UNIVERSITY OF CAPE COAST

IDENTIFICATION OF THE VECTOR AND LEISHMANIA SPECIES IN ENDEMIC CUTANEOUS LEISHMANIASIS COMMUNITIES IN THE HO MUNICIPALITY

BY

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Thesis submitted to the Department of Biomedical Sciences of the School of Allied Health Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfilment of the requirements for the award of Doctor of Philosophy degree in Parasitology

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DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature: ..... Date: 5-05-2022

Name: Edna Dzifa Doe

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

Leishmaniasis is a parasitic neglected tropical disease which is caused by protozoan parasites. These parasites are transmitted by the bite of infected female sand flies (vectors) that feed on blood. Monitoring of these sand flies is significant for leishmaniasis control. This study assessed the relationship of species of sand flies, *Leishmania* spp. and human host in endemic communities of the Ho Municipality. Structured questionnaires were administered to individuals in the selected endemic communities. Sand flies were collected, and morphologically identified using Centre for Disease Control light and sticky paper traps and taxonomic keys respectively. Cytochrome *c* oxidase I gene was used to confirm the sand flies that were morphologically identified. Initial PCR amplification of cytochrome *b* and mincircle genes were carried out. DNA sequencing was carried out to identify blood meal source and *Leishmania* DNA in sand flies. A phylogenetic tree was constructed and a pairwise heat map was generated to examine the patterns of relatedness/ similarity amongst the *Leishmania* spp. detected. The results obtained from the knowledge, attitude and practice analysis showed that 88.29% of the study participants had a reasonable knowledge of the disease and its local name. The study participants were of the view that the disease was mainly treated with a particular type of herb - *Hyptis suaveolens* (L) Poit. Eleven different species of sand flies were morphologically identified. Of the female sand flies species identified, 23.14% had taken a blood meal. DNA sequencing results indicated that four of the female sand flies had fed on house mouse (*Mus musculus)* and human blood. It can be concluded that *L. major, L. amazonensis and L. donovani* were identified as the possible parasites circulating in the endemic communities.

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DEDICATION

WO1 (rtd) Edward & Mrs. Rosina Doe

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LIST OF ABBREVIATIONS

CDC	-	Centre for Disease Control and Prevention
CL	-	Cutaneous leishmaniasis
CO1	-	Cytochrome *c* oxidase subunit I gene
Cyt *b*	-	Cytochrome *b* gene
DNA	-	Deoxyribonucleic Acid
GHS	-	Ghana Health Service
KAP	-	Knowledge, Attitude, Practice
kDNA	-	Kinetoplast DNA
MCL	-	Muco Cutaneous leishmaniasis
MoH	-	Ministry of Health
mtDNA	-	Mitochondrial DNA
NTD	-	Neglected Tropical Diseases
PAHO	-	Pan American Health Organization
PCR	-	Polymerase Chain Reaction
RFLP	-	Restriction Fragment Length Polymorphism
VL	-	Visceral leishmaniasis
WHO	-	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Protozoan parasitic diseases remain a major public health problem in the tropical regions of the world according to the World Health Organization (WHO, 2018a). The public health impacts of theses protozoan parasitic diseases have been grossly underestimated, mainly due to lack of awareness of its serious effect on health (Bhargava and Singh, 2012; WHO, 2018a). Leishmaniasis, one of such protozoan parasitic diseases is documented as being part of the twenty neglected tropical diseases (NTDs) listed by WHO, (2018a). The settings in which the disease is found ranges from rainforests in tropical zones of Central America into temperate regions of South America, the tropics and sub tropics of Africa and extend into Southern Europe (particularly the Mediterranean area). and to deserts in Western Asia and the Middle East (Alvar *et al.*, 2012; Eshetu, Awekew, and Bassa, 2016; Steverding, 2017). The disease has a strong link to poverty (Feasey, Wansbrough-Jones, Mabey, and Solomon, 2010) poor housing (Alvar, Yactayo, and Bern,. 2006), lack of financial resources (Okwor and Uzonna, 2016), malnutrition and population displacement (WHO, 2018b).

Leishmaniasis is a complex vector borne disease which is caused by more than twenty diverse species (Cobo, 2014) of obligate intracellular protozoa parasites of the genus *Leishmania* (Azevedo *et al.*, 2012). Martins, Barreto, Lauris, and Martins, (2014) reported that, these *Leishmania* parasites are divided into Old World and the New World species. The Old World species in most cases cause ulcers that are self-limiting (Hepburn, 2000). The New World species cause a wide spectrum of conditions from not posing any serious health threat to severe

mucosal lesions (Shin, Lee, Cho, and Park, 2013). These *Leishmania* parasites are transmitted by the bite of infected females of about thirty species of sand flies which are considered to be vectors (Cobo, 2014; Kakarsulemankhel, 2011; Sunyoto *et al.*, 2018). The reservoir hosts of these sand flies is reported to include animals such as canids, rodents, marsupials, hyraxes, or human beings (Akhoundi *et al.*, 2016). The risk of transmission of the disease peaks from dusk to dawn (Stark and Vidyashankar, 2017). This is so because, the sand fly is typically active during that time.

According to the World Health Organization Global Health Observatory Data, (2016a), leishmaniasis is prevalent in more than ninety eight countries world wide. Between twelve to fifteen million people are infected (Alvar *et al.*, 2012; Torres-Guerrero *et al.*, 2017) and more than three hundred and fifty million people are at risk of the disease (Akhoundi *et al.*, 2016; Pavli and Maltezou, 2010). It is estimated that between nine hundred thousand and one million seven hundred thousand people are infected every year, but only a small fraction of them develop the disease over time (Alvar *et al.*, 2012; WHO, 2016a) whilst between twenty thousand and thirty thousand people may eventually die from the disease (WHO, 2017a). Majority of the new cases however occurred in thirteen countries: Afghanistan, Algeria, Bangladesh, Bolivia, Brazil, Columbia, Ethiopia, India, Iran, Peru, South Sudan, Sudan and Syria (WHO, 2016a). The incidence are probably underrated in most countries, firstly because cases are not documented and reporting is only compulsory in thirty three of the more than ninety eight countries affected (Clem, 2010). Therefore the actual burden are most likely much higher than these estimates (Alvar *et al.*, 2012; McGwire and Satoskar, 2014). Secondly, because leishmaniasis is a disease associated with

poverty, it is typically kept hidden by the affected individuals and their families due to the stigma and thus results in high morbidity (Al-Kamel, 2016).

There are three main clinical manifestation of the disease. The first is cutaneous leishmaniasis (CL) which is localized with single to multiple skin ulcers (Stark and Vidyashankar, 2017). The second is mucocutaneous leishmaniasis (MCL) which is cutaneous leishmaniasis with mucosal involvement and the third is visceral leishmaniasis (VL) which is systemic and the most severe (Steverding, 2017). The most common form of the disease globally is cutaneous leishmaniasis with seven hundred thousand to one million three hundred thousand new cases occurring annually worldwide by estimation (WHO, 2016b). It is prevalent in many regions of the world and considered by the World Health Organization as a category I emerging and uncontrolled disease with a capability to adapt to varying environments (de Vries, Reedijk, and Schallig, 2015). It is endemic in eighty seven countries worldwide including twenty countries of the New World and sixty seven countries of the Old World (WHO, 2014). More than 90% of the cutaneous leishmaniasis cases have been documented to occur in Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, Pakistan, Peru, Saudi Arabia, Sudan, and Syria (Sheikh, Amir, Amir, and Amir, 2020; WHO, 2016b).

The distribution of each of the *Leishmania* species globally informs the type of disease that occurs in an area (Alemayehu and Alemayehu, 2017). For instance, *Leishmania major* causes cutaneous leishmaniasis in Africa, the Middle East and parts of Asia (Akhoundi *et al.*, 2016) whilst *Leishmania tropica* causes this disease in the Mediterranean and parts of Asia (Alemayehu and Alemayehu, 2017; Kumar, 2013). About fifty three *Leishmania* parasite species have been

described in the world (Akhoundi *et al.*, 2016)). Of the total, thirty one of them have been identified to be parasites of mammals and twenty species are pathogenic to humans (Alvar *et al.*, 2012; Torres-Guerrero *et al.*, 2017).

Two genera of sand flies have been described in the transmission of the disease in the Old World, namely *Phlebotomus* and *Sergentomyia* (Boakye, Wilson, and Kweku 2005). The known vectors of leishmaniasis are *Phlebotomus* species of sand flies (Boakye *et al.*, 2005). For instance, in the North of Africa, cutaneous leishmaniasis cases are caused by three parasite species: *Leishmania infantum*, *Leishmania major* and *Leishmania tropica* (Kimutai *et al.*, 2009). These three *Leishmania* species are transmitted by different *Phlebotomus* species which prevail under different biological climates (Aoun and Bouratbine, 2014; Kimutai *et al.*, 2009). In East Africa the aetiological agents include *Leishmania aethiopica, Leishmania major* and *Leishmania tropica* which are also transmitted by different *Phlebotomus* species (Jirata *et al.*, 2006; Ngure *et al.*, 2009; Olobo-Okao and Sagaki Patrick, 2014; Tonui, 2006). Only *Leishmania major* had been reported in West Africa as the causative agent of cutaneous leishmaniasis, however, it has been reported to be transmitted by both *Phlebotomus* and *Sergentomyia* species in some countries (Berdjane-Brouk *et al.*, 2012; Kimutai *et al.*, 2009).

Again in the North of Africa cutaneous leishmaniasis is highly predominant mainly in Algeria, Libya, Morocco and Tunisia whilst the number of cases is quiet low in Egypt (Alvar *et al.*, 2012). In Algeria, Libya and Morocco, the development of new endemic foci with a drastic increase in incidence had been reported since the 1980s (Kimutai *et al.*, 2009). According to WHO, only Ethiopia and Sudan report cases of cutaneous leishmaniasis in East Africa (WHO,

2017a). Relatively, there have been low prevalence rate of the disease in West African countries including Burkina Faso, Cameroon, Ghana, Mali, Niger, Nigeria and Senegal (Kimutai *et al.*, 2009). Although the first work that was published reporting that leishmaniasis was present in Niger was in 1911, other cases of the disease have been consequently reported largely from Cameroon (Rageu, 1951), Mali (Lefrou, 1948), Nigeria (Dyce-Shar, 1924) and Senegal (Riou and Advier, 1933) all in the West African region. Burkina Faso, Gambia, Guinea and Mauritania in the sub-region had also documented cases (Boakye *et al.*, 2005). As a result of this, cutaneous leishmaniasis was suggested to be endemic in a belt running in the West from Mauritania, Gambia through Senegal to Nigeria and Cameroon in the East. Even though the cutaneous leishmaniasis belt then cut across the northern part of Ghana (described as an arid sahel-savannah region) which is typically associated with the transmission of the disease (Fryauff *et al.*, 2006), there has not been evidence of transmission of the disease in these areas. Boakye *et al.*, (2005) reported that the disease had not been documented in Ghana until the year 1999, when some lingering sores diagnosed as cutaneous leishmaniasis caused by *Leishmania major* were reported in the then Ho District of the Volta Region. This District, now a Municipality is described as a semi-deciduous forest ecosystem where two *Phlebotomus* species have been identified (Boakye *et al.*, 2005; Fryauff *et al.*, 2006). Even though *Phlebotomus duboscqi* was the least abundant of the seventeen different sand fly species collected then it also further constituted less than 0.1% of the total catch (Fryauff *et al.*, 2006). This was obviously inadequate to yield a profile of seasonal abundance, though it had been documented to transmit the *Leishmania* parasite in most parts of West Africa. Further, Boakye *et al.*, (2005) reported that in 1997

and 2002, the sand fly species that was collected in the Upper East Region specifically Navrongo comprised of fourteen different species. Thirteen of these were species of *Sergentomyia* which were not generally regarded as likely vectors for transmitting leishmaniasis to humans (Maia and Depaquit, 2016; Ready, 2013).

Majority of the suspected vectors documented in Ghana have been various *Sergentomyia* species. For instance, in the Ghanaian study three man biting *Sergentomyia* species: *Sergentomyia schwetzi, Sergentomyia clydei* and *Sergentomyia adleri* were identified from the then Ho district between 2004-2005 (Boakye *et al.*, 2005). The numbers of *Sergentomyia schwetzi* captured was overwhelming as compared to *Phlebotomus duboscqi*, which is the suspected primary vector. Research carried out in Ghana, Mali and Tunisia have suggested the possible involvement of *Sergentomyia hamoni* and *Sergentomyia ingrami* (Nzelu *et al.*, 2014), *Sergentomyia darlingi* (Berdjane-Brouk *et al.*, 2012) and *Sergentomyia minuta* (Jaouadi *et al.*, 2015) respectively as vectors of leishmaniasis. Jaouadi *et al.*, (2015) reported that *Sergentomyia* species appear to be able to colonise different environmental conditions and biotypes.

In the transmission of leishmaniasis, the existence of animal reservoir hosts is very significant. This is because they provide sources of parasite for vector infection (Alemayehu and Alemayehu, 2017). Largely, each species of *Leishmania* has one or more primary reservoir hosts (Roque and Jansen, 2014). Animal hosts for *Leishmania* species that infect man are usually rodents and dogs (Boakye *et al.*, 2005). Even though dogs are considered the most significant domestic reservoirs of *Leishmania* species, the role of other domesticated mammals such as cats as reservoirs have also been reported (WHO, 2010). To

maintain the transmission of *Leishmania* in an area, the competence of the reservoir host species and how accessible the *Leishmania* parasite is to the vector (Hailu, Yimer, Mulu, and Abera, 2016) are important factors to be considered. In Ghana there has not been any evidence of reservoir host reported yet (Kweku *et al.*, 2011; Seake-Kwamu, 2010).

Kebede *et al.*, (2016) reported that knowing the impact of leishmaniasis and the human behaviour surrounding it helped improve its control and treatment. Thus, the health education for high risk population has a crucial role in preventing the disease. To achieve this, the awareness and the attitude of the group that play major roles in training and educating the community should be improved (Ahmadi, Ghafarzadeh, and Jalali, 2012). There is therefore the utmost need for the assessment of the level of knowledge, attitude, and practice of people living in leishmaniasis endemic communities in Ghana to be able to suggest control strategies to help curb the transmission of the disease.

Moreover, leishmaniasis is being reported in hitherto non-endemic areas of the world where new species of the vectors are emerging. To help evaluate the risk of spread of leishmaniasis, a proper identification of sand flies in an area is paramount (Dokianakis *et al.*, 2018). Sand fly species are conventionally identified using morphological features (Dvorak *et al.*, 2014). This approach is quite laborious, involes a lot of time and demands a certain degree of expertise. It can lead to incorrect epidemiological predictions (Dokianakis *et al.*, 2018). Molecular techniques have been developed to improve the problems with identification of sand fly species. These techniques have become a vital tool regarding how these vectors are distributed geographically (Depaquit, 2014).

Identification of the source of blood meal is important for determining the vectorial capacity and the host preferences of the sand fly (Abbasi, Cunio, and Warburg, 2009; Boakye *et al.*, 1999). The study to examine the source of blood meal of sand flies has had great significance, both ecologically and epidemiologically (Abbasi *et al.*, 2009; Abbate *et al.*, 2020; Alfonso, Miranda Chaves, and Rangel, 2012). Where, for the female sand fly species essential proteins are needed to complement its egg production. This it does by engorging on the vertebrate host blood meal (Azizi, Askari, Kalantari, and Moemenbellah-Fard, 2016). In this regard, particular sand fly species are drawn to certain animal host species or group while most others tend to be universal (Sales *et al.*, 2015).

In nature, sand flies become possibly infected with unpredictable doses of leishmania parasites (Stamper *et al.*, 2011). This dose combined with the immunity of the sand fly (Kelly *et al.*, 2017), parasite virulence (Louradour *et al.*, 2017) and the blood meal has an effect on the development of parasite in the gut and subsequent transmission (Courtenay *et al.*, 2017). Where it has been documented that the rate of sand fly infection with *Leishmania* even in endemic areas is generally negligible (0.01–1%) (Kato *et al.*, 2007).

Moreover, a report put together by the Ghana Health Service Public Health Division (GHS, 2015) regarding the master plan for neglected tropical disease programme indicated that the key strategies used for leishmaniasis was case detection and management. This report further indicated that it aimed to increase public awareness in the endemic areas of Ghana. Therefore, based on the paucity of data on the persistence of the transmission of the disease in the endemic communities in Ghana, it is imperative to carry out this research to be able to come out which effective control strategies that can be adapted.

1.2 Statement of the Problem

It can be deduced from the background that, over the last decades since leishmaniasis first emerged in the Volta region of Ghana,it has persistantly affected individuals in the endemic commnuties. Moreover, limited information about the practices and policies carried out in relation to the disease is available. In that regard the statement of problems can be enumerated as:

Firstly, a good estimate of the true burden of leishmaniasis in Ghana cannot be stated confidently because of poor self-reporting and poor surveillance thereby hampering management of the disease. These have led to unaccountable fluctuations in the reported number of cases in Ghana over the past two decades. Such that, an active search in 2012 found cases in the endemic communites (Kwakye-Nuako, 2016) even though the number of cases had been reported to have decreased to zero between 2008 and 2009.

Secondly, it is not clearly established which species of parasites and vector combination are contributing to leishmaniasis in Ghana. As a resullt, national control efforts cannot be targeted directly at the incriminating vector species. It is therefore important to identify and implicate the particular sand fly species that carry and transmit *Leishmania* species in this endemic focus.

Thirdly, the health system is not able to target prevention efforts and community education without a good knowledge of the roles of the vector species involved in the transmission of the disease. Also, there is limited information about the knowledge, attitude and practice of the disease by the population at the endemic focus.

