.1	*S. damnosum* 18S n 28SrRNAps n 5.8SrRNAcs cln1b	0.77	1.05	1.39	0.04	0.02	0.00				
08	EF535155.1	*S. limbatum* isolate3 ITS1ps 5.8SrRNAcs n ITS2ps	0.84	0.92	1.27	0.00	0.04	0.04	0.04			
09	EF535152.1	*S. guianense* isolate2 ITS1ps 5.8SrRNAcs n ITS2ps	0.84	0.92	1.27	0.00	0.04	0.04	0.04	0.00		
10	AF427961.1	*S. roraimense* isolate BRroraim08 18SrRNAps ITS1 5.8SrRNA n ITS2cs n 28SrRNAps	0.91	0.94	1.48	0.04	0.08	0.08	0.08	0.04	0.00	

KEY: ***** = five asterisks to indicate query sequence
ITS1ps = first internal transcribed spacer partial sequence
5.8SrRNAps = 5.8S ribosomal RNA partial sequence
ITS2cs = second internal transcribed spacer complete sequence
n = and
28SrRNAps = 28S ribosomal RNA partial sequence
18SrRNAps = 18S ribosomal RNA partial sequence
cln2b = clone 2b
cln1b= clone 1b

Table 4.5b: Estimates of Evolutionary Divergence between selected sequences from Amplicon 2 ITS1 Query Sequence BLASTN Result by Pairwise Distances

S/N	Accession	Name	01	02	03	04	05	06	07	08
01	New Sample	Lane 7 ITS1 Query Sequence*****								
02	AF081895.1	*S. damnosum* from Uganda ITS1ps	2.52							
03	U36205.1	*S.damnosum* 18S n 28SrRNAps n 5.8SrRNAcs cln1b	3.54	2.58						
04	U306207.1	*S. sirbanum* 18S n 28SrRNAps n 5.8SrRNAcs cln1a	3.54	2.58	0.00					
05	U306204.1	*S.damnosum* 18S n 28SrRNAps n 5.8SrRNAcs cln1a	3.52	2.47	0.06	0.06				
06	AF081900.1	*S.damnosum* from Uganda ITS1ps	3.34	1.45	3.41	3.41	2.55			
07	AF081899.1	*S. damnosum* from Uganda ITS1ps	3.34	1.45	3.41	3.41	2.55	0.00		
08	AF081901.1	*S. pandanophilum* from Uganda ITS1ps	3.66	1.56	3.59	3.59	3.81	1.94	1.94	

KEY: ***** = five asterisks to indicate query sequence
ITS1ps = first internal transcribed spacer partial sequence
5.8SrRNAps = 5.8S ribosomal RNA partial sequence
ITS2cs = second internal transcribed spacer complete sequence
n = and
28SrRNAps = 28S ribosomal RNA partial sequence
18SrRNAps = 18S ribosomal RNA partial sequence
cln1b = clone 1b
cln1a = clone 1a

Table 4.5c: Estimates of Evolutionary Divergence between selected sequences from Amplicon 2 ITS-2 Query Sequence BLASTN Result by Pairwise Distances

S/N	Accession	Name	01	02	03	04	05	06
01	New Sample	Lane 12 ITS2 Sample Query Sequence*****						
02	U36205.1	*S. damnosum* 18S n 28S rRNAps n 5.8S rRNAcs cln1b	4.14					
03	U36204.1	*S. damnosum* 18S n 28S rRNAps n 5.8S rRNAcs cln1a	3.36	0.06				
04	DQ869241.1	*S. damnosum s. l.* Pm21 5.8S rRNA n ITS2ps	2.93	3.18	3.16			
05	DQ869240.1	*S. damnosum s. l.* Pm18 5.8S rRNA n ITS2ps	2.93	3.18	3.16	0.00		
06	AY625917.1	*S. damnosum* cytoforms Kulfo isolate Ku3 5.8S rRNA n ITS2ps	2.93	3.18	3.16	0.00	0.00	

KEY: ***** = five asterisks to indicate query sequence
18S = 18S ribosomal RNA partial sequence
28SrRNAps = 28S ribosomal RNA partial sequence
5.8SrRNAps = 5.8S ribosomal RNA partial sequence
ITS2ps = second internal transcribed spacer partial sequence
n = and
cln1b = clone 1b
cln1a = clone 1a

Table 4.5d: Estimates of Evolutionary Divergence between selected sequences from Amplicon 2 16S rRNA Query Sequence BLASTN Result by Pairwise Distances

S/N	Accession	Name	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19
01	New Sample	Lane 22 16SrRNA*****																			
02	AY077709.1	*S. damnosum*	2.77																		
03	AF416976.1	*S. soubrense*	2.88	0.02																	
04	AF466184.1	*S. pandanophilum*	2.77	0.01	0.03																
05	AF081909.1	*S. pandanophilum*	2.77	0.01	0.03	0.00															
06	AY885648.1	*S. kilibanum*	2.77	0.04	0.06	0.03	0.03														
07	GQ866020.1	*S. chalongi*	3.71	2.50	2.60	2.50	2.50	2.53													
08	GQ866017.1	*S. tani*	3.95	2.51	2.61	2.51	2.51	2.54	0.44												
09	GQ866000.1	*S. grossifilum*	3.89	2.61	2.71	2.61	2.61	2.65	0.06	0.08											
10	AB056731.1	*S. mie*	2.75	2.78	2.70	2.74	2.74	2.74	2.34	2.50	2.44										
11	AB056730.1	*S. mie*	2.75	2.77	2.70	2.74	2.74	2.74	2.34	2.50	2.44	0.00									
12	U17732.1	*S. meridionale*	4.05	2.98	2.91	2.98	2.98	2.94	2.59	2.74	2.53	2.71	2.71								
13	GQ866015.1	*S. yuphae*	3.48	2.71	2.81	2.71	2.71	2.71	0.06	0.11	0.13	2.17	2.17	2.55							
14	GQ865998.1		2.77	2.72	3.86	3.72	3.72	3.77	0.17	0.23	0.23	2.29	2.29	2.80	0.13						
15	AB093124.1		3.26	2.17	2.42	2.17	2.17	2.17	2.12	2.44	2.55	1.14	1.14	2.24	2.12	2.61					
16	AB093122.1	*S. sabahense*	3.26	2.17	2.42	2.17	2.17	2.17	2.12	2.44	2.55	1.14	1.14	2.24	2.12	2.61	0.00				