Fourthly, blood feeding females of *Phlebotomus* species are generally thought to be the only and proven natural vectors of protozoan *Leishmania*

species. However, elsewhere there have been detection of *Leishmania* DNA in species of the genus *Sergentomyia* (Berdjane-Brouk *et al.*, 2012; Campino *et al.*, 2013; Latrofa *et al.*, 2018; Maia and Depaquit, 2016). This finding defies the belief and view that *Leishmania* is solely transmitted by species of the genus *Phlebotomus*. Additionally, the population of sand flies in nature are usually found as separated subunits as a result of ecological, behavioural or genetic diversification (Belen, Kucukyildirim, and Alten, 2011). These populations show different phenotypic and genotypic features from each other depending on gene flow, and diversifying selection (Hedrick, 2005). It is logical to expect that for communities known to be endemic for *Leishmania* infection, new vector species such as *Sergentomyia*, in addition to the previously identified species may have become vectors of the disease overtime. These observations emphasize the need to study the endemic focus and examine all possible vector(s) responsible for disease transmission.

1.3 Research Questions

1. How will the knowledge, attitude and practice with regards to leishmaniasis in the endemic community be enhanced regarding identifying what is known and done about the disease?
2. What will the identity of the captured sand flies in the endemic communities suggest?
3. How will the infection rate of the different species of sand flies be evaluated?
4. How will the probable host involved in the transmission of the disease in the endemic community be examined?

1.4 Research Aim

To assess the relationship of species of sand fly, *Leishmania* parasites and human host in the endemic communities in the Ho Municipality.

1.4.1 Specific objectives

The study sought to:

1. Assess the community knowledge, attitude and practice to be informed of what is known and done with regards to the mode of transmission, prevention and control of the disease.
2. Establish the identity of captured sand flies to provide the relative abundance of sand flies in the endemic communities.
3. Measure the infection rate of the sand flies in the endemic commnuties.
4. Identify the probable hosts involved in the transmission of leishmaniasis by blood meal analysis.

1.5 Significance of the study

Leishmaniasis is one of the neglected tropical diseases reported in Ghana. Over the years, since it was first documented in Ghana, it has continually been reported in this moist semi-deciduous forest ecosystem and resulted in stigmatization of the individuals who have been affected. As a result of the stigmatization that the affected individuals experience and go through, they end up not going about their daily activities which generate income for them and also help build the economy. Hence, this leads to low productivity on the part of the affected individual. Further, health authorities have not intensified the education in the said communities to help control the sand fly (vector) and as such the disease. This study sought to add to knowledge by identifying the sand fly

species, detect the *Leishmania* parasite in the vector and determine the infection rate of the vectors. As this is expected to help inform control strategies.

1.6 Organization of Thesis

The thesis is organized into five chapters. The Chapter one is the introduction and includes background to the study, statement of problem, the research questions, the research aim and objectives, significance of the thesis, and organisation of the thesis. Chapter two reviews relevant literature concerning the study. Chapter three talks about the materials and methods that describe the procedures used in the course of the study. Chapter four details the findings and attempts at discussing the findings in relation to the reviewed literature. Chapter five gives summary, conclusion, limitations and some recommendations.

CHAPTER TWO

LITERATURE REVIEW

2.1 Global Burden of Leishmaniasis

Leishmaniasis is one of the neglected tropical diseases which is widely distributed around the world affecting men, women and children alike (Torres-Guerrero *et al.*, 2017). The fact that leishmaniasis occurs as syndromes (McGwire and Satoskar, 2014) coupled with the lack of surveillance and reporting in the countries most affected by the disease (Alvar *et al.*, 2012) significantly limits the determination of the actual burden of the disease. Overall, an estimated one million three hundred thousand new cases of the diseases occurred annually worldwide in 2016 (WHO, 2017a). Of these, an estimated three hundred thousand cases was visceral leishmaniasis and one million was cutaneous leishmaniasis (WHO, 2018a).

Globally, the disease has affected more than twelve million people (Figure 2.1) and this has induced significant morbidity and mortality throughout the world (Akhoundi *et al.*, 2017; Alvar *et al.*, 2012; Eshetu *et al.*, 2016). In sub saharan Africa, data on the burden of leishmaniasis is critically lacking (Sunyoto *et al.*, 2018; WHO, 2017a). Nevertheless, leishmaniasis is reported to be endemic in countries mostly in the North, Central, East, the Horn of Africa and West Africa (Boakye *et al.*, 2005) and more that three hundred and fifty million people are at risk of the disease (Alvar *et al.*, 2012). Most of the reported cases of the diseases in these regions has been cutaneous leishmaniasis.

In Ghana, since the disease was first reported in 1999 by Boakye *et al.*, (2005) in a moist semi deciduous ecosystem in the Volta region of Ghana, a number of studies (Fryauff *et al.*, 2006; Kimutai *et al.*, 2009; Kwakye-Nuako,

2016; Kweku *et al*., 2011; Mosore, 2016; Nzelu *et al*., 2014, 2015) have been carried out in the endemic communities with different objectives in mind. However, over the decades the burden of leishmaniasis on the people in the endemic commnuties has been very devastating.

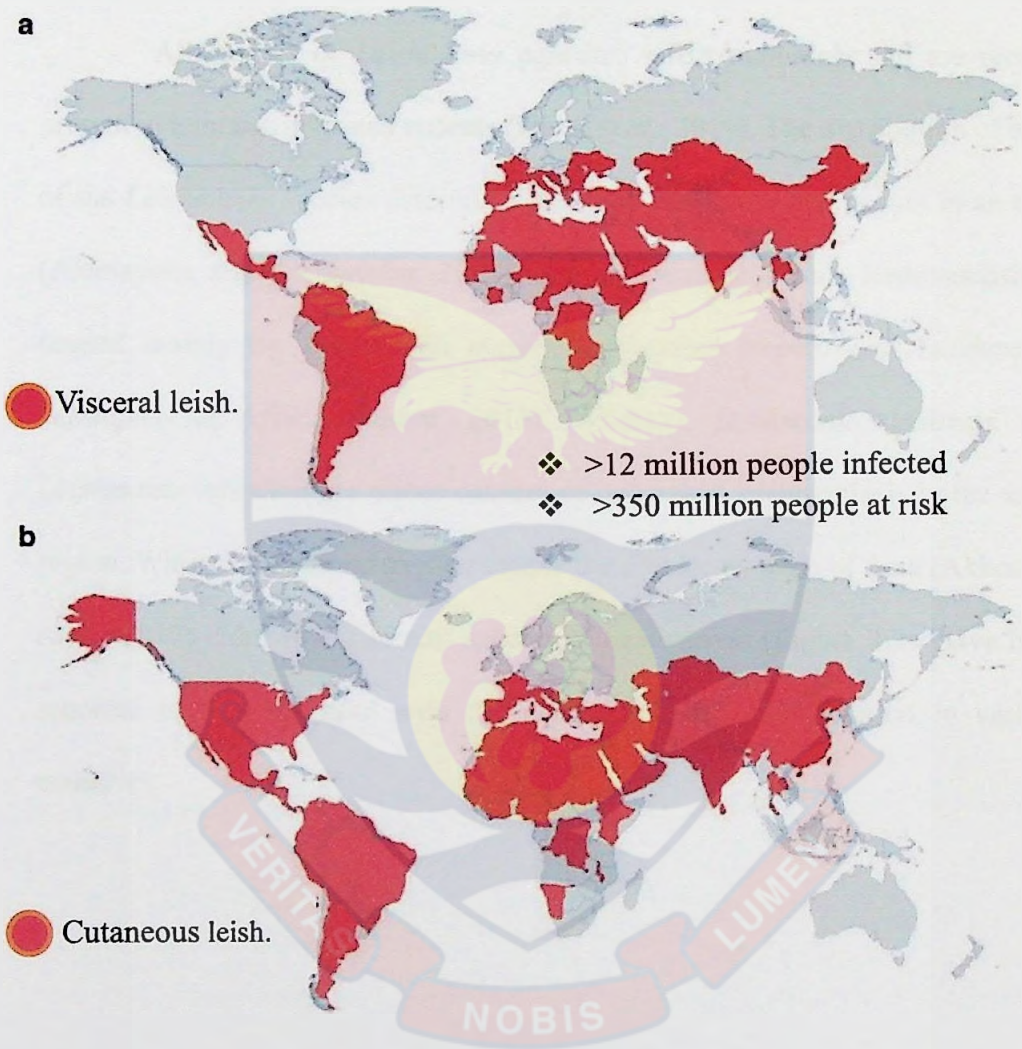


Figure 2.1: Global distribution of leishmaniasis

a. countries reporting cases of VL and b. countries reporting cases of CL (Source: https://link.springer.com/article/10.1007/s00436-021-07139-2)

2.2 The *Leishmania* Parasite and Life Cycle

To better understand the interrelationship between the vector of leishmaniasis and its reservoir host, knowledge of the *Leishmania* parasite and its

life cycle is paramount. The *Leishmania* parasite is acquired by the female sand fly when it feeds on an infected mammalian host, or feed on animal host (Steverding, 2017). Since the *Leishmania* parasite is heteroxenous, it is able to colonize two hosts at two different stages of its life cycle; an invertebrate and a vertebrate host (Toz *et al.*, 2013).

All species of *Leishmania* parasites infect mammals and are mostly present in humans, dogs and rodents (Hirve *et al.*, 2016). The distribution of each of the *Leishmania* species determines the type of disease that occurs in an area (Alemayehu and Alemayehu, 2017). For instance, cutaneous leishmaniasis is caused mainly by *Leishmania major, Leishmania tropica and Leishmania aethiopica* in Africa (Kumar, 2013). Whereas, *Leishmania donovani* and *Leishmania infantum* are minor causers of cutaneous leishmaniasis in the same region. Whilst *Leishmania tropica* causes the disease in parts of Asia (Akhoundi *et al.*, 2017). Globally, different species of *Leishmania* (Figure 2.2) have been reported to be associated with the diverse clinical manifestation in various countries.

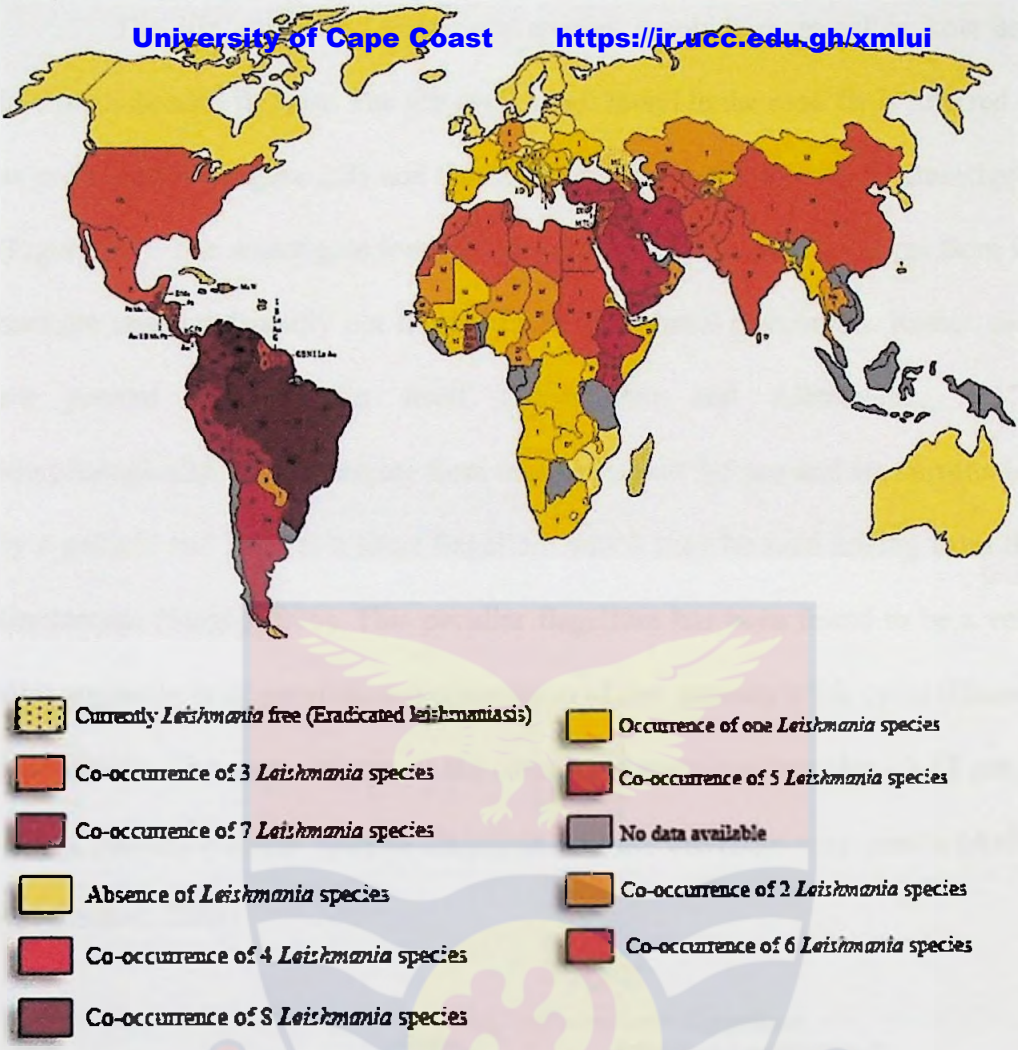
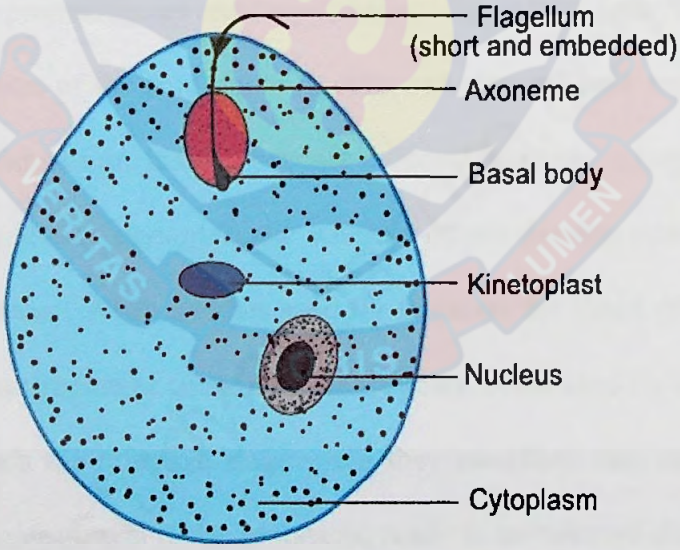


Figure 2.2: Map showing the distribution of *Leishmania* species pathogenic to humans

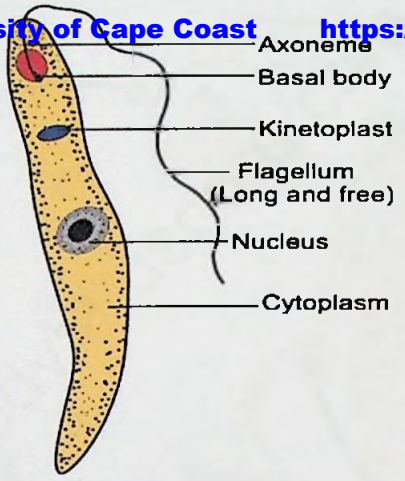
(Source: Akhoundi *et al.*, 2017)

The species of *Leishmania* recorded in Ghana as the possible causative agent of cutaneous leishmaniasis was *Leishmania major* (Kweku *et al.*, 2011). However, Kwakye-Nuako *et al.*, (2015) reported a new species of *Leishmania* causing cutaneous leishmaniasis to be members of the *Leishmania enriettii complex.* It is also worth mentioning that majority of *Leishmania* parasites are more restricted regarding the range of species of sand fly that is able to transmit them (Pratlong *et al.*, 2013).

The life cycle of *Leishmania* species involves mammalian host and intermediate sand fly host. The life cycle stage found in the sand fly is referred to as promastigote (Figure 2.3) and that found in the vertebrate host is amastigote (Figure 2.3). The amastigote form of the parasite that the sand fly takes from its host are characteristically not located in the peripheral circulation. Rather they are present in the skin itself (Alemayehu and Alemayehu, 2017). Morphologically, the amastigote form measure about 3-5 μm and are surrounded by a pellicle and possess a short flagellum which may be seen arising from the kinetosome (Singh, 2006). This peculiar flagellum has been found to be a very vital organelle in its survival and completion of the parasite's life cycle (Gluenz *et al.*, 2010). The promastigote on the other hand measures between 10-15 μm in length, possess a single anterior flagellum and are therefore very motile (Arfan and Simeen, 2008).



A LEISHMANIA: AMASTIGOTE FORM



B LEISHMANIA: PROMASTIGOTE FORM

Figure 2.3*:* The amastigote form [A] and the promastigote form [B] of *Leishmania* species
(Source:http://www.studyandscore.com/studymaterial-detail/leishmania-general-characters-distribution-and-life-cycle)

The life cycle of *Leishmania* (Figure 2.4) begins when an infected female sand fly takes blood meal from its host. It releases promastigotes through the proboscis into the skin of its host during the process. Macrophages then phagocytize the promastigotes and they transform into amastigotes. These results in the proliferation of the amastigote in cells and macrophages and during this time, the symptoms of the disease become evident. However, a large proportion of infected people stay asymptomatic whereas others develop one of the three forms of the disease. When another sand fly feeds on the blood of the infected host, it ingests macrophages containing amastigotes. In the sand fly's mid gut, the amastigotes reach the infective stage when they transform into promastigotes. These promastigotes travel to the proboscis, ready to be released during the next blood meal.

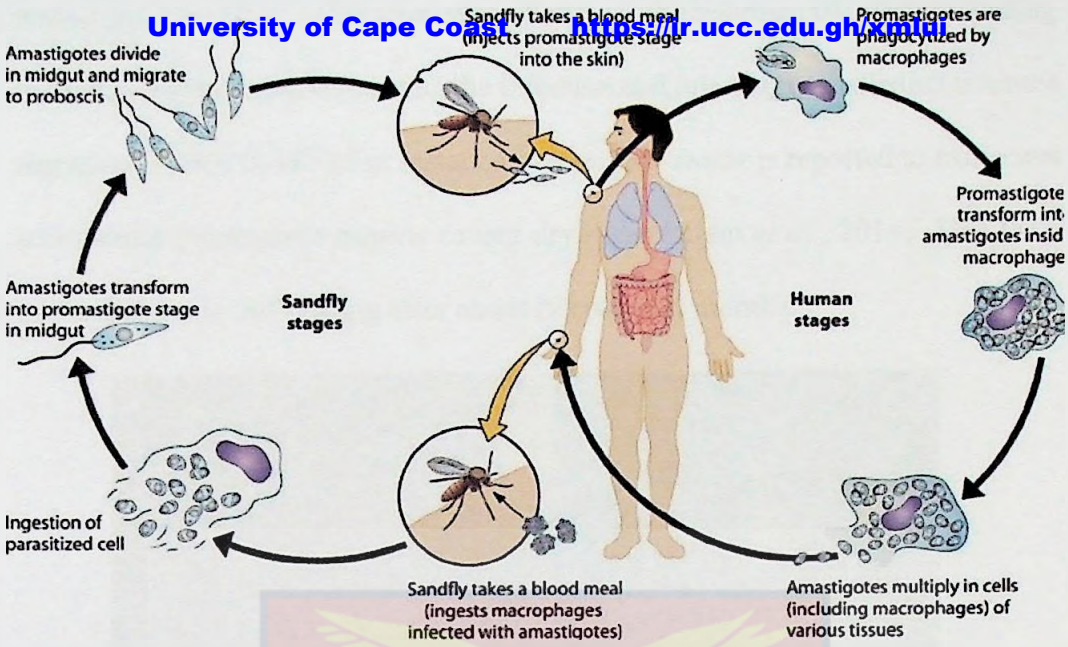


Figure 2.4*:* Life cycle of *Leishmania* species

(Source: Esch and Petersen, 2013)

2.3 Pathogenesis and Clinical Manifestations of Leishmaniasis

Leishmaniasis is categorized by clinical manifestation into three main categories: cutaneous, mucocutaneous and visceral leishmaniasis (Steverding, 2017; WHO, 2017b).

Cutaneous leishmaniasis is also known as oriental sore, tropical sore, chiclero ulcer, Aleppo boil and Delhi boil (Calvopiña *et al.*, 2013). It usually occurs around the exposed parts of the body such as face,neck, and forearms (Figure 2.5) which are susceptible to sand fly bite and often results in the formation of ulcers or nodules around exposed areas (Güran, 2019). The site of the sand fly bite begins with a papule which then grows in size and after sometime it eventually turns to crust form which may also ulcerates. During this phase, a rapid and intense neutrophil infiltration is followed by release of macrophages that phagocytize the promastigotes and transform them into amastigotes (Hurrell *et al.*, 2015). The typical symptom of skin lesion is seen at this stage. It is worth

noting that the clinical characteristics of cutaneous leishmaniasis vary according to the *Leishmania* species causing the infection and this triggers a distinct immune response (WHO, 2018b). For instance *Leishmania major* is reported to cause wet sores while *Leishmania tropica* causes dry sores (Alam *et al.*, 2014). This form of the disease is self healing after about twelve (12) months.

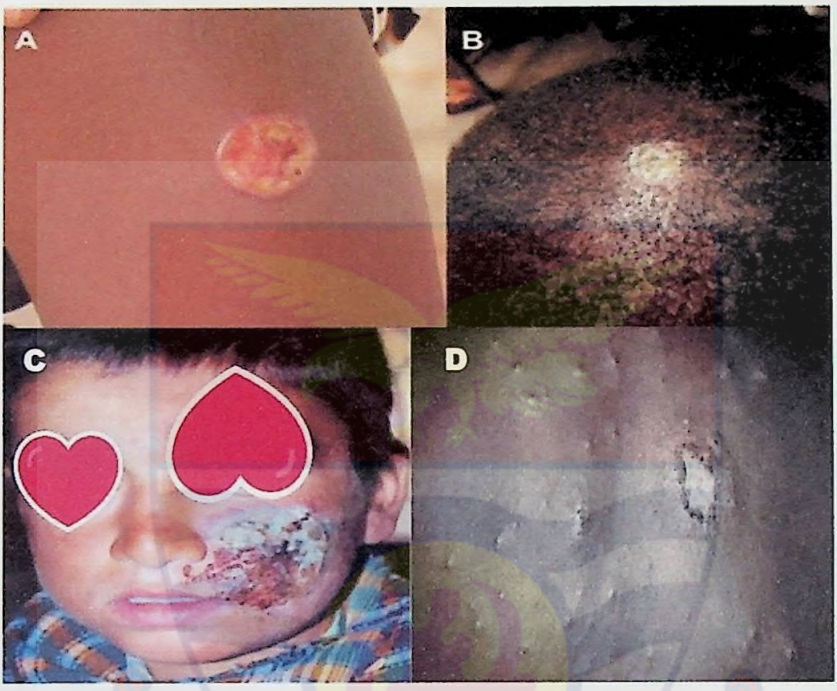


Figure 2.5: Lesions of cutaneous leishmaniasis
(Source:Couppie *et al.*, 2004; Kwakye-Nuako *et al.*, 2015)

Mucocutaneous leishmaniasis, also called Espundia affects the mucous membranes (McGwire and Satoskar, 2014). The disease results in the disfiguring and destruction of tissue of the nose, mouth oro- and naso-pharynx and eyelids (Figure 2.6) and can progress to affect respiratory functions (Torres-Guerrero *et al.*, 2017). It is reported to be caused mostly by *Leishmania braziliensis* (McGwire and Satoskar, 2014). It can develop while skin lesions are still present and the initial signs are usually erythema and ulcerations at the nares, followed by destructive inflammation, with ulcers and nodules that can spread to involve the nasal septum, and in some cases, the oral cavity, pharynx or larynx (Goto and

Lauletta Lindose, 2012). Mucocutaneous leishmaniasis does not heal spontaneously.

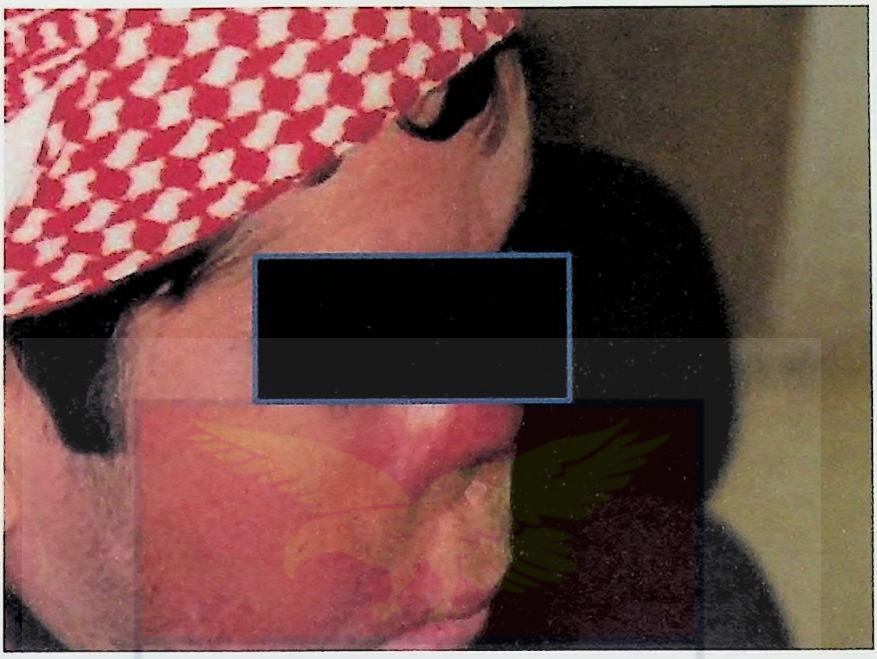


Figure 2.6: A boy suffering from muco-cutaneous leishmaniasis.
(Source: (WHO, 2018b) Photos on vector borne disease)

Visceral leishmaniasis is also known as kala-zar due to the characteristic darkening of the skin (Ardic, Ardic, and Gudel, 2018). It is the most serious form of the disease and it affects several internal organs usually the spleen, liver, and bone marrow (Figure 2.7) (Cobo, 2014). Other symptoms associated with this form of disease are abdominal distension with splenomegaly and hepatomegaly, decreased appetite, prolonged undulant fever, signs of anaemia, and weight loss (McGwire and Satoskar, 2014). The spectrum of visceral leishmaniasis varies from asymptomatic to life-threatening degree in severity (Stark and Vidyashankar, 2017). It has been reported to be caused by *Leishmania donovani* and *Leishmania infantum* (Pratlong *et al.*, 2013). This form of the disease might lead to death of the affected individual if left untreated.

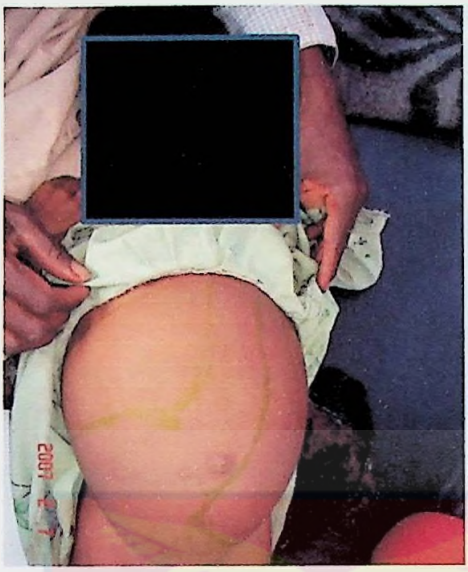


Figure 2.7: A girl suffering from visceral leishmaniasis with markers showing signs of liver and spleen enlargement.
(Source: (WHO, 2018b)

2.4 Diagnosis of Leishmaniasis

The parasitological diagnosis of leishmaniasis is by microscopic examination, cultivation, PCR and serological methods (Ardic *et al.*, 2018). For the diagnosis of visceral leishmaniasis, the confirmation of *Leishmania* infection is by visualisation of the parasites in a tissue smear such as a splenic aspirate, bone marrow or liver biopsy by microscopy (Singh, 2006; de Vries *et al.*, 2015). Additionally, serological methods are being used for the diagnoses of visceral leishmaniasisis (Ardic *et al.*, 2018). In the case of cutaneous leishmaniasis, diagnosis entails microscopic examination of the stained skin scraping or fluid from lesions and identification of amastigotes in Giemsa or Leishman stained reagent (Stark and Vidyashankar, 2017). The microscopic examination is a diagnostic routine technique (León *et al.*, 2017). Though microscopy is the gold standard, its sensitivity is low and variable (Goto and Lauletta Lindoso, 2012). It

is therfore not able to morphologically distinguish *Leishmania* species from each other under the light microscope (Hodiamont *et al.*, 2014: Wall *et al.*, 2012). The set back with this approach is that it is unable to distinguish asymptomatic cases from disease cases. Thus, to increase sensitivity, parasites are grown in culture media - Novy MacNeal Nicolle and isolated. Over the years molecular methods such as PCR have increasingly become appropriate for the diagnosis of this disease (Muñoz *et al.*, 2019).

2.5 Detection of *Leishmania* DNA

The detection of *Leishmania* DNA either in the sand fly or tissue sample is carried out by Polymerase Chain Reaction (Aransay. Scoulica, and Tselentis, 2000; Phumee *et al.*, 2013; Remadi *et al.*, 2020; Srisuton *et al.*, 2019). This technique has been reported to be the most sensitive and specific. However, the efficacy of the assays used depend on the selected target loci for amplification whether conserved or variable and the number of target genes used (Kalle, Kubista, and Rensing, 2014). This technique has been shown to be an important tool for *Leishmania* DNA detection in the sand fly (de Assis *et al.*, 2010; Coura-Vital *et al.*, 2011; Mohammadiha *et al.*, 2013; Solano-Gallego *et al.*, 2011). However, several other molecular methods have been developed on several *Leishmania* target sequences (Mary, Faraut, Lascombe, and Dumon, 2004) to detect the *Leishmania* DNA. For instance, the conserved region of *Leishmania* kinetoplast DNA (kDNA) minicircle has been used as a specific target for conventional or quantitative PCR assays (Mohammadpour *et al.*, 2019). Indeed, kDNA is the most common target used for *Leishmania* species identification.

Several other molecular techniques, biological samples and target genes have been used in detecting *Leishmania* DNA (Maia *et al.*, 2009). Additionally,

other molecular tests that target different gene sequences have also been documented over the years (Monroy-Ostria *et al.*, 2014). However, the ribosomal DNA internal transcribed spacer 1 sequence or sequences within the kinetoplast DNA of the genus *Leishmania* as the main target is mostly used (Odiwuor *et al.*, 2011; Toz *et al.*, 2013). Since the rate of sand fly infection with *Leishmania* even in endemic areas is generally negligible (0.01–1%) (Kato *et al.*, 2007) the use of other molecular approaches for the detection of *Leishmania* species in sand flies is greatly recommended. The use of genetic targets such as minicircle kinetoplast DNA (kDNA), is documented as being very sensitive and specific for the amplification of DNA fragments of *Leishmania* species (Jara *et al.*, 2013). Noteworthy that if *Leishmania* DNA is identified in the sand fly it does not necessarily conclude that the sand fly species is a vector. This is because the assay is not able to differentiate between the presence of *Leishmania* amastigotes from an infected blood meal and a *Leishmania* promastigote (Aransay *et al.*, 2000). Thus, for identifying the sand fly species as a vector, PCR alone cannot be used as a tool.

Screening of sand fly for *Leishmania* DNA only indicates the sand fly has *Leishmania*. However, to zero in on which species of sand fly it carries, DNA sequence analysis of targets that have been amplified by PCR are subjected to DNA sequencing (Al-Huchaimi *et al.*, 2018; Bennai *et al.*, 2018; Fotakis *et al.*, 2019; Llanes-Acevedo *et al.*, 2016; Nzelu *et al.*, 2014). Over the years, the development of this high-speed analytical technique referred to as next-generation sequencing has allowed high-throughput analysis of biological information (Ohashi, Hasegawa, Wakimoto, and Miyamoto-Sato, 2015). Moreover, the sequencing technology has been extensively used for the

identification of *Leishmania* species and to differentiate species of parasites by phylogenetic analysis (Akhoundi *et al.*, 2017; Rogers *et al.*, 2011).

2.6 Emerging Strains of *Leishmania* species

Akhoundi *et al.*, (2016), Alvar *et al.*, (2012) and Torres-Guerrero *et al.*, (2017) reported that twenty (20) species of *Leishmania* are pathogenic to humans. The distribution of each of these *Leishmania* species globally determines the type of disease that occurs in an area. For instance, *Leishmania donovani* caused visceral leishmaniasis in Asia and Africa while *Leishmania major* caused cutaneous leishmaniasis in Africa in human (Alemayehu and Alemayehu, 2017). However, over the years new emerging strains of *Leishmania* have been reported to cause particular form of the disease.

In Ghana, Kwakye-Nuako *et al.*, (2015) from his study reported that *Leishmania enrietii* complex was accounting for cutaneous leishmaniasis in the endemic communities of the Volta region. Stikingly, this parasite has been isolated from a guinea pig in Brazil (Espinosa *et al.*, 2018). This suggest that members of *Leishmania enriettii* complex are able to infect a wide range of different hosts, from rodents to humans. This means there is the possiblity that there might be other emerging parasites in the endemic communities which must be researched into.

2.7 Biology of the Sand fly

The morphological structure of the sand fly (Figure 2.8) has been characterized as being a tiny insect ranging from 1.5-3 mm in length, which possesses dark and large eyes, long antenna and long legs, with its mouthparts oriented downwards (Claborn, 2010). They live for about forty days (Von Stebut, 2015). In respect of their feeding, both the male and female adult sand flies have

been shown to obtain carbohydrate nutrition from plant juices (Depaquit *et al.*, 2010). However, female sand flies, just as is known for female *Anopheles* mosquitoes, also need blood meals to complement the essential proteins that is required for their development and the production of eggs during their cycle (Azizi, Moemenbellah-Fard, Kalantari, and Fakoorziba, 2012). They thus are claimed to feed on a wide variety of vertebrate hosts for their blood meal; these may include amphibians, birds, cats, dogs, livestock, reptiles and rodents (Muñoz *et al.*, 2019). Males are not involved in the transmission cycle of the parasite since male sand flies are not blood feeders.

The sand fly's role in the transmission of leishmaniasis is uniquely associated with its characteristics and needs for survival. Specifically, its confirmed low and weak flight pattern, flying close to the ground (Claborn, 2010), indicates that it does not travel long distances to reach its source of blood meal. Therefore, within the endemic areas the transmission is often irregular (Svobodova *et al.*, 2006). Additionally, the sand fly's ability to inhabit domesticated animal shelters and in the earthen floor of human habitation (Dokianakis *et al.*, 2018) enhances the proximity to its hosts and thereby improves the likelihood of transmission. However, they are able to meet their need for blood meal by nocturnal biting; different species have been shown to bite at different times of the night (Adam, Hassan, Abdelnour, and Awadallah, 2017; Aklilu *et al.*, 2017; Gebresilassie *et al.*, 2015; Ready, 2013).

Figure 2.8*:* Blood fed female sand fly

(Source: Maroli *et al.*, 2013)

2.8 The Vector and Potential Vectors

There are more than eight hundred species of sand flies that have been grouped into five genera: *Phlebotomus, Sergentomyia, Lutzomyia*, *Brumptomyia*, and *Warileya* (Kumar, 2013; Maroli *et al.*, 2013; Ready, 2013). *Phlebotomus* and *Sergentomyia* species are grouped under the Old World whilst *Lutzomyia*, *Brumptomyia*, and *Warileya* are grouped under the New World (Akhoundi *et al.*, 2016). Of these eight hundred species, ninety eight are suspected to be vectors of human leishmaniasis and *Phlebotomus* species make up forty two (Al-Huchaimi *et al.*, 2018).