17	AB093119.1	*S. aeneifacies*	3.26	2.17	2.42	2.17	2.17	2.17	2.12	2.44	2.55	1.14	1.14	2.24	2.12	2.61	0.00
18	AB093110.1	*S. tumpaense*	3.16	1.96	2.17	1.96	1.96	1.96	1.92	2.31	2.44	1.04	1.03	2.60	1.92	2.51	0.03
19	AB093106.1	*S. dumogaense*	3.16	1.96	2.17	1.96	1.96	1.96	1.92	2.31	2.44	1.04	1.03	2.60	1.92	2.51	0.03

KEY: ***** = five asterisks to indicate query sequence
16SrRNA = 16S ribosomal RNA

Table 4.6: Summary of suspected Simulium species from Phylogenetic Analyses of NRG

Gene Occurrence	Accession	Species Name	Species Group	SOT	Species Localities
ITS2; ITS1	U36204.1	*S. damnosum* clone 1a	Savanna	II	s. a. n. w/a
ITS2; ITS1; ITS	U36205.1	*S. damnosum* clone 1b	Savanna	III	s. a. n. w/a
ITS	U36206.1	*S. sanctipauli*	Forest	I	s. a. n. w/a
ITS1	U36207.1	*S. sirbanum* clone 1a	Savanna	I	s. a. n. w/a
ITS	U36210.1	*S. sirbanum* clone 2b	Savanna	I	s. a. n. w/a

Key: SOT= Species Occurrence Tally
s. a. n. w/a= Species available in Nigeria and other West Africa
ITS = internal transcribed spacer
ITS1 = first internal transcribed spacer
ITS2 = second internal transcribed spacer
red square = show markers with the highest species occurrence
green square = show markers with the higher species occurrence
blue square = show markers with the least species occurrence
value of the square for species occurrence = red square > green square > blue square

CHAPTER FIVE

5.0 DISCUSSION

The Gurara River area was chosen as the onchocerciasis transmission study focus of the study because of the evidence/claim of *S. damnosum* complex (black flies) activities around the river (Personal Communication).

The experience of the entomological collections of adult female *S. damnosum* complex was in line with observation by Adeleke *et al.*, (2011), who reported seasonal fluctuations of *S. damnosum* complex along river Osun. The first entomological collections of adult female *S. damnosum* complex from this study focus along river Gurara during December 2015 were very low with total of three collections for that day per person. The second visit for entomological collections of adult *S. damnosum* complex was at the peak of the raining season during first week of June 2016. In a nutshell, the over flooding of the entire river bank made both breeding sites and fly catching points to be submerged by flood. However, the third visit by the third week of June to last week of July 2016 for entomological collections was with more fruitful efforts with a high collection of adult female *S. damnosum* complex. The high catch of adult female *S. damnosum* complex population within the study period of vector flies collections may indicate high prevalence around the time and may be responsible for the severity of biting nuisance likely to sustain human onchocerciasis transmission in that environment (Garms *et al.*, 2015).

Lambertson *et al.* (2015) highlighted that different vector species influenced different transmission of onchocerciasis whether mild or severe, through biting and parous rates including vector competence of transmitting the disease, hence; they suggested it should be included in transmission models.

Earlier studies have shown that most of adult female *S. damnosum* complex of the rain forest regions are vectors and carrier of the "forest-strain" of the parasite (*Onchocerca volvulus*) which transmit the milder onchocerciasis of skin lession. While most of those from the savannah regions are known to be vector and carrier of the "savanna-strain" of the parasite (*O. volvulus*) presenting the more severe form of onchocerciasis that result into ocular morbidity (Adeleke *et al.*, 2011; Ezugbo-Nwobi and Eneanya, 2013).

Nevertheless, cytological analyses of polytene chromosomes of these insect vectors had been the old method capable of classifying unambiguously the sibling species of *S. damnosum s. l.* into six siblings in Nigeria. In West Africa, the nine sibling species of *S. damnosum* complex that have been taxonomically identified and documented include *S. sirbanum, S. damnosum sensu stricto, S. dieguerense, S. sanctipauli, S. soubrense, S. squamosum, S. yahense, S. leonense,* and *S. konkorense* (Ibeh *et al.*, 2006). The commonly employed method of identification is restricted to the larvae, and cannot be used to distinguish the adult female *S. damnosum* complex, which serve as actual "culprit-vector" of the concerned *O. volvulus* parasites that transmit human onchocerciasis.