Although it has been reported by the WHO that each sand fly species characteristically transmits only one species of parasite (WHO, 2010), later studies have indicated that each sand fly species is able to support the development of multiple *Leishmania* species and are referred to as permissive vectors (Alexandre *et al.*, 2020; Dostálová and Volf, 2012). Sand flies of the genus *Phlebotomus* are the only proven vectors of leishmaniasis (Alemayehu and Alemayehu, 2017). This claim is based on the fact that over the years, only

species of the genus *Phlebotomus* have been reported to take blood meals of mainly mammals and sometimes of birds, and thus are thought to transmit *Leishmania* in the Old World (Akhoundi *et al.*, 2016; Blavier *et al.*, 2019). For instance, *Phlebotomus duboscqi* has been reported to be the main vector of leishmaniasis in Ghana, Senegal, The Gambia (Boakye *et al.*, 2005) and Mali (Anderson *et al.*, 2011). Additionally, *Phlebotomus rodhaini* has been reported in Ghana (Mosore, 2016), Mali (Anderson *et al.*, 2011) and Sudan (Elnaiem *et al.*, 2011) as a vector of leishmaniasis.

Despite the fact that this is not confirmed, there are pieces of evidence in favour of the notion that another genus of sand fly, specifically, *Sergentomyia* may be vectors of leishmaniasis. These evidence include the view that in the many leishmaniasis foci in Africa where *Sergentomyia* are dominant species it is because these species can better withstand the biotopes and the environment they inhabit (Senghor *et al.*, 2011). Further, the classical *Phlebotomus* species are scarce or absent (Akhoundi *et al.*, 2016). Another body of evidence is that although most species of *Sergentomyia* have been reported to likely feed on cold-blooded vertebrates and are herpetophilic (González *et al.*, 2017). some species occasionally bite and feed on humans (Bennai *et al.*, 2018; Jaouadi *et al.*, 2013; Sadlova *et al.*, 2013; Senghor *et al.*, 2016). Again, some of them have been found infested with *Leishmania* that are pathogenic to humans. For example, *Sergentomyia clydei* (Ayari *et al.*, 2016), *Sergentomyia darlingi* (Berdjane-Brouk *et al.*, 2012), *Sergentomyia minuta* (Maia et al., 2015), *Sergentomyia schwetzi* (Senghor *et al.*, 2016) have all been reported to feed on mammals, including man. *Leishmania* DNA and/or *Leishmania* parasite have been detected in species of *Sergentomyia* in other countries (Figure 2.9). What cannot be categorically said

is whether these species of *Sergentomyia* sand flies could have vectorial competence for some *Leishmania.* Other authors have also stated that leishmaniasis could be transmitted by sand flies contrary to their usual vectors in the Old World as well as in the New World (Bongiorno. Habluetzel, Khoury, and Maroli, 2003; Galvis-Ovallos *et al.*, 2017).

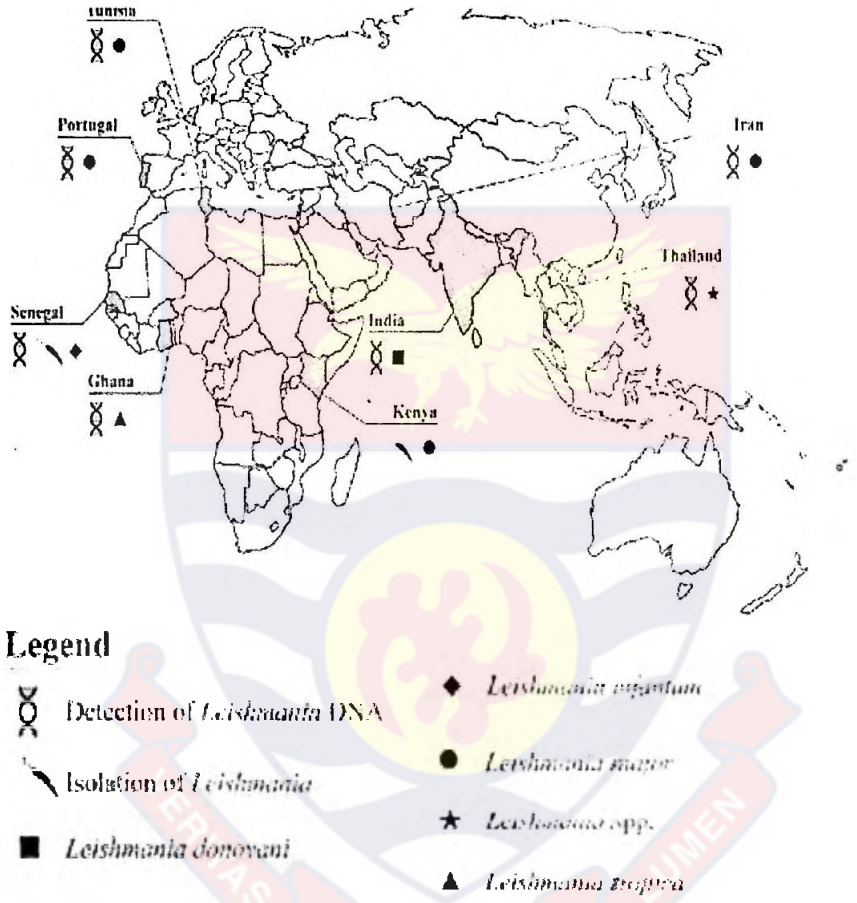


Figure 2.9*:* Map showing *Leishmania* DNA detection and/or parasite in *Sergentomyia* species

(Source: Maia and Depaquit, 2016)

2.9 Sampling Methods for Sand fly

Over the years vector surveillance has been aimed to detect the presence of a vector in a given population or to determine the abundance of the vector in order to estimate the risk of disease transmission in an area (Alten *et al.*, 2015).

In this regard, the sand fly is monitored routinely for the above reason. Thus, to aid in this monitoring, various techniques have shown to help collect these adult sand flies. Notable among these include the Centre for Disease Control and Prevention (CDC) light trap which can be used either with or without CO_2, the human landing collection and sticky paper trap used with or without light (Müller, Revay, and Beier, 2011). Other techniques include the malaise, the Shannon traps, the Disney traps (Hesam-Mohammadi *et al.*, 2014) and aspirator collections (Alten *et al.*, 2015). However, of all these techniques, the CDC light traps have become the standard surveillance technique (Dinesh *et al.*, 2008; Mong'are *et al.*, 2015; Orshan, Szekely, Khalfa, and Britton, 2010) and is generally used for collecting outdoor sand flies. This is so because they are simple to use and less labour intensive than other methods.Additionally, their positioning above the ground poses few problems of standardization. They are also able to operate overnight to collect these sand flies.

The CDC light traps are equipped with incandescent or ultra violet light and as such it is able to significantly catch more sand flies from surroundings several metres away (Müller *et al.*, 2011). The light suction trap is able to easily draw sand flies into the direction of the light source (Junnila *et al.*, 2011). The sticky paper traps made of A4 white plain paper painted with castor oil as adhesive is another technique employed. It is able to intercept sand flies only from their immediate areas and thus yield relatively low numbers of sand flies (Burkett *et al.*, 2007). However, to achieve this the sticky paper is placed near the resting place of the sand flies. Although, the other techniques have been documented to collect sand flies, the CDC light trap and sticky paper trap techniques was employed for collection of sand flies in this study in order to maximize catch.

2.10 Identification of Sand fly

The identification of sand flies in an endemic focus is key step to adapting control strategies. As a result, the sand flies can be identified either morphologically or molecularly. Morphologically, the method has been largely carried out by identifying the internal structures of both the male and female based on diagnostic keys (Al-Mayali and Al-Hassani, 2017; Baharshahi *et al.*, 2016; Singh and Phillips, 2010). This approach has been the accepted practice; it required specific knowledge and skill on the part of the taxonomist. If taxonomic keys were updated regularly then accurate results will be achieved. However, most identification keys that are based on morphological characters are now outdated and are not always reflecting current views of particular groups (Dvorak *et al.*, 2014; Ready, 2013). As a result, when the sand flies to be identified are numerous then, it becomes overly demanding and time consuming (Depaquit, 2014).

Based on the technical limitations associated with the morphological identification techniques, various molecular approaches have been developed (Fujita *et al.*, 2012; Khalid *et al.*, 2010; Mukhopadhyay, Ghosh, and Braig, 2000; Nzelu *et al.*, 2015). These molecular approaches have been documented to tackle such instances where two closely related species of sand fly were differentiated (Dvorak *et al.*, 2014). Of the many molecular approaches to identify sand flies, using standard mitochondrial markers have become popular (Adeniran *et al.*, 2019). Example of such include employing the ribosomal gene sequences namely 18SrDNA (Al-Dakhil, Al-Ajmi, Siddiqi, and Ayaad, 2017) and the D2 domain in 28SrDNA to re-examine the relationships within sand flies (Azpurua, De La Cruz, Valderama, and Windsor, 2010). The use of mitochondrial genes

(cytochrome *b* gene and cytochrome *c* oxidase subunit I gene) and nuclear gene have also been used to resolve intraspecific and sub generic relationships (Gutiérrez *et al.*, 2014; Kuwahara *et al.*, 2009).

Of all the various molecular approaches have been developed and documented (Fujita *et al.*, 2012; Khalid *et al.*, 2010; Mukhopadhyay *et al.*, 2000; Nzelu *et al.*, 2015) to identify species of sand flies, mitochondrial markers have become a popular approach, particularly the use of cytochrome *c* oxidase subunit I (COI) for DNA barcoding (Azpurua *et al.*, 2010; Cohnstaedt *et al.*, 2011; Depaquit, 2014; Gutiérrez *et al.*, 2014; Hebert, Cywinska, Ball, and deWaard, 2003; Pinto *et al.*, 2015). The COI is used because it is documented to be the most conserved gene (Kumar, Srinivasan, and Jambulingam, 2012) and is most widely used in species identification (Kim *et al.*, 2012). Studies have shown that the 5' fragment of the COI appears to be an excellent tool for distinguishing between two closely related species of sand flies (Jinbo *et al.*, 2011; Pinto *et al.*, 2015) that are present in a disease transmission area. Nonetheless, the DNA barcoding approach should complement the conventional method of identification in order to verify questions that may arise from morphological identification (Dokianakis *et al.*, 2018). In this way systematic discrepancies could be fixed and all individual sand flies studied can be sited in their particular taxa (Depaquit, 2014).

2.11 Blood Meal Identification of Sand flies

To aid in the determination of the probable host involved in the transmission of leishmaniasis by blood meal analysis, it is necessary to understand the epidemiology of vector borne disease (Jaouadi *et al.*, 2013). This is because the feeding pattern of such vectors is of immense significance since any data on their host blood meal sources could help identify the potential

reservoir hosts and provide basic epidemiological information for vector control programs (Azizi *et al.*, 2016; Kato *et al.*, 2007). Previously, serological techniques such as the ELISA (Bongiorno *et al.*, 2003), latex agglutination test (Abbasi *et al.*, 2009) and the precipitin test (Nery, Lorosa, and Franco, 2004) were employed to detect the blood meal. However, these techniques have now been observed to take a lot of time and are less sensitive (Maleki-Ravasan *et al.*, 2009). DNA based molecular techniques have gained approval and are very specific and sensitive (Guo *et al.*, 2012). These different molecular approaches have thus been employed to identify the feeding sources of the sand fly to properly evaluate their blood feeding preferences (Baum *et al.*, 2015). Molecular methods with quiet a high degree of sensitivity and specificity have been developed for identifying blood meal source (Paiva-Cavalcanti, Regis-da-Silva, and Gomes, 2010), Such methods are based on the polymerase chain reaction such as PCR Restriction Fragment Length Polymorphism (PCR-RFLP) amplification (Paiva-Cavalcanti *et al.*, 2010; Quaresma *et al.*, 2012). Additionally, mitochondrial DNA (mtDNA) cytochrome *b* gene is widely used in identifying the blood meal of insect species (Che Lah, Yaakop, Ahamad, and Md Nor, 2015) where the cytochrome *b* gene (cyt b) which codes for an electron-transporting protein is utilised. This is so because the mitochondrial DNA contains a high proportion of evolutionary nucleotide substitutions making it particularly valuable in discriminating between closely related vertebrates (Guo *et al.*, 2012). Also, the availability of a conserved region of the gene in vertebrates also makes the cytochrome *b* gene a good candidate for PCR blood meal identification (Ernieenor Faraliana, Mariana, and Tze Ming, 2013; Hadj-Henni *et al.*, 2015). The cytochrome *b* gene does this by identifying engorged females containing digested blood in their abdomen

accurately. Furthermore, Hadj-Henni *et al.*, (2015) in their study concluded that the cytochrome *b* gene was better able to identify the source of blood meal in *Culicoides* than the vertebrate prepronociceptin gene (PNOC). Quaresma *et al.*, (2012) reported that the most specific method to identify blood meal source is DNA sequencing of amplified DNA. Therfore, sequencing of these amplified DNA regions is the ideal approach since the primers used can amplify conserved homologous DNA fragments from diverse potential of vertebrate blood sources (Kent, 2009).

2.12 The Reservoir Host

The presence of reservoir hosts is very important in the spread of leishmaniasis since they provide a source of the parasites from vector infection (Boakye *et al.*, 2005). These reservoir hosts are required to maintain the life cycle of the *Leishmania* species. Roque and Jansen, (2014) reported that every species of *Leishmania* had one or more primary reservoir hosts. Therefore, many species of wild and domestic mammals have been documented as hosts and/or reservoirs of *Leishmania* species. For instance, dogs and mongooses (Rohousova *et al.*, 2015), foxes, rock hyraxes and rodents (Lemma *et al.*, 2017), armadillos, bats, jackals, kangaroos, monkeys, porcupines, primates, squirrels and wolves (Akhoundi *et al.*, 2016; Alemayehu and Alemayehu, 2017) and cats (Roque and Jansen, 2014) have been reported as reservoirs in different localities. Indeed some domesticated animals like the dog have been the most reported species in the epidemiology of the disease (Eshetu *et al.*, 2016). The presence and frequency of these animals in an endemic area may have significant consequence on the pressure of the disease on humans. However, only local studies that involve

ecological and parasitological analysis can ascertain whether an animal is playing a role as reservoir in a given endemic area (Roque and Jansen, 2014).

Few studies have been carried out on flying animals to establish whether they are reservoir host, yet, the *Leishmania* parasite have been isolated from the blood of bats (Berzunza-Cruz *et al.*, 2015). Noteworthy, the reservoirs of *Leishmania major* in West Africa have been rodents (Kone *et al.*, 2016). For instance, *Leishmania major* have been isolated from two species of hedgehog: *Atelerix algirus* and *Paraechinus aethiopicus* in Algeria (Tomás-Pérez *et al.*, 2014). On the other hand, in North East Africa, specifically Sudan *Canis familiaris* was reported as the reservoir host for *Leishmania donovani* (Hassan *et al.*, 2009). The question arises as to how there is transmission of the disease in Ghana when no reservoir host has been documented yet (Kweku *et al.*, 2011; Seake-Kwamu, 2010).

2.13 Treatment and Control of Leishmaniasis

Leishmaniasis is the only neglected tropical disease, which is being treated by non-leishmanial drugs (Croft and Yardley, 2002). Thus far, no drug or effective vaccine has been made available that will induce long term protection and also ensure immunity that will be effective against any form of human leishmaniasis (Ghorbani and Farhoudi, 2017; Kobets, Grekov, and Lipoldova, 2012). Nonetheless, Alidadi and Oryan, (2014) and Mutiso *et al.*, (2013) reported that several vaccine preparations were in more or less advanced stages of testing. Moreover, since there is no confirmed reported anti-leishmanial vaccine in clinical use, the treatment of visceral leishmaniasis relies entirely on chemotherapy (Gupta and Nishi, 2011). Hitherto, individuals suffering from cutaneous leishmaniasis can be treated with topical applications (de Vries *et al.*,

2015). Alternative treatment protocols include antifungal agents such as fluconazole, itraconazol and ketoconazole (González, 2013). Another way of treating leishmaniasis is the use of controlled release systems, such as liposomes and nanoparticles (de Menezes *et al.*, 2015). However, not all lesions require treatment.

On the African continent and specifically in Ghana where access to health care is poor or unavailable, many communities resort to medicinal plants for treatment (Darko, 2012). Essential oils and extracts of a large number of plants have been shown to be effective against different species of *Leishmania* (Monzote *et al.*, 2014; Rodrigues *et al.*, 2013). Studies carried out by Ogeto *et al.*, (2013) have shown the anti-leishmanial activity of *Aloe secundiflora* plant extracts against *Leishmania major*. The anti-leishmanial activity of some of these plants has been attributed to the presence of the compounds such as alkaloids, chalcones, triterpenoids, naphthoquinones, quinones, terpenes, steroids, lignans saponins, and flavonoids (Oryan, 2014). A study by (Yamamoto *et al.*, 2014) demonstrated that without causing toxic effects, oleanolic and ursolic acid that contained fractions from *Baccharis uncinella* leaves eliminated the promastigote and amastigote forms of *Leishmania amazonensis* and *Leishmania braziliensis*. In Ghana, there is limited report of tests carried out on any Ghanaian medicinal plant for anti *Leishmania* activity. There is also no documented evidence of any plant medicine that has been used to treat leishmaniasis (Amoa-Bosompem, 2016).

The control of leishmaniasis mostly depend on the control of the vector, in some cases, management of reservoir hosts and the early diagnosis and treatment of human cases (Otranto and Dantas-Torres, 2013). Thus, facts on possible sand fly vectors as well as their associated *Leishmania* species is of

essence in order to control the transmission of *Leishmania* in an endemic area (Nzelu *et al.*, 2014). The sand fly is reported to be very vulnerable to insecticide that contain d-limonene and linalool as components (Sarwar, 2015). Therefore, the most widely used intervention for controlling sand flies include practices such as spraying houses with such insecticides. Bed nets could provide considerable protection from sand flies at night. Such bed nets which may or may not be treated with pesticide have demonstated to be effective in areas where sand flies rest mostly indoors after feeding (Yaghoobi-Ershadi *et al.*, 2006). Also, the use of repellents that contain N,N-diethyl-3-methylbenzamide (DEET) as a defensive mechanism against bites of sand flies had shown to be an efficient control method mostly in regions with health facilities that are poor (Alexander and Maroli, 2003).

In Ghana, there has been limited control program for leishmaniasis if any. However, there was mass spraying with insecticides when leishmaniasis was first detected in Ghana and this was suppose to have played a role in the control of leishmaniasis (Laudau, 2008) although it is far from sufficient. A report sited by Seake-Kwamu, (2010) stated that there was no leishmaniasis reservoir control program then since no reservoir host had been reported..

Leishmaniasis as a neglected tropical disease, a zoonotic, and a vector-borne disease, faces challenges to its control globally. As a neglected tropical disease, it is reported to mostly afflict the poorest populations (Feasey *et al.*, 2010; Molyneux, Savioli, and Engels, 2017). As a result it does not have access to funding or the stability reserved for research and development of diseases such as cancer, diabetes or HIV (Molyneux *et al.*, 2017). Kamhawi, (2017), reported that as a zoonosis, its control is hampered by the presence of various animal

reservoirs, which forms barriers to its elimination. Then as a vector-borne disease, essential aspects of both sand fly and human behaviours challenge vector control efforts (Cameron *et al.*, 2016). In addition to these, the emergence of new disease manifestations, new vectors or new potential reservoirs (Pothirat *et al.*, 2014; Senghor *et al.*, 2016) have been recently reported and this may pose further challenges for control efforts.

2.14 Knowledge, Attitude and Practices (KAP) towards Leishmaniasis

Ahmadi *et al.*, (2012) reports that people's awareness of this disease has not been desirable. For instance, reporting of the disease is compulsory only in thirty three countries out of the over ninety eight affected by leishmaniasis, thus, a substantial number of cases are never reported (Weerakoon *et al.*, 2016). However, in order to carry out successful control programs, it is important to know the risk factors associated with it so that implementers of the program can understand the disease with regards to the knowledge, attitudes, and practices (KAP) of the population. Moreover, the adoption of preventive measures strongly depends on the attitudes and behaviours of the population at risk (Akram, Khan, Qadir, and Sabir, 2015). Alemu *et al.*, (2013) reported in their study that for the success of control and prevention programs of any disease, the most significant requirement was the participation of the community. As a result, co-operation of the population that is affected was crucial in the execution and use of activities for the program. Additionally, to achieve this goal, it is crucial to discern the knowledge and attitude gaps in the targeted population before establishing health education interventions (Dawaki *et al.*, 2016).

A number of such KAP studies have been carried out in Western Asia where the findings have helped to improve control of leishmaniasis (Akram *et al.*,

2015; Hejazi *et al.*, 2010). In sub Saharan Africa, KAP studies carried out mainly in Ethiopia (Alemu *et al.*, 2013; Kebede *et al.*, 2016; López-Perea *et al.*, 2014) revealed lack of knowledge among communities in relation to the sand flies and leishmaniasis. These studies also revealed the lack of management of the disease and as such activities towards behavioural change and social mobilization was suggested.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical Issues

Ethical approval was obtained from the Institutional Review Board of University of Cape Coast (UCCIRB/CHAS/2017/32). The Ho Regional Ghana Health Service, heads of family and leaders of the endemic communities were spoken to and they gave their consent before the study began. After the potential study participants had been briefed about the study, informed consent (APPENDIX A) was sought for before proceeding with the questionnaire administration. The questionnaires was administered by a one-on-one interview, in such a manner as to ensure that no other person heard the participant and the researcher.

3.2 Study Area

The study was conducted within the Ho Municipality. This Municipalty is located in the middle zone of the Volta region particularly, in the south eastern part of Ghana. (Figure 3.1). Its total land area is 2,361 square kilometres and it lies between latitude 6°36′43″N and longitude 0°28′13″E and the elevation of the villages is 150m /490ft above sea level (Ghana Statistical Service, 2014). The Municipality is characterized by a moist semi-deciduous forest ecosystem (Fryauff *et al.*, 2006). The vegetation of the Municipality is mainly forest and forest savannah. The major economic activities of the population are agriculture, trading and animal farming like goat and fowl breeding (Kwakye-Nuako, 2016). There are two main seasons: wet and dry (Kwakye-Nuako, 2016). The wet season encompasses the major rains from March to August and the minor from October

to December (Mosore, 2016). Most of the houses have been constructed from mud and bricks and are separated by closely planted banana and plantain trees which provides warmth and mosture for breeding of the sand fly. The population of the Municipality according to 2010 Population and Housing Census (Ghana Statistical Service, 2014) stands at 177,281 with 47.28% (83,819) males and (52.72%) 93,462 females.

The outbreak of cutaneous leishmaniasis was reported in 1999 in the communities within this moist semi-deciduous forest ecosystem, which was not typically associated with leishmaniasis. The study was carried out in selected endemic communities in the Ho Municipality of the Volta region of Ghana where there had been reported incidences of the disease over the years (Boakye *et al.*, 2005; Fryauff *et al.*, 2006; Kwakye-Nuako, 2016; Kweku *et al.*, 2011; Mosore, 2016; Nzelu *et al.*, 2014; Villinski *et al.*, 2008). It was carried out in two parts; the first part involved questionnaire administration to individuals who had given their consent to participate in the study. Whilst the second part involved collection of sand flies by siting of the CDC light traps and sticky paper traps around the dwellings of inhabitant of the community and subsequent laboratory analysis. During the questionnaire administration, individuals from nine (9) communities namely; Klefe Atsatime (6.618294 °N, 0.444726°E), Klefe Dome (6.621197°N, 0.443164°E), Klefe Demete 6.61678°N,0.437906 °E), Taviefe-Dzefe (6.660296°N, 0.471817°E), Taviefe Avenya (6.664015°N, 0.473331°E), Matse Havi (6.688649°N, 0.482185°E), Dodome Dogblome (6.760774°N, 0.51546°E), Lume Atsyiame (6.619918°N, 0.464762°E), Dodome Awuiasu (6.767553°N, 0.514789°E) were engaged. This was so because data collection depended on visits by community health nurses on their routine visitation. However, the CDC

light traps and sticky paper traps were sited in four (4) endemic communities namely; Klefe (6.621197°N, 0.443164°E), Lume Atsiame (6.619918°N, 0.464762°E), Dodome Dogblome (6.760774°N, 0.51546°E) and Dodome Awuiasu (6.767553°N, 0.514789°E). This was so because these communities represent areas in the Municipality where cases of cutaneous leishmaniasis had been reported (Ghana Health Services, 2013). Additionally, Kwakye-Nuako, (2016) had reported a new strain of *Leishmania* species in these four villages.

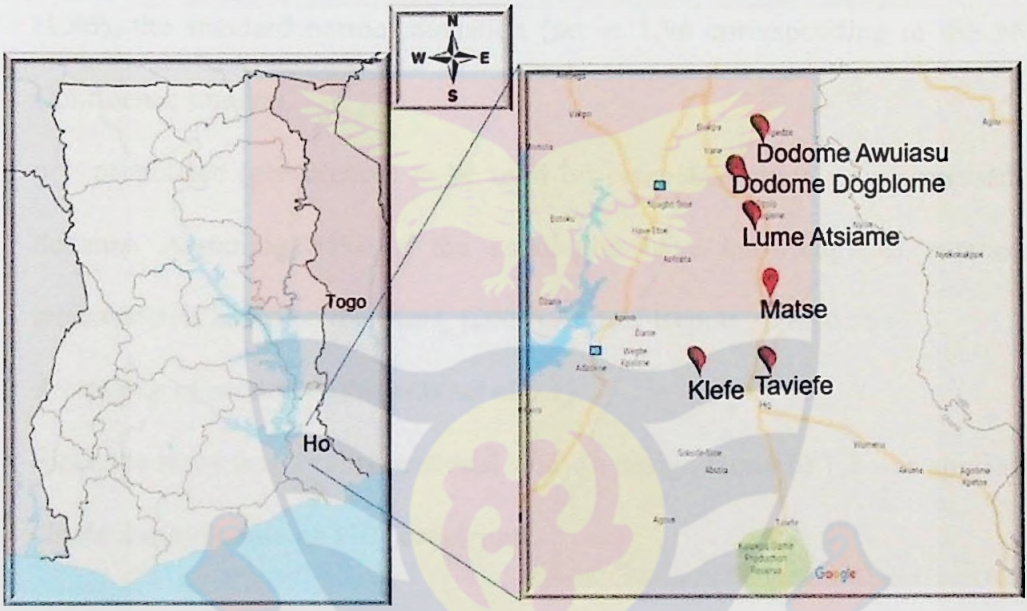


Figure 3.1*:* A map of Ghana showing the study sites

(Source: https://www.onestopmap.com/ghana/ghana-36/)

3.3 Study Design and Sample Size Determination

A community based cross-sectional study was conducted between November 2017 and March 2018 among the inhabitants of the endemic areas in the Ho Municipality. This was carried out using structured questionnaires. It aimed to assess the burden of disease in the community by getting information regarding their knowledge, attitude and practice of the disease. This study intended to help come out with control strategies in curbing the disease.

The sample size was calculated using the WHO recommended statistical formula for health studies: the random sample size equation (1) and a modification (2) since the population was less than 10,000. Where.

$N = Z^2p\ (1- p)/d^2$ -----(1)

$S = N/1+N/population$ -----(2)

N = number of study participants enrolled in the study

Z = test statistics which allows to calculate the results with 95% confidence (1.96), the standard normal deviation (set at 1.96 corresponding to the 95% Confidence Interval,

p = proportion (prevalence) to be used on estimates which was expressed in decimal. Assuming 55% of the population have knowledge of cutaneous leishmaniasis based on the MoH, (2004) Annual Report., p is 0.55.

d = degree of accuracy (this was set at 0.1)

Since the study was a cross sectional study, a design factor of 1.2 was applied to obtain a sample size of 110 participants.

3.4 Questionnaire Development

The study questionnaires (APPENDIX B) were developed by following Frary's guidelines on questionnaire construction (Frary, 1998). The study questionnaire comprised of six parts. Part A related to sociodemographic background of study participants, part B on knowledge regarding CL, part C experience with the disease, part D on attitude towards CL, and Part E on practice related to CL prevention. The questionnaire was pretested. Pretesting of the questionnaire was done on ten (10) randomly selected study participants in the communities. The questionnaire was finalized after ambiguous and unsuitable questions were modified based on the result of the pretest. Also, the pretest helped

to improve the phrasing of questions in the questionnaire. Generally, knowledge of the disease was assessed by asking questions like symptoms and vector of the disease. Whereas, attitudes and practices were assessed by the responses of the study participants related to the seriousness of the disease, preventive measures of the disease and information source.

3.5 Collection of Sand flies

In November and December 2017, and between January – August 2018, the sand flies were collected. Locations for siting of the CDC light traps and sticky traps (Figure 3.2) were randomly selected for sand fly collections in the selected endemic communities listed above. These communities are characterized by moist semi deciduous forest ecosystem which is typically dominated by plantain and banana trees. The CDC light traps captured the sand fly by attraction while the sticky paper traps captured by interception. The two traps were used in order to maximize the catch. The sticky paper traps were constructed from A4 white paper with castor oil as the adhesive and a wooden stick perforated through for support.

The selected endemic communities represent areas in the Municipality where cases of cutaneous leishmaniasis caused by *Leishmania (Mundinia) enriettii* complex member had been reported (Ghana Health Services, 2013; Kwakye-Nuako *et al.*, 2015). A total of four CDC light traps and twenty (20) sticky paper traps were set from 6:00pm to 6:00am each day for collection of the sand flies. The sand flies that were collected with the CDC light traps were freeze-killed at -20° C and sorted out into labelled 1.5 ml eppendorf tubes containing silica gel for dry preservation. Sand flies that was collected with the sticky traps were preserved in 70% ethanol. The tubes were secured in sealed sample

collection bags and this was transported to the entomology laboratory at the Noguchi Memorial Institute for Medical Research for morphological identification.

A B

Figure 3.2: CDC Light trap in use [A] and sticky paper trap in use [B]

3.6 Dissections and Morphological Identification of Sand flies

All sand flies captured were separated into either male or female on the basis of morphology of their reproductive organ observed under a stereomicroscope (Olympus SZ60, Thermo Fisher Scientific, (U.S.A). This is according to the morphological taxonomic keys. Males had no pathogenic importance and were not identified and were thus excluded from the study. Each female sand fly was dissected by first removing the head and last three abdominal segments with a pair of dissecting pins. The head and the last three abdominal segments were placed in a labelled 0.2 ml sterile micro tube containing 2 drops of clearing medium made up of chloral hydrate and phenol for about 48 hours. The two drops are just enough to make the internal organs (cibarium, pharynx and spermathecae) visible. The thorax and the upper abdominal segments were kept in another 0.2 ml sterile micro tube with the same labelling as that for the head and last three abdominal segments. After the clearing process, the head and

last three abdominal segments were placed on a glass slide and a drop of mounting medium (8 g Arabic gum, 70 g chloral hydrate, 10 ml distilled water, 5 ml glycerine and 3 ml glacial acetic) was added. Making sure the head was placed with proboscis facing upward and last abdominal segments in a lateral position; a glass coverslip was used to cover the sample. The slide with the fixed sand fly was allowed to clear and dried using Slide Warmer Model XH-2001, (Thermo Fisher Scientific, U.S.A) at about 55-60°C for about 2 weeks and then observed under an optical microscope for species identification.

Sand flies were identified and grouped into species using taxonomic keys (Abonnenc, 1972). Morphological features used in the identification of species included the presence of cibarium, cibarial teeth, pharynx and spermatheca. An Olympus BH-2 mounted camera connected to a monitor utilizing software was used to capture images of the features of the different species. In the identification process, three main features were considered; the cibarium, pharynx and spermatheca. The cibarium is a structure that lies between the pharynx and the proboscis in the head. Present in the cibarium are cibarial teeth which are unique for various species. The pharynx also located in the head and usually a bottle or lamp-glass shaped is a posterior continuation of the cibarium consisting of a dorsal and ventral plate. The spermatheca, found only in the females varies in size and shape for different species. Generally, *Phlebotomus* species have a cibarium which lacks cibarial teeth and a pigmented patch whereas *Sergentomyia* species have both the cibarial teeth and pigmented patch present.

3.7 DNA Extraction of Sand flies

DNA were extracted from each sand fly using the potassium acetate extraction method described by Aransay *et al.*, (2000) with slight modifications.

This method was used because it has been reported to yield enough DNA to test a large number of markers and it has been recommended in the cases when many markers are to be tested on the same samples (Fraga *et al.*, 2011; Madhan, Sugumaran, Kalaiyarasu, and Bubna, 2009). This method had also been reported for the DNA extraction from mosquitoes and triatomines with excellent results (Fraga *et al.*, 2011).

The individual sand fly was macerated with plastic pestle in 1.5 mL labelled tubes. One hundred and fifty microliters (150 µL) of extraction buffer, which was made up of 1% sodium dodecyl sulphate (SDS), 25 mM NaCl and 25 mM EDTA was added, and tubes were placed in a water bath at 65 °C for 30 minutes. Following the addition of 100 µL of 3 M potassium acetate (pH 7.2), the homogenates were incubated on ice for 30 minutes and then centrifuged for 15 minutes at 13,000 rpm. The supernatant was recovered, and DNA was precipitated by the addition of 600 µL of 100% ethanol. The homogenate was placed in the freezer at 30 minutes for 1 hour, then centrifuged at 13000 rpm for 25 minutes, after which the ethanol was discarded and the resulting pellet air dried for 10 minutes. The DNA pellets was re-suspended in 50 µL of 0.5X Tris-EDTA (TE) (pH 8.0). Five-microliter (5 µL) portions of the DNA extracted were used for PCR amplification.

3.8 Molecular Identification of Sand flies

The morphologically identified sand flies were confirmed by molecular method. This was achieved by using the mitochondrial cytochrome *c* oxidase gene subunit I (COI). PCR was performed on the DNA extracts from the individual sand flies. The COI gene, which is widely used for DNA barcoding,

was used in selected samples to confirm sand flies that had been morphologically identified to species level. The COI was amplified using primers referenced by Gutiérrez *et al.*, (2014). DNA extracts from individual sand flies were used as template and the primers LCO1490 forward: 5'-GGTCAAATCATAAAGATATTGG- '3 and HCO2198 reverse: 5'-TAAACTTCAGGGTGACCAAAAAATCA- '3 (Macrogene, Korea) was used for the amplification 658 bp (Gutiérrez *et al.*, 2014).

The amplification used 0.125 µL of One Taq DNA polymerase [1.25 U/50 µL] (New England Biolabs, Inc.) in a total reaction volume of 25 µL. This contained 5 µL of DNA, 0.5 µL dNTPs (dATP, dCTP, dGTP and dTTP) [10 mM], 5 µL of buffer (containing 1.8 mM of MgCl) [5X], 0.5 µL of each primer [10µM], and sterilized water to make up the final volume. The thermal cycling conditions previously described by (Gutiérrez *et al.*, 2014) was used with slight modifications. An initial denaturation at 95°C for 5 min, amplification was performed with 37 cycles consisting of denaturation at 94°C for 30s, annealing at 53°C for 45s, extension at 68°C for 1 min 30s, followed by a final extension at 68°C for 10 min and 4°C as holding temperature. For each PCR reaction, a negative control containing sterilized distilled water instead of DNA was included. The positive control DNA was previously identified sand fly obtained from the Noguchi Memorial Institute for Medical Research. The reaction was carried out in the BIORAD System Peltier Thermal Cycler (SN DY004411).