On the other hand, morphological identifications of the adult female *S. damnosum* complex can be distinguished or discriminated into either *S. damnosum* sub-complex "i. e., savanna group consisting of *S. damnosum sensu stricto, S. sirbanum* and *S. dieguerense*" or "forest group consisting of *S. squamosum*; *S. yanhense*; and *S. sanctipauli* sub-complex with about eight species". So, the old and most practical procedure of morphological identifications routinely available to distinguish between the species of adult female *S. damnosum* complex still remains the assessment of micro-morphological characters (Ebido *et al.*, 2011) with the use of stereo microscope as employed in this study. This best known morphological identification procedure

by World Health Organization Onchocerciasis Control Programme (WHO-OCHRT, 2004) involving West African countries to the West of Nigeria was adopted along with that of Wilson *et al.*, (1993 and 1994) in this study, where virtually all the twenty selected adult female *S. damnosum* complex were broadly classified as "*S. damnosum* sub-complex" i. e., "savanna group".

Moreover, it has been shown that many closely related species of blood sucking insect vectors with varying ecology and host preferences are nearly inseparable morphologically (Walton *et al.*, 2008).

Furthermore, several previous studies on morphological characters of *S. damnosum* complex also revealed that the savanna cytospecies could be easily separated from the forest dwelling species using the colour of the wing turfs, fore coxa and abdominal tergite. And that the use of morphological characters could further allow the identification of *S. yahense* with 99% specificity (Wilson *et. al.*, 1993; Mafuyai *et. al.*, 1996b;; Adeleke *et. al.*, 2011; Garms *et. al.*, 2015).

Onchocerciasis is a nationwide public health problem in terms of geographical spread, geopolitical zone locations and importance in Nigeria (Edungbola *et al.*, 1987). Researchers in Nigeria have also ascertained the prevalence of onchocerciasis by entomological evaluation and morphotaxonomy in the determination of the vector flies' parousity, infectivity rate and type (Ibeh *et al.*, 2005; Adeleke *et al.*, 2011; Sam-Wobo *et al.*, 2013).

The high proportion of nulliparous adult female *S. damnosum* complex observed in this present study correlated with a recent study by Sam-Wobo *et al.*, (2013) in parts of Oyo and Ogun States; and between the bordered rivers of Nigeria and Republic of Benin. The abundance of savanna group of the adult female *S. damnosum* complex in all the examined 20 samples was an

indication that the infection with *O. volvulus* from this group of vectors in study focus is that of ocular morbidity of blinding onchocerciasis. Meanwhile, the absence of L_3 parasite stage of *O. volvulus* in all the dissected head regions of the vector flies neutralized the wearisomeness of the ocular onchocerciasis in this study focus as there was a zero transmission potential as regards the calculated monthly transmission potential (MTP; appendix III). Thereby, suggesting a very low or no microfilarial load in the residents or dwellers around the study focus which may be as a result of the effectiveness of ivermectin microfilaricidal chemotherapeutic treatment going on for the past years in the area (NITR Sight Safer).

The dwindling pool of taxonomists is a key inherent limitation in morphology-based identification systems of *S. damnosum* complex which signal the need for a new solution to taxon recognition of adult female *S. damnosum* complex in Nigeria and elsewhere on the globe.

Considering the need to distinguish and identify adult female *S. damnosum* complex accurately, efforts have been made to develop DNA-based methods to distinguish the sibling species. This effort has resulted in the isolation of three DNA probes, which when used together allow the six sibling species to be divided into three groups consisting of two species each (Post and Flook, 1992; Wilson *et al.*, 1994). Also, genomic identification system, which permits life's discrimination through the analysis of a small segment of the genome, is one of the promising approaches to diagnosis of biological diversity. This concept has already gained broad acceptance among those working with the least morphologically tractable groups such as viruses, bacteria and protists (Allander *et al.*, 2001, Hamels *et al.*, 2001). Meanwhile, the problems inherent in morphological taxonomy are general enough to motivate the diffusion of this approach to all life. In fact, there are a growing number of cases in which DNA-based

identification systems have also been applied to higher organisms too (Bucklin *et al.*, 1999; Trewick, 2000; Vincent *et al.*, 2000).

The biological samples of adult female *S. damnosum* complex and the immature larvae stages for this study were stored in 80% isopropanol and carnoy's fixative (ethanol : acetic acid – 3:1) respectively. It was expected that these storage reagents will permeate the tissues of these biological samples to stabilize and protect the integrity of their nucleic acid. Thereby, it minimized the need to process samples immediately or to freeze samples in liquid nitrogen. On occasion where samples could not be use within a week when stored at room temperature or at 4^0C within few weeks of not more than four, the samples were stored indefinitely at -20^0C to make them suitable for further downstream applications in PCR amplifications and DNA sequencing.

The purity of the extracted gDNAs (mean = 1.8) was within acceptable range and this was further confirmed by the agarose gel electrophoresis. The purity was assessed by the ratio of the absorbance at A_{260}/A_{280}nm on a NanoDrop Spectrophotometer (GeneQuantpro RNA/DNA Calculator) of which 1.4 – 2.3 was the range contamination evaluation. Determination of the concentration of nucleic acid purity (mean = 91.91ng/µl) are of importance to downstream protocols, techniques and applications in molecular biology as inaccurate quantification can increase variability in such downstream assays, thereby leading to reduced result confidence.

In a nutshell, efficient and effective nucleic acid extraction methods are of paramount importance in molecular biology, more so the ability to extract pure DNA efficiently is critical and yet overlooked (Amberg *et al.*, 2015; Narayan *et al.*, 2016).

In the work described herein, morphological identification and transmission/infectivity potentials was assessed along with the molecular approaches. So, the DNA nucleotide sequence differences

among the sibling species of the adult female *S. damnosum* complex was exploited by using DNA-based assays, PCR-mediated protocols, DNA sequencing, BLASTN and multiple sequence alignment (MSA); and phylogenetic and molecular evolutionary analyses that may be capable of classifying and distinguishing the various sibling species of *S. damnosum* complex.

The data of the double bands of amplicon for lane 2 of ITS gene was a clear indication that the two pooled morphologically identified species may not be the same as claimed by their morphological identities. This contradiction to morphological identifications brings out the significance of DNA-based identifications of adult female *S. damnosum* complex as better off than the morphological identifications of adult female *S. damnosum* complex which were laid with ambiguity of species small size and structural homogeneity (Adler *et. al.,* 2004). It was of a unique note that ITS of nuclear ribosomal gene was able to clearly distinguished lane 2 (sample containing extracted gDNA from two pooled morphologically identified adult female *S. damnosum* complex) into two different band sizes of approximately 450bp and 250bp. Hence, the different band sizes of 450bp and 250bp for ITS gene for amplicon 2A and 2B respectively; and 550bp for ITS gene of amplicon 3 indicated the ability of the ITS gene to be use in discriminating between different sibling species of the *S. damnosum* complex. The ITS showed the best identification utility among the ITS, ITS1, and ITS2 employed in this study.

On the other hand, the ITS data obtained with different base pair sizes of 250bp, 450bp, and 550bp from this study contradicted the findings of Brockhouse *et al.,* (1993) that previously worked on the larval stage given band sizes of 780bp and 900bp for monomorphic species of *S. squamosum* and *S. damnosum sensu stricto* respectively; and band sizes of 700bp, 835bp, and 875bp for the polymorphic species of *S. sirbanum* in their work. This noted difference could be as a result of the developed adaptive resistance by the *S. damnosum* complex to the on-going

ivermectin microfilaricidal treatment which may result into further polymorphisms within the *S. damnosum* complex from this study focus.

The PCR amplification of the first internal transcribed spacer (ITS-1) region of the nuclear ribosomal DNA repeat using primer from Tang *et al.*, (1996a) was analyzed by agarose gel electrophoresis. The data of ITS-1 amplification gave single band of amplicon of uniform band size of approximately 350bp for amplicon 1 – 4. Even with those samples of ITS-1 ribosomal gene from lanes 1 and 2 containing two morphologically identified adult female *S. damnosum* complex. Except, lane 5 that was the negative control where no band of amplicon was observed. The implication was that those forward and reverse primers used has lesser discriminating capacity to distinguish between sibling species of *S. damnosum* complex on PCR-mediated assay when compared with ITS.

The PCR amplification of the second internal transcribed spacer (ITS-2) region of the nuclear ribosomal DNA repeat using primer from Thanwisai *et al.*, (2005) was analyzed by agarose gel electrophoresis. The data of ITS-2 amplification gave single band of amplicon with uniform band size of approximately 500bp. While, no band of amplicon was observed as expected for lane 5 that was the negative control. The implication was that those forward and reverse primers used were not good marker to distinguish between sibling species of *S. damnosum* complex as ITS gene did.

Comparing the trend of PCR amplification of the nuclear ribosomal genes among ITS, ITS-1, and ITS-2 utilized in this study, the ITS amplification has a best utility for distinguishing between the sibling species of adult female *S. damnosum* complex characterized from this study than others. The order of their discriminating values in this present study based on PCR-

mediated assays and phylogenetic/molecular evolutionary analyses is ITS>ITS-2/ITS-1 and ITS>ITS-1>ITS-2 respectively.

The PCR amplification of 16S rRNA gene showed the same trend as observed for both ITS-1 and ITS-2. Single band of amplicon of uniform band size of approximately 500bp was observed for the PCR amplification of 16S ribosomal RNA of the mitochondrial ribosomal RNA repeat analyzed by agarose gel electrophoresis.

Theoretically, DNA sequencing methods and techniques that are used for the determination of the order of nucleotide bases (adenine, guanine, cytosine, and thymine – A, G, C, and T) in a particular DNA molecule. In this present study, the BigDye termination sequencing method was used with the chain terminator dideoxyribonucleotides (ddNTPs) in a single reaction using the forward primers only, rather than the four reactions in the labeled primer method. Each of the four ddNTPs chain terminator was labeled with a fluorescent dye which emits light at different wavelength.

After obtaining the DNA nucleotide sequences, the data of sequence similarities/homologies and differences generated from the BLASTN (somewhat similar sequence program) result selected nucleotide sequences from MSA of MEGA 6.06 were used to develop phylogenetic tree for members of *S. damnosum* complex. When looking at MSA, it is useful to consider different aspects of the sequences, which include identity, similarity, and homology. Identity means that the sequences have identical residues at their respective positions. On the other hand, similarity has to do with the sequences being compared having similar residues quantitatively. For example, in terms of nucleotide sequences, pyrimidines are considered similar to each other, as are purines. So, similarity ultimately leads to homology, in that the more similar the sequences

are, the closer they are to being homologous. Thus, similarity in sequences can then go on to help to find common ancestry (Pearson, 2013) in molecular phylogenetics of *S. damnosum* complex.

MEGA is a windows-based program (and can also be run under Mac OSX or Linux using Windows emulators) with a full graphical user interface. It includes distance, parsimony and likelihood methods of phylogeny reconstruction. It incorporates the alignment programme of Clustal W and can retrieve data from GenBank (Tamura *et al.*, 2013). The alignment explorer is a tool for building and editing MSA in MEGA, with either the Clustal W or Muscle algorithms.