3.9 Genetic Variability of Sand fly 18S rRNA gene

To investigate the genetic variability, the 18S ribosomal ribonucleic acid (rRNA) region was amplified by PCR using specific primers (Al-Dakhil *et al.*, 2017). DNA extracts from individual sand flies were used as template and the

primers Sand F1 forward: 5'- AGGCTCATTCAGTCGCTTTC-3' and Sand R1 reverse: 5'-TGCAAGCTTATGACTCACACTT-3' (Macrogene, Korea) were used for the amplification of a 750 bp fragment (Al-Dakhil *et al.*, 2017). The 750 bp segment was amplified in a 25 µL reaction volume. This reaction volume contained 5 µL of DNA, 0.5 µL dNTPs (dATP, dCTP, dGTP and dTTP) [10 mM], 5 µL of buffer (containing 1.8 mM of MgCl) [5X], 0.75 µL of each of primer [10 µM], 0.25 µL of One Taq DNA polymerase [1.25 U/50 µL] (New England Biolabs, Inc.) and sterilized water to make up the final volume. The modified thermal cycling conditions previously described by Al-Dakhil *et al.*, (2017) was used. An initial denaturation at 95°C for 5 mins, amplification was performed with 40 cycles consisting of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, extension at 68°C for 2 mins, followed by a final extension at 68°C for 10 min and 4°C as holding temperature. For each PCR reaction, a negative control containing sterilized distilled water instead of DNA was included.

To carry out the PCR-RFLP of the 18S rRNA gene of the sand flies, the PCR products were digested with *RsaI* restriction endonuclease (Promega). This enzyme was chosen for the digestion because its recognition site was found within the sequences of the gene of interest. It also showed adequate differentiation after contacting an in silico analysis. The RFLP was used to detect differences, if any, among the individual sand flies, based on the cuting at different points within the lengths of the 18S rRNA gene PCR products by the *RsaI* restriction enzyme.

For the RFLP analysis, a control reaction for digesting the DNA products was set which yielded results. The reaction mix in a total volume of 20 µL contained 5 µL of the PCR product, 5 units of the restriction enzyme, 2 µL of 10X RE buffer, 0.2 µL of acetylated BSA and sterile distilled water. Samples

were digested for 1 hour at 37°C. The digested PCR products were separated by electrophoresis on 2% agarose gel in 1X TAE buffer at 80V for 1 hour to produce a DNA fragment pattern. The pattern was identified by staining with ethidium bromide (Sigma) and visualized under ultraviolet light. Band sizes were determined and compared with markers in the 100 bp/200 bp DNA Ladder (Bio Labs).

3.10 Blood Meal Identification of Sand flies

Identification of blood meal in the individual sand flies was performed by PCR amplification of the cytochrome *b* gene (cyt b). This was conducted on the one hundred and twenty five (125) species of female sand flies that was engorged . A 359 bp fragment of the *cyt b* gene was amplified using primers previously described by (Steuber, Abdel-Rady, and Clausen, 2005). DNA extracts from individual sand flies were used as template and the primers cyt *b* forward: 5'- CCATCCAACATCTCAGCATGAAA-3′ and cyt *b* reverse 5'-GCCCCTCAGAATGATATTTGTCCTCA-3′ (Macrogene, Korea) was used for the amplification of 359 bp of the conserved region of the *cyt b* gene (Steuber *et al.*, 2005). The 359 bp *cyt b* segment was amplified using 0.125 µL of One Taq DNA polymerase [1.25 U/50 µL] (New England Biolabs, Inc.) in a total reaction volume of 25 µL. This consisted of 5 µL of buffer (containing 1.8 mM of MgCl) [5X], 0.5 µL of 10 mM dNTPs (dATP, dCTP, dGTP and dTTP), 0.5 µL of each of primer [10 µM], 5 µL of DNA and sterilized water to make up the final volume. The thermal cycling conditions used was: an initial denaturation at 95 °C for 10 mins, amplification was performed with 40 cycles consisting of denaturation at 94°C for 30s, annealing at 52°C for 30s, extension at 68°C for 45s, followed by a

final extension at 68°C for 5 mins, and 4°C as holding temperature. For each PCR, a negative control containing sterilized distilled water instead of DNA was included. The positive control was DNA extracted from the blood of mosquito. The reaction was carried out in the BIORAD System Peltier Thermal Cycler (SN DY004411) .

3.11 *Leishmania* Detection in Sand flies

Detection of *Leishmania* DNA in the individual sand flies was carried out using conventional polymerase chain reaction (PCR) amplification. The PCR assay used amplified conserved regions, using as target DNA, a minicircle of kDNA (120 bp) for identification of the genus *Leishmania* using mincr1 and mincr2 primers. A 120 bp fragment of the minicircle DNA of the parasite kinetoplast was amplified using previously described primers (Mosore, 2016): mincr1 forward: 5'-GGGGAGGGGCGTTCTGCGAA-3' and the mincr2 reverse: 5'-CGCCCCCTATTTTACACAACCCC-3' primers (Macrogene, Korea).

The polymerase chain reaction (PCR) was performed in a 25 µL reaction volume. This contained 5 µL of DNA, 0.5 µL dNTPs (dATP, dCTP, dGTP and dTTP) [10 mM], 5 µL of buffer (containing 1.8 mM of MgCl) [5X], 0.75 µL of each of primer [10 µM], 0.25 µL of One Taq DNA polymerase [1.25 U/50 µL] (New England Biolabs, Inc.) and sterilized water making up the final volume. The modified thermal cycling conditions (Mosore, 2016) included an initial denaturation at 94°C for 5 mins, amplification was performed with 35 cycles consisting of denaturation at 94°C for 30s, annealing at 60°C for 30s, extension at 68°C for 1 min, followed by a final extension at 68°C for 10 mins and 4°C as holding temperature. For each PCR, a negative control containing sterilized

distilled water instead of DNA was included. DNA extracts from *Leishmania major*, *Leishmania braziliensis*, *Leishmania donovani* and *Leishmania infantum* were used as PCR positive controls. The reaction was carried out in the BIORAD System Peltier Thermal Cycler (SN DY004411).

3.11.1 Pairwise Identity Map

A pairwise heat map which is a graphical representation of the data that utilize colour coded systems was generated. This map presented the individual values contained in a matrix as colours. The purpose was to examine patterns of similarity and/ relatedness amongst the parasites detected. Colour intensity of each square showed the correlation between the variables on each axis. The colour scale red-yellow-green was used from the highest (similar) to the lowest (dissimilar) in that order. This colour scale applied a colour gradient to the range of cells. The colour indicated where each cell value falls within the range.

3.12 Gel Electrophoresis

The amplified PCR products (5µL) were mixed with blue loading buffer (Promega) and subjected to electrophoresis on a 2% agarose (Sigma) gel in 1X (Tris-acetate) TAE buffer stained with ethidium bromide (10 mg/mL) at 80V for 1 hour, with a 100 bp/ 200 bp DNA Ladder (Bio Labs) provided as molecular weight size standard. The DNA bands were visualized under ultraviolet light and subsequently photographed. By comparing the DNA product bands with bands from the known molecular weight markers, the sizes of each product were determined.

3.13 Sequencing and Phylogenetic Analysis of PCR Products

The PCR amplified products obtained using the COI primers (658bp), sand fly primers (750bp), blood meal primers (359bp) and the mincir primers (120bp) were sequenced. This was after the gel electrophoresis was carried out, and thereafter sand flies species that showed intense DNA band at the required base pair was randomly selected for the sequencing. The sequencing employed the Sanger method in both forward as well as reverse directions using an Applied Bio systems (ABI) ABI 3500XL Genetic Analyser (NimaGen BV. Nijmegen). The same primers used for the PCR reactions were used. The derived sequences from both strands were aligned using the DNASTAR (Lasergene) software. Sequences were then compared with the GenBank database using NCBI BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi), aligned and analysed in MEGA version 7. BLASTN was opted for because it is a sensitive programme that can be used to align genomic DNA sequences. This was utilised by comparing the nucleotide sequences of the sand flies to one another. After conducting the BLASTN, there were a lot of sequences identified in the GenBank. Nonetheless, only species that showed the highest value of identification and query cover was selected. This is bearing in mind that only sequenced strain will show up in the search considering the closest database matches at species level.

Based on this, the sand fly COI gene sequences were analysed with those of HQ585351.1, AB759971.1, JX105037.1, MH577096.1 and AB787194.1. The sand fly 18S rRNA gene was analysed with AJ2444414.1, AJ244397.1 JQ929125, AJ391739.1, AJ391738.1, AJ244425.1 and AJ244421.1. The cytochrome *b* gene for blood meal was analysed with LC088152.1, JX266260, HQ180173.1, and AY509658.1. The *Leishmania* species minicircle DNA of the

parasite kinetoplast gene was analysed with HQ600661.1, KC869685.1, KY698860.1, AF308682.1 and AJ010080.2.

3.14 Data Analysis

The data collected in relation to the questionnaire was entered into Statistical Package for the Social Sciences (SPSS) version 25, cleaned, validated and analysed. The demographic characteristics of the study participants were summarized using medians and means.. Questions with correct and wrong responses were scored 1 and 0 respectively. Frequency and percentage were used to express the level of community member's KAP. Where percentage above 90% was graded as excellent, 70% to 89% was graded as satisfactory, 60% to 69% was graded as very good, 50% to 59% was graded as good and below 50% was graded as poor. ANOVA was used to determine the statistical differences in percentage of flies collected in terms of months, species and communities.

Results obtained from Sanger sequencing was checked for homology by using the bioinformatics tool BLAST from NCBI and DNASTAR software (Lasergene) employed to assemble the data. MEGA 7 program was also used to conduct the statistical analysis. Neighbour joining method was employed in building relationship between the blasted sequence product and the reference sequence.

CHAPTER FOUR

RESULTS AND DISCUSSION

This study generally aimed at investigating the vector, the parasite and the probable host regarding cutaneous leishmaniasis in the endemic communities in the Ho Municipality. The first part of this chapter reports the results obtained from the various methods that was employed to achieve the research objective while the other part of the chapter discusses the outcome, referencing relevant literature.

4.1 Results

4.1.1 Demographic Information

A total of 111 individuals participated in the questionnaire study. Of these, 70 (63.06%) were females and 41 (36.94%) were males. Most of the study participants were in the age range of 31 - 40 (23.42%) with the youngest age being 10 and the oldest age being 76. The mean age was 38.0 years and the median age was 37. Majority of the study participants (87.39%) were engaged in one form of work or the other. Of these, 29.73% (33) were engaged in farming, and students made up 19.82% of the participants. Overall, more than half were self employed. Additionally, 91.0% had obtained formal education. Details of the distribution of the demographic information are provided in the APPENDIX C.

4.1.2 Knowledge on Cutaneous Leishmaniasis

Among the 111 study participants, majority - 81.98% (91) had knowledge about the disease and 88.29% (98) knew the local name of the disease to be agbamekanu. Most of the study participants (95.50%) that had heard of the disease, had first-hand knowledge from the community in which they lived. As little as, 19.82% of the study participants knew that a fly (insect vector)

transmitted the parasite causing the disease, however. they could not confirm this to be the sand fly. On the other hand, as many as 80.18% of the study participants had no idea about the mode of transmission of the disease. With regards to the symptoms associated with leishmaniasis, 48.65% reported that it was associated with itching and painful swelling sore, 34.23% reported only deep painful sore and 17.12% reported they had no idea (APPENDIX C).

4.1.3 Attitude Towards Cutaneous Leishmaniasis

Regarding the attitudes associated with the disease, 17.12% indicated low productivity and stigmatization, whilst 55.86% indicated severe pain, big sore and swelling as being the major problems they had encountered in association with the disease. Although, 47.75% did not indicate which section of the population was most affected by leishmaniasis, 46.85% reported that everybody was equally affected. A section of the participants (65.5%) indicated that the disease was not associated with spirituality (APPENDIX C).

4.1.4 Practices in Relation to Cutaneous Leishmaniasis

With regards to practice towards prevention of the disease in the endemic communities, 39.64% of the study participants stated that they always used bed net they acquired from the open market in their homes and 23.42% indicated they had never used bed net before. Additionally, 87.39% never entertained dogs, a potential reservoir, in their communities, because it is revered in their community as a god. Furthermore, most of the study participant stated wearing protective clothes and using repellent (18.02 %), spraying and weeding (23.42 %) around their homes and sleeping under bed net (9.01%) as their way of

prevention. Despite these, 49.55% of the study participants did not know the mode of prevention of the disease (APPENDIX C).

In respect of treatment, the study participants sought for the topical application of extracts obtained from *Hyptis suaveolens (L) Poit* (identified by the botanist at the University of Ghana –Department of Botany) (APPENDIX E) to the sore (open lesion). Overall, 30.12% of study participant reported intense pain when the herbal extract was applied to their sore (lesion). Cost and availability of the herb were not mentioned as barriers because the wild herbs grew in the community, therefore it was freely obtained and easily accessible.

4.1.5 Collection and Distribution of Sand flies

A total of 727 sand flies were captured during the sampling period from the stated endemic communities in the Ho Municipality. Females made up 553 (76.07%) of the sand flies collected whereas males made up 174 (23.93%). The monthly distribution of the sand fly collected and the relative abundance is presented in Table 4.1. No sand fly was captured in the months of November and December '17 and June and July '18. More sand flies was captured in May followed by March and April. In the months that the sand fly was captured more females were captured as compared to the males. There was no significant difference ($p > 0.05$) in the percentage of flies collected in terms of months and communities.

Table 4.1: Monthly distribution and relative abundance of sand fly

Month/Year	Sand fly	
	Female	Male
Nov-Dec '17	0	0
Jan'18	72	24
Feb'18	56	19
Mar'18	123	37
Apr'18	48	18
May'18	209	66
Jun'18	0	0
Jul'18	0	0
Aug'18	45	10
Total	553	174
Relative abundance (%)	76.07	23.93

4.1.6 Morphological Identification of Sand flies

Overall five hundred and fifty three (553) female sand fly was collected. Out of this three hundred and sixty three (363) representing 65.64% was morphologically identified under the microscope and grouped into species using taxonomic keys (Abonnenc, 1972) and the remaining one hundred and ninety (34.36%) was visually identified as male and female by the expert on the field. The morphologically identified female sand flies belonged to two (2) genera with 3.31% belonging to the genus *Phlebotomus* and 96.69% to the genus *Sergentomyia*. The genus *Phlebotomus* included *Phlebotomus rhodaini* while the genus *Sergentomyia* included, *Sergentomyia africana, Sergentomyia similima, Sergentomyia ghesquierei, Sergentomyia ingrami, Sergentomyia schwetzi, Sergentomyia antennata, Sergentomyia hamoni, Sergentomyia dureini, Sergentomyia buxtoni* and *Sergentomyia collarti* (Figure 4.1 and APPENDIX D).

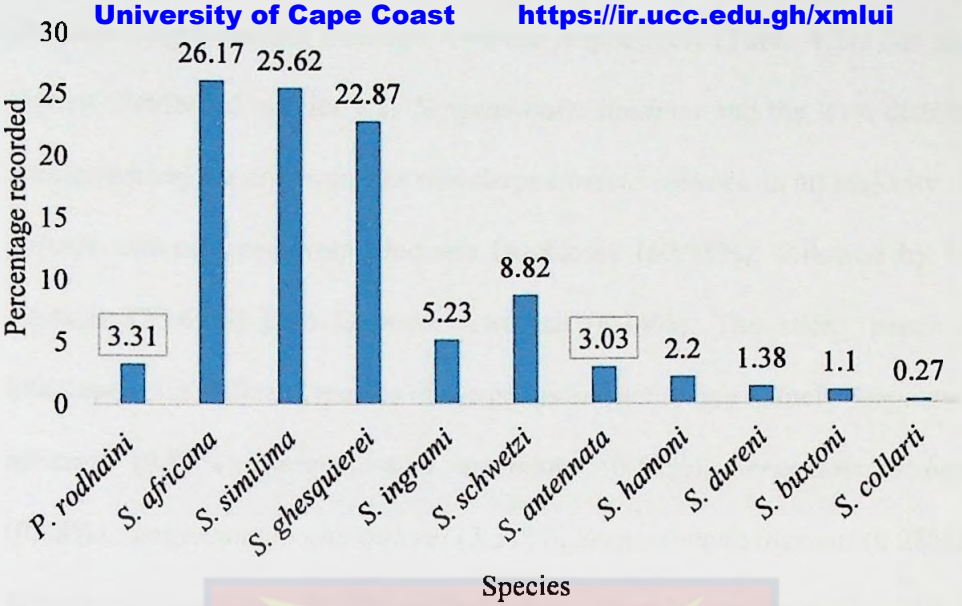


Figure 4.1: Sand fly identified in the study sites

Of the morphologically identified sand fly species, again the month of May recorded the highest number representing a total of 37.79%, followed by March representing a total of 22.24% (Figure 4.2).

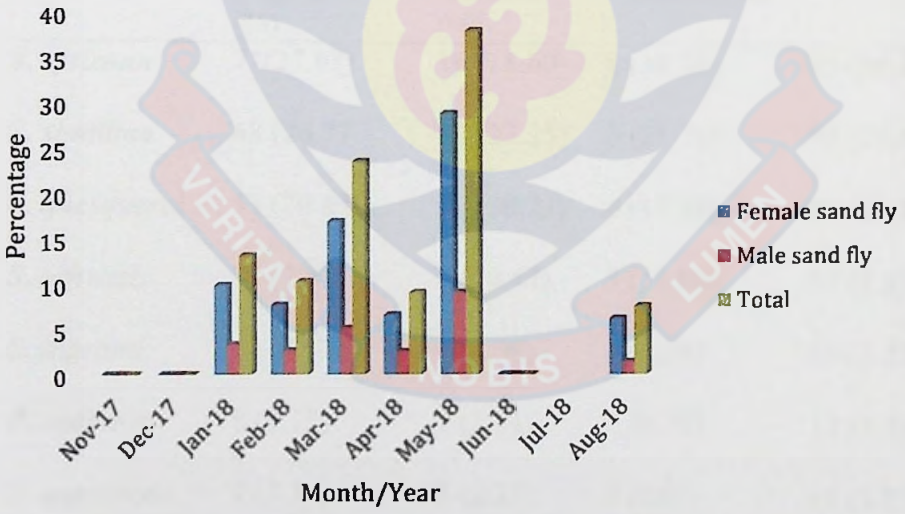


Figure 4.2: Monthly distribution of sand fly collected

Sergentomyia africana was the most abundant species identified and it was distributed across the three communities. *Sergentomyia africana* included 16/86 (18.60%), 71/254 (27.95%) and 8/24 (34.78%) from Lume Atsiame,

Dodome Dogblome and Dodome Awuiasu respectively (Table 4.2). The second highest distributed species was *Sergentomyia similima* and the least distributed species among the communities was *Sergentomyia collarti*. In all majority of the species was captured from Dodome Dogblome (69.98%), followed by Lume Atsiame (23.69%) then Dodome Awuiasu (6.34%). The sticky paper traps intercepted six different species of the genus *Sergentomyia* namely *Sergentomyia africana* (0.83%), *Sergentomyia antennata* (0.55%), *Sergentomyia buxtoni* (0.28%), *Sergentomyia ghesquierei* (3.31%), *Sergentomyia ingrami* (0.28%) and *Sergentomyia schwetzi* (0.55%) in the Dodome Dogblome village. The CDC light traps on the other hand attracted all the eleven different species of sand flies identified in the study.

Table 4.2: Distribution of female sand flies in the endemic communities

Sand fly species	Communities			
	Dodome Dogblome (%)	Lume Atsiame (%)	Dodome Awuiasu (%)	Total (%)
S. africana	71(27.95)	16 (18.60)	8 (34.78)	95 (26.17)
S. similima	68 (26.77	20 (23.25)	5 (21.74)	93 (25.62)
S. qhesquerei	53 (20.87)	26 (30.23)	4 (17.39)	83 (22.87)
S. schwetzi	20 (7.87)	9 (10.47)	3 (13.04)	32 (8.8)
S. ingrami	12 (4.72)	6 (6.98)	1 (4.35)	19 (5.23)
P. rodhaini	8 (3.15)	2 (2.33)	2 (8.70)	12 (3.31)
S. antennata	9 (3.54)	2 (2.33)	0 (0.0)	11 (3.03)
S. hamoni	6 (2.36)	2 (2.33)	0 (0.0)	8 (2.2)
S. dureini	4 (1.57)	1 (1.16)	0 (0.0)	5 (1.38)
S. buxtoni	3 (1.18)	1 (1.16)	0 (0.0)	4 (1.1)
S. collarti	0 (0.0)	1 (1.16)	0 (0.0)	1 (0.3)

Typically, *Phlebotomus* are usually larger bodied with lighter integument whilst *Sergentomyia* are smaller with a darker cuticle. Key characters such as the pharynx, cibarium and spermathecae were considered during the morphological identification process. The characteristic features of the collected sand fly species and the images that was generated under the microscope from the morphological identification are presented in Table 4.3 and Figure 4.3 respectively.

Table 4.3: Characteristic features of the collected sand fly species

Sand fly species	Morphological characteristics		
	Cibarium	Pharynx	Spermathecae
P. rhodaini	lacks rows of teeth	weakly distended	long tubular
S. africana	well-developed set of teeth	hour-glass shaped	double walled
S. antennata	lateral teeth	heart-shaped	long
S. buxtoni	mushroom shaped cibarium patch	diamond shape	wide tubular
S. collarti	fence-like cibarial teeth	narrow	elongated tubular
S. dureni	median teeth	extended	tubular
S. ghesquierei	31-40 teeth	Lamp-glass shaped	tubular
S. hamoni	long, sharp cibarial teeth	Broad-heart shaped	long
S. ingrami	two sets of teeth	narrow shaped	elongated capsule
S. schwetzi	tightly packed median teeth	distended posteriorly	tubular
S. similima	teeth are equal sized, pointed, fence-like	heart shaped	tubular spermatheca with ducts separated at the base

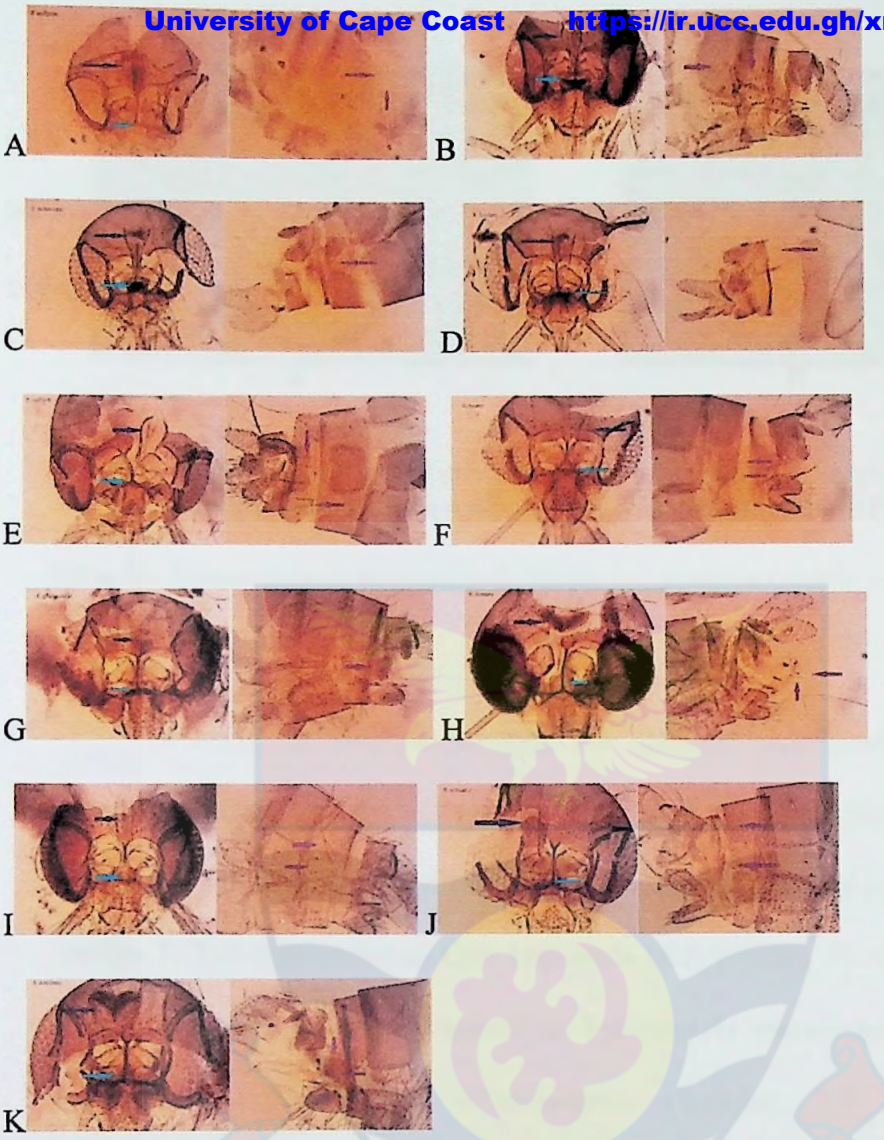


Figure 4.3: Images of pharynx (blue), cibarium (cyan) and spermathecae (purple) of the morphologically identified sand fly species *Phlebotomus rodhaini* [A], Sergentomyia africana [B], Sergentomyia antennata [C], *Sergentomyia buxtoni* [D], *Sergentomyia collarti* [E], *Sergentomyia dureni* [F], *Sergentomyia ghesquierei* [G], *Sergentomyia hamoni* [H], *Sergentomyia ingrami* [I], *Sergentomyia schwetzi* [J] and *Sergentomyia similima* [K]

4.1.7 Molecular Identification of Sand flies

For each of the different species morphologically identified, the PCR amplified products showed bands of molecular weight 658 bp as shown in Figure 4.4 and the APPENDIX E.

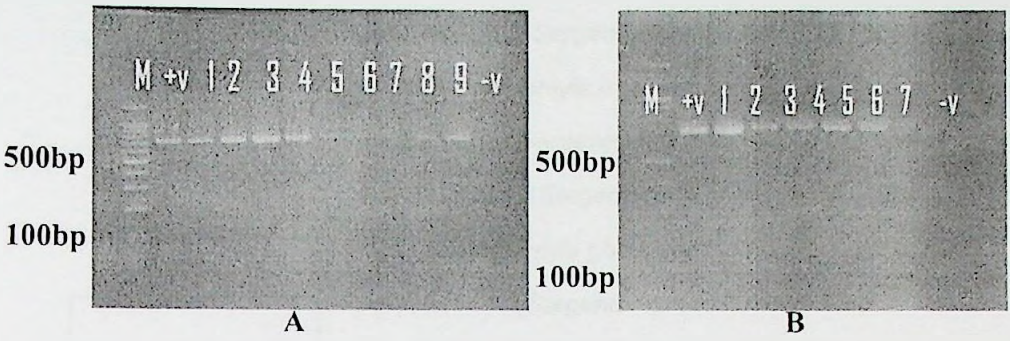


Figure 4.4: Agarose gel electrophoresis of PCR products of *Sergentomyia similima* [A] and *Phlebotomus rodhaini* [B] after amplification of COI gene (658bp).
Lane M: Molecular weight marker (100bp). +v, positive control. -v, negative control

However, to ascertain whether the obtained sequenced product was congruent with the morphologically identified sand flies, the obtained sequences was compared with the GenBank database, aligned and analysed in MEGA version 7. The mitochondrial COI sequences (APPENDIX F) obtained varied from 655 to 682 bp by direct sequencing. The sand fly species that yielded good sequence data include: C1 (*Phlebotomus rhodaini*), C3 (*Sergentomyia ghesquierei*), C4 (*Sergentomyia ingrami*), C5 (*Phlebotomus rhodaini*), C6 (*Phlebotomus rhodaini*) and C7 (*Phlebotomus rhodaini*). (Figure 4.5). After multiple alignments of study sequences and those obtained from the BLAST and in addition to reference sequences obtained from GenBank the obtained sequence confirmed the morphologically identified sand fly species.

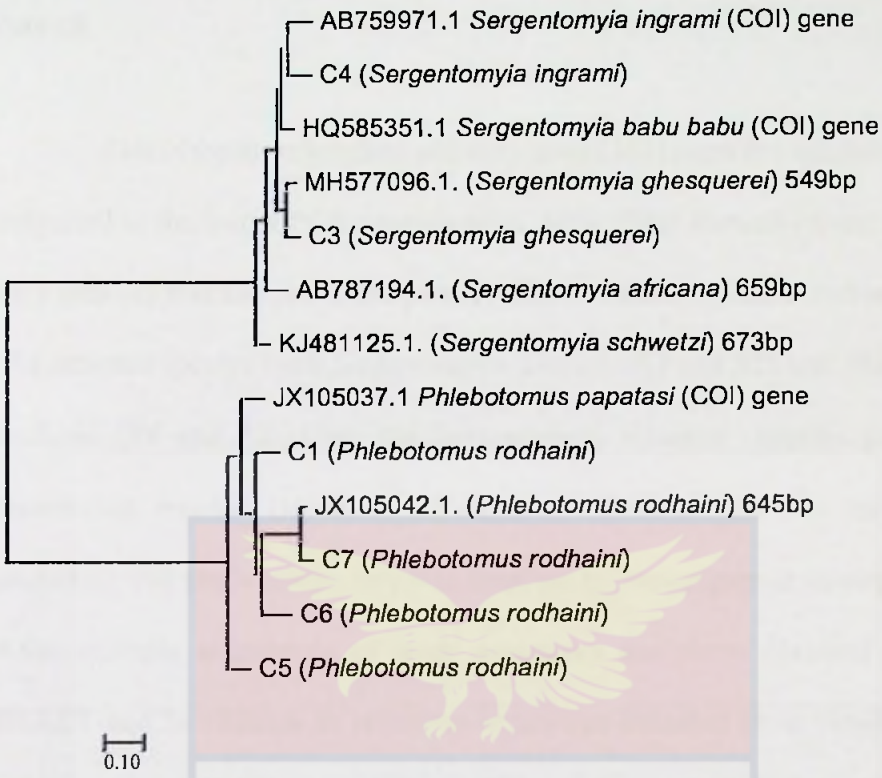


Figure 4.5: Neighbour Joining tree using mitochondrial COI gene among sand fly species.
AB759971.1, MH577096.1 and JX105042.1 are the reference sequences

For the genetic variability of the collected sand fly (18S rRNA), the intial amplification by PCR using specific primers, Sand F1 and Sand R1 was succesful (Figure 4.6 and APPENDIX E).

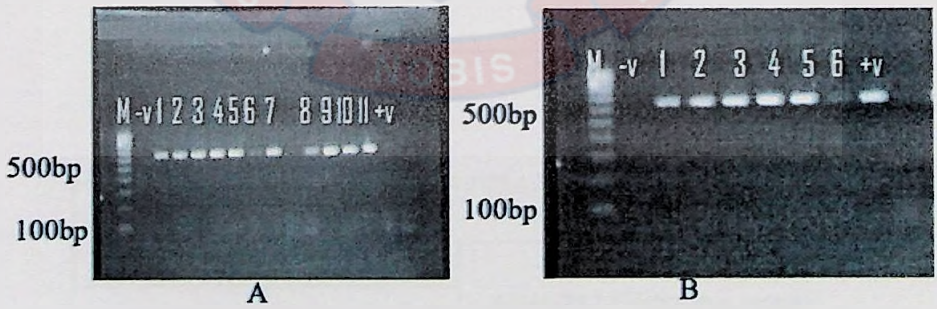


Figure 4.6: Agarose gel electrophoresis of PCR products of *Sergentomyia similima* [A] and *Phlebotomus rodhaini* [B] after amplification of 18S rRNA gene (750bp).

Lane M: Molecular weight marker (100bp). +v, positive control. -v, negative control

Out of the three hundred and sixty three (363) sandflies species that were subjected to the initial PCR amplification, 80% (290) showed bands. Of these, only four (4) that showed intense bands were randomly selected and sequenced. The selected species were *Sergentomyia schwetzi* (S1 and S2) and *Phlebotomus rodhaini* (P1 and P2). Only the *Sergentomyia schwetzi* species gave DNA sequencing results. However, *Phlebotomus rodhaini* did not form contiq assembly. The phylogenetic tree was obtained by the neighbour joining method. After multiple alignments of study sequences and those obtained from the BLAST and in addition to reference sequences obtained from GenBank, the neighbour joining tree was contructed. This tree was constructed with the species of sand flies that was generated from the BLASTN based on the total score and query cover (Figure 4.7).

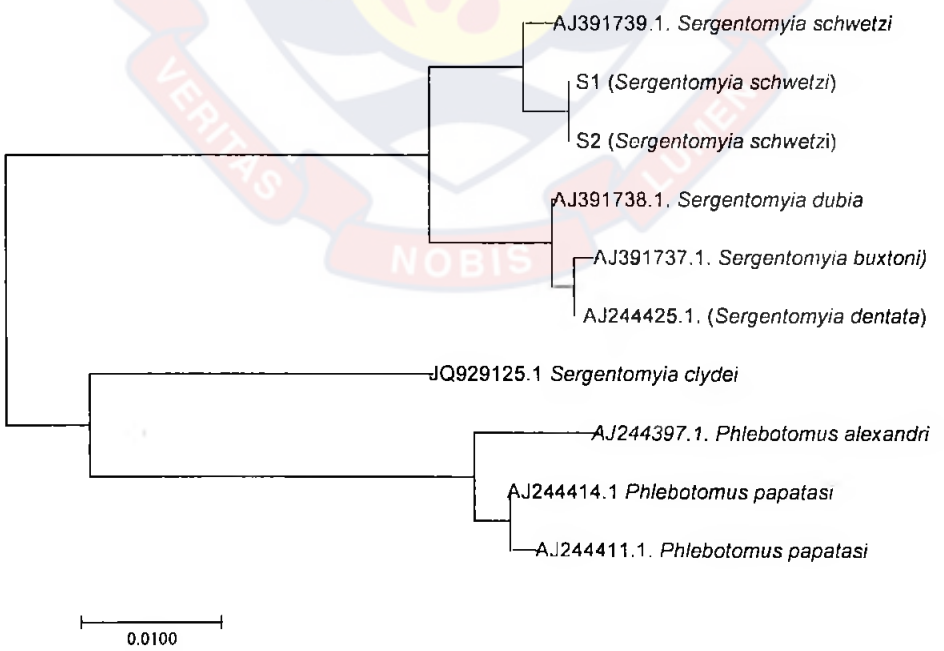


Figure 4.7: Neighbour joining tree using 18S rRNA gene among selected sand fly species.
S1 and S2 are the sequences of the study.

In order to establish a genetic basis for the precise taxonomic identity of the sand fly species, analysis of the 18S rRNA gene was carried out using PCR–RFLP assay. There was no digestion of 18S rRNA gene PCR products with *RsaI* restriction enzyme. The expected cleavage products for the sandfy, were at least two fragments (150 and 600 bp) with the enzyme *RsaI*. This cleaving would have meant there is a recognistion site for the enzyme in the sequence of this particular species of sand flies.

4.1.8 Identification of Sand fly Blood Meal

The female sand flies were grouped as non-engorged and engorged. The non-engorged totalled 65.56% (238/363) while the engorged totalled 34.43% (125/363). Majority of the engorged sand flies were collected at Dodome Dogblome. After amplification of the cytochrome *b* gene by PCR on all the morphologically identified female sand flies, 67.2% (84/125) of the engorged female sand flies showed presence of blood meal by having the DNA product of 359 bp amplified (Figure 4.8 and APPENDIX E). The remaining 32.8% (41/125) were negative despite the presence of blood in their abdomen during the morphological identification. These remaining 32.8% that were ne gative despite the presence of blood in their abdomen included *Sergentomyia africana* (20/41), *Sergentomyia similima* (12/41), *Sergentomyia ingrami* (5/41) and *Sergentomyia schwetzi* (4/41). Overall, out of the three hundred and sixty-three (363) female sand flies, 84 representing 23.14% showed presence of blood meal by amplification at 359bp. The species of sand fly that showed the presence of blood

meal included *Phlebotomus rodhaini, Sergentomyia ghesquierei, Sergentomyia similima, Sergentomyia africana, Sergentomyia antennata, Sergentomyia ingrami, Sergentomyia hamoni and Sergentomyia schwetzi* (Table 4.4).