MSA can be used to create a phylogenetic tree (either rooted or unrooted-cladogram or phenogram). This is made possible by two reasons: the first is because functional domains that are known in annotated sequences can be used for alignment in non-annotated sequences. The second reason is that conserved regions known to be functionally important can be found. And this makes it possible for MSA to be used to analyze and find evolutionary relationships through homology between sequences. Also, point mutations and "indels" (insertion or deletion) events can be detected (https://www.quora.com>bioinformatics...) as well.

Before phylogenetic analysis was embarked upon, there was need to carry out MSA as one of its main applications lies in phylogenetic analysis. MSA was used for reasons such as to detect the regions of variability and homology/similarity among the DNA sequences, to provide stronger evidence than pairwise similarity for structural and functional inferences, and to serve as the first step in phylogenetic reconstruction, in RNA secondary structure prediction, and in building profiles (probabilistic models) for DNA signals (Pearson, 2013).

Computational algorithms of MEGA 6.06 was used in the present study to produce the MSA and analyze the alignments because more than two or three sequences of biologically relevant length would be difficult to align by hand. From the resulting MSA, sequence homology was inferred

and phylogenetic analysis was conducted to evaluate the sequences that shared evolutionary origins.

All searches for the query DNA sequences used in the NCBI BLAST were limited to records matching entrez query – txid 7190 of black flies. Graphic summary of the BLASTN result for amplicon 2A of ITS gene showed that no significant similarity was found in the NCBI GenBank database for it. Hence, no further phylogenetic analysis could be performed on it. The graphic summary and alignments portions of the BLASTN result for amplicon 2B of ITS gene showed distribution of the top 117 BLAST hits on 100 subject sequences, and 9 relevant species were selected to make 10 with the consensus based on identities ≥72% for further phylogenetic analysis. The graphic summary and alignment portions of BLASTN result for amplicon 3 of ITS gene showed distribution of the top 101 BLAST hits on 100 subject sequences. But only one relevant species of identity of 83% could be selected from the alignments, thereby, limiting the capacity for phylogenetic analysis. As such, phylogenetic tree could not be constructed on the pairwise alignment because at least 3 taxa will be required for analysis (Tamura *et. al.*, 2013).

The graphic summary and alignment portions of BLASTN result for amplicon 2 of ITS-1 gene gave distribution of the top 115 BLAST hits on 100 subject sequences, where 7 relevant species were selected to make 8 with the consensus based on identities ≥73% for phylogenetic analysis.

While the graphic summary and alignment portions of BLASTN result for amplicon 2 of ITS-2 gene showed distribution of the top 88 BLAST hits on 74 subject sequences, where 5 relevant species were selected to make 6 with the consensus based on identities ≥71% for phylogenetic analysis.

The graphic summary and alignment portions of BLASTN result for lane 22 of 16SrRNA gene gave distribution of the top 103 BLAST hits on 100 subject sequences, where 18 species were

selected to make 19 with the consensus based on identities ≥80% for constructing phylogenetic tree.

Historically, the primary interest in constructing trees was the pattern of evolutionary relationships itself, or simply the topology of the tree. More recently however, phylogenetic trees are being generated to derive information regarding the processes responsible for the observed pattern of evolutionary relationships, and the tree topology becomes the framework upon which further inference can be drawn. As such, phylogenetics facilitates analysis of gene duplications, rates of evolution, polymorphisms, recombination, divergence of lineages and population demographics (Holder & Lewis, 2003; Bos & Posada, 2005).

Several methods of estimating phylogenetic trees are available. Some of the more commonly used methods include neighbor joining (NJ) (Saitou & Nei, 1987), maximum parsimony (MP) (Fitch, 1971; Farris, 2008) and maximum likelihood (ML) (Beerli & Felsenstein, 2001). Later, more new methods that employ a Bayesian statistical approach (Ronquist, 2004) have been successfully implemented, and these methods have quickly generated much interest (Huelsenbeck *et al.*, 2001). While several differences exist, one common feature that unites NJ, ML and Bayesian methods is the use of explicit statistical models of nucleotide evolution.

However, the MEGA version 6.06 used in this study has minimum evolution (ME) and unweighted pair group mean average (UPGMA) packages in addition to ML, NJ, and MP available for estimating phylogenetic tree. While, ML was specifically chosen for analysis and building phylogenetic tree in this study because of the very weak similarities discovered from the obtained MSA.

Estimation of phylogenetic relationships among genes can help predict which ones might have similar functions (e.g. ortholog detection). Today the use of molecular data such as DNA (as

seen from this study) and protein sequences has mostly outshined the traditional morphological features of classifying organisms in phylogenetics. Phylogenetic trees usually illustrate the evolutionary relationships among groups of organisms, or among a family of related nucleic acid or protein sequences. So, phylogenetic tree is of help in analyzing the changes that have occurred in evolution of different organisms (Pearson, 2013).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

In this study, the morphological identities of 20 selected adult female *S. damnosum* complex were investigated alongside with the transmission/infectivity potentials of 156 observed parous vector flies from the 522 caught flies. This was consequently followed by PCR-mediated molecular assays and DNA nucleotide sequencing of the PCR amplicon before subjecting them to phylogenetic and molecular evolutionary analysis to characterize the adult female *S. damnosum* complex. The DNA-based identification tool involved the nuclear ribosomal gene of ITS, ITS-1 and ITS-2 markers and mitochondrial ribosomal gene of 16S rRNA marker.

BLASTN was carried out for all the 8 successful DNA nucleotide sequence individually by using the database of NCBI GenBank, and all searches were limited to records matching entrez query-txid 7190 of black flies in NCBI GenBank database. Meanwhile, only the close and related species of relevant significance with a good E-value and appropriate identity of not less than 70% were considered and selected for phylogenetic and molecular evolutionary analysis out of the revealed sequences. Any BLASTN results that yielded nucleotide base lengths less than 100bp were not selected from the search to generate phylogenetic trees.

6.2 CONCLUSION

The study revealed zero transmission/infectivity potentials as well as morphological identities as *S. damnosum* sub-complex (otherwise called savanna group) for the investigated vector flies.

The two bands of amplicon with approximately 250bp and 450bp for amplicon 2 of ITS nuclear ribosomal gene revealed the best PCR-mediated molecular identification utility as the previously

morphological identified two pooled vector flies were further reflected as different species on the account of the obtained two PCR amplicon.

Furthermore, the fact that the nucleotide query sequence of one of the unique double bands of amplicon (amplicon 2A of approximately 250bp) for ITS nuclear ribosomal gene did not give any significant similarity when BLASTN was carried out was another evidence that it may be a new cryptic species or that there is no deposition of its sequence in the NCBI GenBank database before the period of this research study.

However, phylogenetic and molecular evolutionary analyses of the selected sequences from the MSA of ITS (amplicon 2B), ITS-1 (amplicon 2), and ITS-2 (amplicon 2) molecular data produced an informative phylogenetic tree with consistence of *S. damnosum* with accession number U36205.1 in each of the three clades of phylogenetic trees analyzed by ML method. So, the implicated *S. damnosum* clone 1b (with accession number U36205.1) from phylogenetic and molecular evolutionary analyses may be suspected to be *S. damnosum sensu stricto* considering the fact it is the most appropriate sibling species member of *S. damnosum* sub-complex than others. More so, the occurrence of *S. damnosum* clone was implicated five times, while *S. sirbanum* and *S sanctipauli* were for two times and one time respectively. Among these three phylogenetically implicated vector flies *S. sanctipauli* belongs to the forest group which was of the least probability while the higher and highest probabilities were for *S. sirbanum* and *S. damnosum sensu stricto*.

Nevertheless, this present study on "Molecular Characterization of Adult Female *S. damnosum* Complex in an Endemic Focus of Gurara River of Kagarko LGA, Kaduna State, Nigeria" demonstrated to some extent the value and advantage of the use of molecular method of characterization over the morphological identification method. The used molecular methods in

this study involves DNA-based techniques, PCR-mediated assays, DNA sequencing, and other bioinformatics techniques such as BLASTN alongside with phylogenetic and molecular evolutionary analyses of generated MSA to clarify gene flow of nuclear ribosomal gene and mitochondrial ribosomal gene for molecular characterization of adult female *S. damnosum* complex. The ITS gene demonstrated highest degree of identification utility than others (ITS-1 and ITS-2 for nuclear ribosomal gene and 16S rRNA for mitochondrial ribosomal gene). In a nutshell, NRG (with specific reference to ITS marker) demonstrated better identification utility than MRG, considering the topology of the constructed phylogenetic trees. Hence, the order of their discriminating value based on PCR-mediated protocol, and phylogenetic analyses is ITS>ITS2/ITS1>16SrRNA and ITS>ITS-1>ITS-2>16SrRNA respectively.

In conclusion, the implicated sibling species from molecular and phylogenetic data are *S. damnosum* clone and *S. sirbanum* (both savanna group members) with intrusion of *S. sanctipauli* (as a forest group member). Thus, the molecular data further corroborated the cytological analyses that have for long presented *S. damnosum sensu stricto*, *S. sirbanum* and *S. dieguerense* as the sibling species members of the *S. damnosum* sub-complex inhabiting the savanna region in West Africa. Secondly, based on the molecular identification by PCR amplification of the ITS gene, a new cryptic species of adult female *S. damnosum* complex has been identified from this study.

6.3 RECOMMENDATIONS

1. It is recommended from this present study that the molecular approach of characterization should be used together with the morphological identification method of the Onchocerciasis sibling vector flies (*S. damnosum* complex), as it gives a more advantage over the later.

2. Also, it is recommended from this present study that the ITS gene of the used molecular method demonstrated the best species characterization utility for *S. damnosum* complex amongst ITS, ITS1, ITS2 and 16SrRNA genes.

3. Molecular method of characterization of *S. damnosum* complex is therefore recommended as a required alternative method to compliment the morphological identification method for delineating molecular differences between species by length or sequence polymorphism.

3. Further intensive multi-gene molecular systematic investigations will be recommended for comprehensive understanding of the genus taxonomy of these vector sibling species and evolution of *S. damnosum* complex with different onchocerciasis transmission potentials.

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APPENDICES

Appendix I: List of Black fly Documented Vernacular/Common Names in Nigeria

Black flies Vernacular Names in Nigeria as compiled by John B. Davies
(Based on the list published in 2006 Studia Dipterologia Supplement, 44: 129 – 135)

S/N	VERNACULAR NAME	LANGUAGE	AREA	SPECIES NAME
1	Abunbu	Tiv	Benue, Manor	S. d. s. l
2	Akinrindi	Gwandara	Niger, Abuja Emirate	+
3	Akurundi	Koro		+
4	Angabu	Bassa	Niger, Gwagwalada	+
5	Bopi or Bopwi	Gwari	Niger, Izom, Gwachipe	+
6	Bumpu	+	Niger, Lafiagi, Kataeregi	+
7a	Bumpwi	+	Niger, Abuja	+
7b	+	+	Benue, Uke	+
8	Cikwibi (a)	Hausa	Niger, Abuja	+
9	Dori	Chamba	Adamawa, Kogin Baba	+
10a	Filfiluwa	Hausa	Adamawa, Serti	+
10b	+	+	Niger/sokoto, zema	+
11	Ita	Gara	Kabba, Ogugu	+
12	Kurukundi	Hausa	Niger, Izom, Bwari	+
13	Kurukundu	+	Niger, Kagara, Kusheriki, Tegina	+
14	Kurukuru	+	Niger, Zungeru	+
15	Kuskuru	+	Zaria, Kudaru, Saminaka	+
16	Kyowa	Tiv	Benue, Kastina Ala	+
17	Luntulumu	Bangawa	Niger, sokoto, Zema	+
18	Makurgi	Nupe	Niger, Lafiagi, Ebba, Kutiwenji	+
19	Mukwai	Fulani	Adamawa, Serti, Beli	+
20	Shakwibi (a)	Hausa	Niger, Abuja, Izom, Mandalla	+
21	Yandori	Jukun	Benue, Takum	+

NOTE: S.d.s.l. = *Simulium damnosum sensu lato*
+ = present as the same as above

Appendix II: Immature stages prospection for *S. damnosum* complex at Gurara River



Appendix III: Black Fly Monthly Transmission Potential Calculation

MTP = £WTP / No of weeks during which captured was realized × No of weeks / month

No = Number

£ = Sum

MTP = Monthly Transmission Potential

WTP = Weekly Transmission Potential

WTP = (No of L3 head in the week / No of flies dissected in the week) × (No of flies captured in the week / No of catching days in the week)

Source: WHO-OCHRT, 2004.

Appendix IV: Materials and Methods Miscellaneous Apparatus, Reagents and Assay kit

The followings are the miscellaneous apparatus utilized in this study: Sterile micro-plastic mortar and sterile micro-plastic pestle, micropipette (20µl, 200µl, 1000µl) and corresponding disposable sterile pipette tips, sterile micro-centrifuge tubes and sterile eppendorf tubes, tube rack, cold pack rack, permanent marker, sterile fine forceps, beaker for used pipette tips and collection tubes, timer, tissue paper and gloves, safety goggle/glass, electrophoresis tank, gel tray, sample comb and insulation tape, lab coat and lab notebook.

The reagents, solvents and solutions used in the study are as follows: Lysis buffer/DNA extraction buffer (10mM NaCl, 20mM Tris-HCl, pH 8.0, 1mM EDTA – Etylene Diamine Tetra Acetate), 3M sodium acetate, Proteinase K solution (10mg/mL of proteinase K in 50mM Tris-HCl, pH 7.5; store at 4^0C), Protein Precipitation Buffer-Phenol equilibrated with 0.1M Tris-HCl, pH 8.0 and Chloroform:Isoamyl Alcohol – (Phenol: Chloroform: Isoamyl Alcohol – 25:24:1), absolute Isopropanol and 80% Isopropanol, DNA Precipitation buffer – Ice cold 100% ethanol, desalting solution – cold 70% ethanol, and 70% ethanol for wiping the table surface, etc., deionized distilled water, normal saline, Giemsa stain, chloroform, TE buffer (10mM Tris-HCl: 0.1mM EDTA, pH 8.0), Electrophoresis buffer – 1x TAE buffer (40mM Tris-Acetate, 1mM EDTA), 5x Gel loading dye, Ethidium bromide (10mg/ml stock solution), Agarose, 10x Gel loading buffer (0.25% bromophenol blue, 0.25% Xylene cyanol, 15% Ficoll – type 400 in 10x TAE buffer), DNA molecular weight marker, RNase A (10mg/mL; Dissolve in 10mM Tris-HCl, pH 7.5, 15mM NaCl; heat at 100^0C for 15min; cool at room temperature and store as aliquots at $–20^0$C), PCR buffer, Taq polymerase, dNTPs (deoxynucleotide triphosphate), forward and reverse primers.

The assay kits used for PCR amplicon purification are from Bioland Scientific LLC (Easyprep^{TM} Genomic DNA Miniprep kit; Easyprep^{TM} PCR Cleaning kit; and Easyprep^{TM} Gel Extraction kit).

Appendix V: PCR Master-mix Components

Components	Volume for one Reaction (µl)	Volume for 6 Reactions (µl)
Premix containing PCR Buffer, dNTPs, Taq Polymerase	Pre-mix	Pre-mix
Forward Primer	1	1x6= 6
Reverse Primer	1	1x6= 6
ddH_2O	16	16x6= 96
Total Volume	18	108

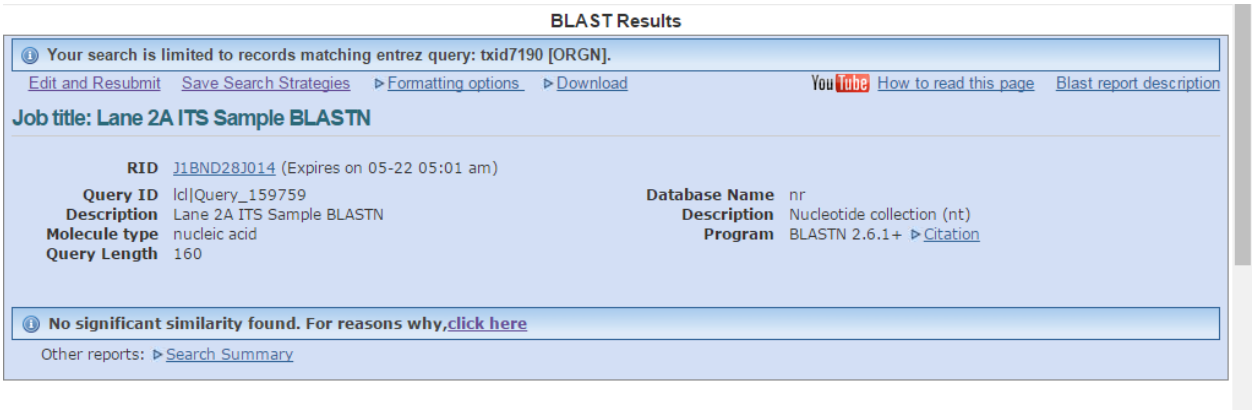
Appendix VI: Two of Chromatograms of DNA Sequencing Results



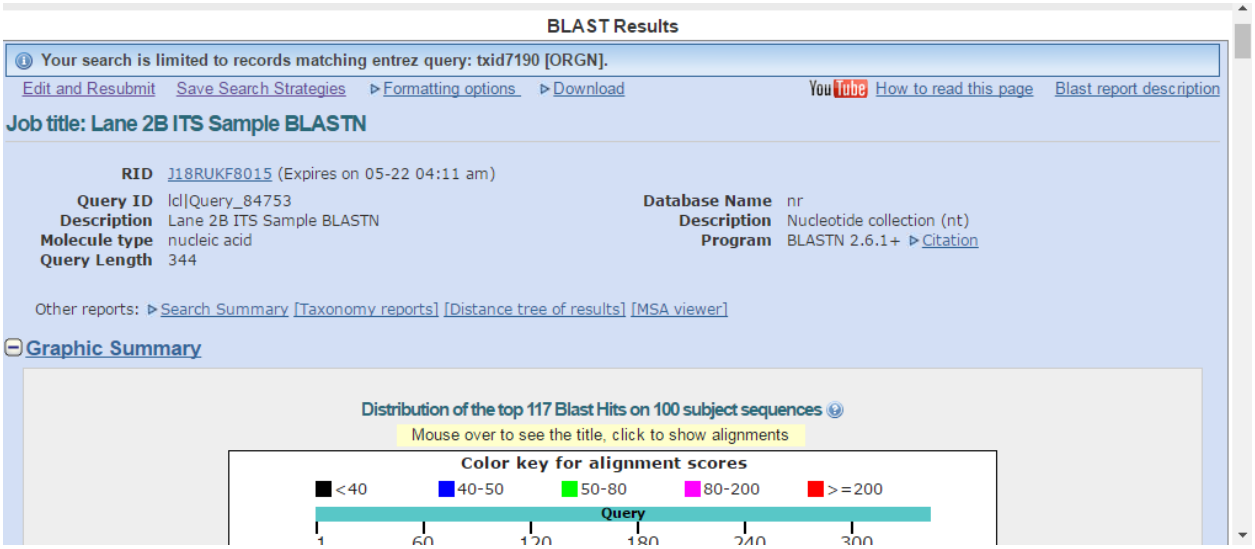


Appendix VII: Graphic Summary of Amplicon 2A and 2B ITS BLASTN Results

Graphic Summary of PCR Amplicon 2A ITS BLASTN result from NCBI GenBank.

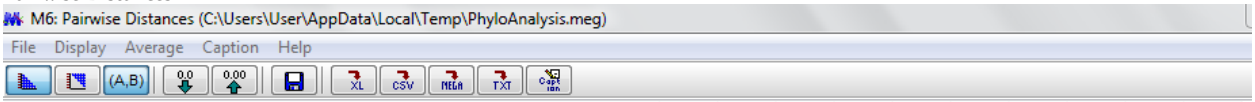


Graphic Summary of PCR Amplicon 2B ITS BLATN result from NCBI GenBank.



Appendix VIII: M6 Graphic Estimates for Amplicon 2B

M6 Graphic Estimates of Evolutionary Divergence between selected sequences from Amplicon 2B Query Sequence of BLASTN Result by Pairwise Distances



	1	2	3	4	5	6	7	8	9	10
1. Lane 2B ITS Query Sequence*****										
2. KF057785.1 S. gravelyi isolate SG10/2012 ITS1ps 5.8SrRNA cs n ITS2ps	1.459									
3. HQ864622.1 S. sp. 6 BR-2011 strain9A 5.8SrRNAps ITS2cs n 28SrRNAps	2.022	1.379								
4. EU429888.1 S. intermedium isolate SiHDam2A3 18SrRNAps ITS1 5.8SrRNA n ITS2cs n 28SrRNAps	0.837	0.918	1.271							
5. U36210.1 S. sirbanum 18S n 28SrRNAps n 5.8SrRNAcs cln2b	0.837	1.081	1.271	0.040						
6. U36206.1 S. sanctipauli 18S n 28SrRNAps n 5.8SrRNAcs	0.771	1.053	1.388	0.040	0.020					
7. U36205.1 S. damnosum 18S n 28SrRNAps n 5.8SrRNAcs cln1b	0.771	1.053	1.388	0.040	0.020	0.000				
8. EF535155.1 S. limbatum isolate 3 ITS1ps 5.8SrRNAcs n ITS2ps	0.837	0.918	1.271	0.000	0.040	0.040	0.040			
9. EF535152.1 S. guianense isolate 2 ITS1ps 5.8SrRNAcs n ITS2ps	0.837	0.918	1.271	0.000	0.040	0.040	0.040	0.000		
10. AF427961.1 S. roraimense isolate BRroraim08 18SrRNAps ITS1 5.8SrRNA n ITS2cs n 28SrRNAps	0.909	0.934	1.477	0.040	0.083	0.082	0.082	0.040	0.040	

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