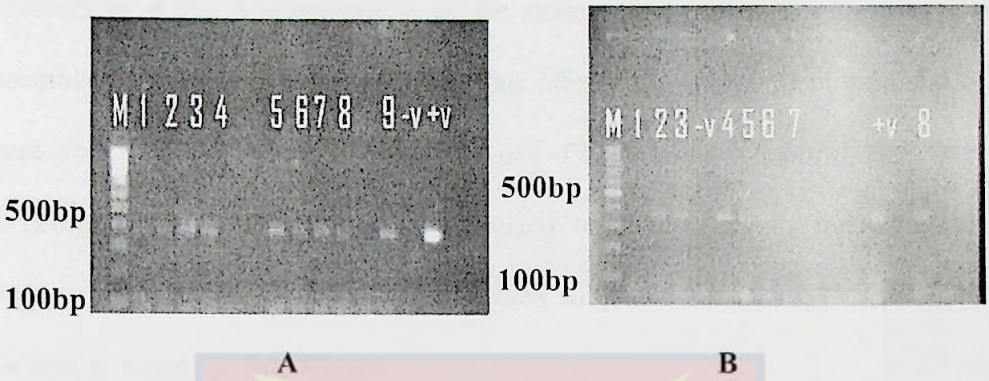


Figure 4.8: Agarose gel electrophoresis of PCR products of *Phlebotomus rodhaini* [A] and *Sergentomyia similima* [B] after amplification of *cyt b* gene (359bp).

Lane M: Molecular weight marker (100bp) PC-positive control of blood from mosquito. NC-negative control

Table 4.4: The percentage of blood meal present in each collected sand fly species

Sand fly species	No. of sand flies identified N = 363	Blood meal identified (%) N = 84
S. africana	95(26.17)	10 (2.75)
S. similima	93(25.62)	20 (5.51)
S. qhesquerei	83(22.86)	27 (7.44)
S. schwetzi	32(8.82)	3 (0.83)
S. ingrami	19(5.23)	5 (1.38)
P. rodhaini	12(3.31)	8 (2.20)
S. antennata	11(3.03)	6 (1.65)
S. hamoni	8 (2.20)	5 (1.38)
S. dureini	5(1.38)	0 (0.0)
S. buxtoni	4(1.1)	0 (0.0)
S. collarti	1(0.28)	0 (0.0)

Twenty (20) of the samples that showed presence of blood meal having the DNA product of 359 bp, was sequenced. Only seven was able to be sequenced representing 8.3%. Unfortunately, of the seven, three could not form contiq assembly. However, in order to have a fair idea of the source of blood meal of these four resulting sand fly species (one *Phlebotomus rodhaini* and three *Sergentomyia similima* species) that generated sequencing results, the nucleotide sequence data obtained was edited and then subjected for BLAST with NCBI nucleotide sequence data library.

After multiple alignments of experimentally generated sequences and those obtained from the BLAST and in addition to reference sequences obtained from the GenBank, the Neighbour-Joining phylogenetic tree that was constructed using MEGA 7 program was presented (Figure 4.9). The phylogenetic tree analysis showed that the resulting four species possess sequences closely related to that of *Mus musculus* (house mouse) and *Homo sapiens* (human).

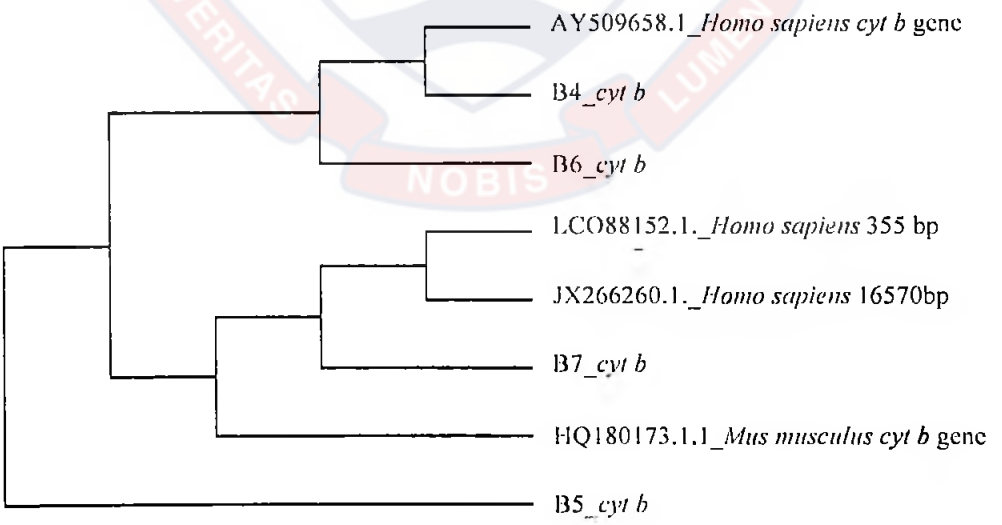


Figure 4.9: Neighbour joining tree using *cyt b* gene among four sand fly species.

AY509658.1, LCO088152.1, JX266260.1 and HQ180173.1 are the reference sequence. B4 (Phlebotomus rodhaini), B5-B7 (Sergentomyia similima) are the sequences of this studies.

4.1.9 *Leishmania* Detection in Sand fly

The extracted DNA from the individual sand flies was screened for *Leishmania* by the amplification of the 120 bp fragment. This was conducted to amplify the conserved regions of *Leishmania* species minicircle DNA of the parasite kinetoplast. Out of the 553 female sand fly species screened, only five (5) amplified for *Leishmania* (Figure 4.10). The infection rate of 0.9% was determined in the collected sand fly species using the minicircle primers.

Figure 4.10: Agarose gel electrophoresis of PCR products showing the presence of *Leishmania* in *Phlebotomus rodhaini* (Lane 1 & 2) and *Sergentomyia similima* (Lane 3 to 5) at 120bp.
Lane M: Molecular weight marker (100bp) PC-positive control (Lm-*L. major*, Lb-*L. braziliensis*, Ld-*L. donovani*, Li-*L. infantum)*. NC- negative control

The five (5) PCR amplified product which indicated the presence of Leishmania DNA; two Phlebotomus rodhaini and three Sergentomyia similima was sequenced and aligned. One of the Phlebotomus rodhaini could not form a contig assembly. The nucleotide sequence data obtained from the four remaining species after editing, subjecting for BLAST and Neighbour joining tree constructed, yielded good results (Figure 4.11) . The phylogenetic tree analysis showed that

Leishmania parasites (L2 and L3) obtained from the vectors was in a way related to Leishmania major whilst L4 and L5 seem to cluster around Leishmania amazonensis. Overall, a positivity rate of 0.17% was determined in Phlebotomus rodhaini and 0.03% was determined in Sergentomyia similima.

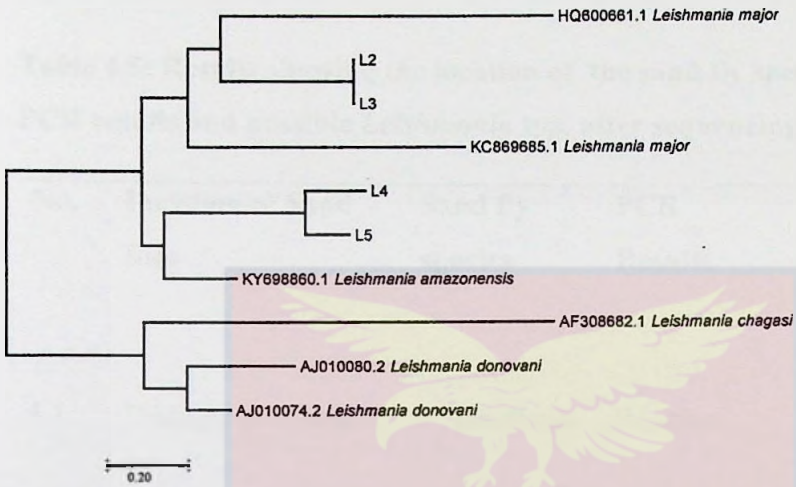


Figure 4.11: Neighbour joining tree using the mincir primers.
L2, L3, L4 and L5 are the sequences of study. HQ600661, KC869685.1, KY698860.1, AF308682.1, AJ010080.2 and AJ010074.2 are the reference sequences.

The heat map (Figure 4.12) that was generated revealed that L2 and L3 were closely related to *Leishmania major*, L4 clustered around *Leishmania donovani* and L5 clustered around and *Leishmania amazonensis*.

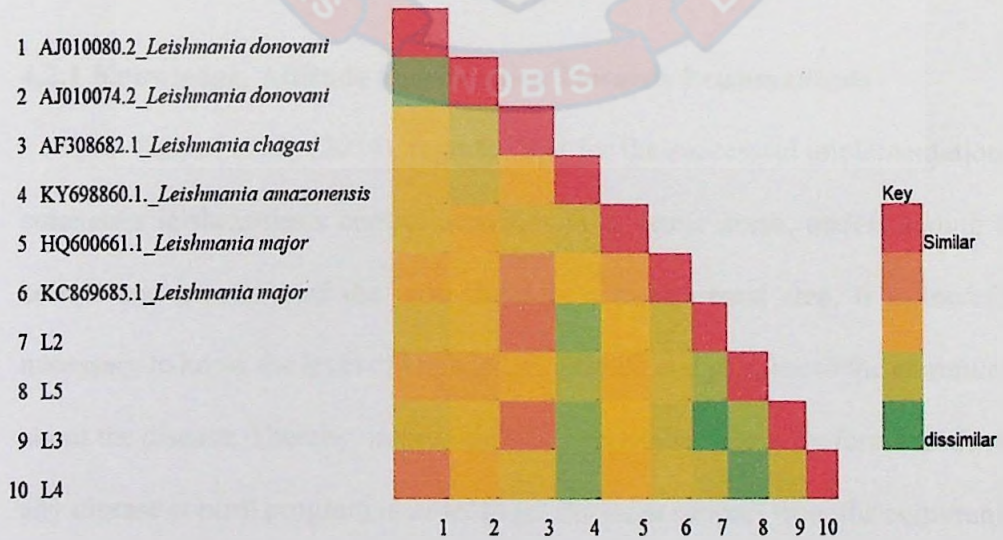


Figure 4.12: Pairwise identity heat map showing the relationship between matrix of *Leishmania* species (L2, L5, L3, L4)
The reference sequences AJ010080.2 , AJ010074.2, AF308682.1, KY698860.1, HQ600661 and KC869685.1.

The location of the five (5) sand flies species that yielded intial PCR amplication results is presented in Table 4.5.

Table 4.5: Results showing the location of the sand fly species and the PCR results and possible *Leishmania spp.* after sequencing

No.	Location of Sand flies	Sand fly species	PCR Results	Possible *Leishmania spp.* (After sequencing)
L1	Dodome Awuiasu	*P. rhodhaini*	Positive	*(No contiq assembly)*
L2	Dodome Dogblome	*P. rhodhaini*	Positive	*L. major*
L3	Dodome Dogblome	*S. similima*	Positive	*L. major*
L4	Dodome Dogblome	*S. similima*	Positive	*L. major, L.amazonensis & L. donovani*
L5	Dodome Dogblome	*S. similima*	Positive	*L. major & amazonensis*

4.2 Discussion

4.2.1 Knowledge, Attitude and Practice Towards Leishmaniasis

Sarkari *et al.*, (2014), reported that for the successful implementation of cutaneous leishmaniasis control activities in endemic areas, understanding the beliefs and practices of the individuals is a fundamental step. It is therefore necessary to know the level of knowledge, attitude and practice of the community about the disease. Thereby, improving it to a satisfactory level before introducing any disease control program in order to get the most support from the community.

Hence, by obtaining these data a successful control program can be planned. Additionally, the results of this study can also help the health authorities for better implementation of the programs related to the control of the disease in the endemic area. The knowledge, attitude and practices in relation to cutaneous leishmaniasis interventions varies among regions and is heavily influenced by sociocultural settings (Kebede *et al.*, 2016). One of the first steps in introducing control programs is to investigate the socio-demographical factors in order to develop rational prevention and control strategies (Amin *et al.*, 2012). To this end, this study provides information on the knowledge, attitude and practices in relation to cutaneous leishmaniasis in the endemic communities of the Ho Municipality of Ghana.

Cutaneous leishmaniasis has been reported to occur in a large extent in endemic areas of the Ho Municipality. This finding provides analysis of KAP on cutaneous leishmaniasis by the study participants in Klefe Atsatime, Klefe Dome, Klefe Demete, Taviefe-Dzefe, Taviefe Avenya, Matse Havi, Dodome Dogblome, Lume Atsyiame, Dodome Awuiasu . Overall, the female to male ratio of the study participants reflected the population data in the Municipality where females are more than males. There was no significant difference ($p>0.05$) between the gender of the study participants and the age with respect to the endemic communities. Furthermore, both male and female provided equal information regarding the disease. Moreover, there was a good representation of the occupation and educational levels known to be present in such rural communities in Ghana. Where according to the Population Census carried out in the communities, majority of the inhabitants of the community practiced farming and trading and this was evident in the results obtained from the questionnaire.

Whether they had ever seen cutaneous leishmaniasis before and from where and whether they, their family members or other persons they know had experienced it, confirmed that the disease was endemic in the communities selected for this study and the study participants are likely to provide responses that will be useful information for effective control strategies..

The general knowledge of the study participants regarding the disease would be considered satisfactory since majority of respondents (88.29%) could identify with the disease with its local name -"agbamekanu". This finding regarding the knowledge of the disease by the study participants is similar to other studies conducted in South Ethiopia (Kebede *et al.*, 2016), Iran (Sarkari *et al.*, 2014), Pakistan (Abazid, Jones, and Davies, 2012) and Isfahan (Hejazi *et al.*, 2010) where majority of study participants indicated they had knowledge of the disease and its local name. The study also revealed that slightly more than 80.0% of the study participants were conversant with the symptoms associated with the disease - itching of the bite area resulting in painful sore. This study further showed that although the study areas was categorized as cutaneous leishmaniasis endemic areas, findings indicate that knowledge on mode of transmission was unacceptably poor (< 20%) and that this knowledge was non-specific, since these study participants could not identify by name, the vector that transmitted the parasite. Sarkari *et al.*, (2014) in their study reported that the knowledge about the vector of the disease in the study area was not satisfactory and in the report of Akram *et al.*, (2015) respondents when asked "do sand flies transmit diseases?, most of the respondents answered "I don't know''. Additionally, (Abazid *et al.*, 2012) reported that a little under half of the respondents to their study implicated sandflies as the vectors of cutaneous leishmaniasis. Lack of information about the

vector for cutaneous leishmanais is a matter of concern for implementation of preventive measures against the disease. This is so because if people do not perceive sandfly to be the vector of disease, they usually will not take proper action to protect themselves from sand fly bites.

The general attitude to the disease was good (55.86%). An interaction with the study participants indicated they had either experienced a bite or a relation had experienced a bite that led to the disease. The subsequent painful sore the study participants experienced resulted in big scars which was the major reason they were stigmatized by friends and family. Such attitudes toward affected individuals prevented them from working, which led to the low productivity. Additionally, most of the participants perceived the disease as a source of worry, however, as many did not consider the disease to have a spiritual cause. This finding was comparable to studies conducted by Kebede *et al.*, (2016) and Nandha, Srinivasan, and Jambulingam, (2014) where the study participants perceived the disease to be a problem. These are perceptions that are most likely to discourage individuals from seeking orthodox treatment.

In respect of practices, particularly regarding the preventive measures, more than half of the study participants did not know a preventive measure. These findings are to be expected, since the findings show that only a few of the study participants (<3.0%) had education on leishmaniasis from the health service and others relied on information from the community. This is suggesting that even though local knowledge, such as the name of the disease and the observed symptoms would be well known by most of the study participants, the lack of information about the vector is a matter of concern if preventive measures against the disease is to be implemented effectively (Akram *et al.*, 2015). Similarly,

studies conducted by Hejazi *et al.*, (2010) and Saberi *et al.*, (2012) reported that the lowest knowledge about the disease regarding practice was about the preventive measures to the disease. Furthermore, in respect of practice towards the prevention of transmission, approximately half of the participants stated actions which were appropriate for the avoidance of bites from insect vector. Although, 49.55% of them indicated they did not know the action to take in relation to avoiding bites, they were sleeping under bed nets, probably as a malaria preventive intervention. The stated frequency of use of bed net was appreciable although poor considering the fact that 23.42% were not using bed net, did not auger well for the prevention of the disease in the community. Similar studies by (Abazid *et al.*, 2012) and López-Perea *et al.*, (2014) also reported the use of bednet by the study participants as a preventive measure.

Although the disease is self-healing after about 6 to 12 months (Goto and Lauletta Lindoso, 2012), clearly, the practices toward treatment are undesirable and will very much increase the morbidity, in other words, these practices may not contribute to managing the disease. This is so, because, the findings show that about half of the participants treated the disease by using herbs (identified by the botanist as *Hyptis suaveolens (L) Poit*) as topical application. This was particularly at the latter stages of the disease and that more than half of the affected individuals sought this herbal treatment within months after the sore had increased in size. The question then arises as to what the said plant is composed of that is able to heal the sore over time. Though sharp pain have been associated with the application of the extract from the herb, its ability to anecdotally heal the lesion, encouraged its persistent use. Similar studies carried out by Prakash *et al.*, (2008) and Nandha *et al.*, (2014) in India, Southern Iran

(Sarkari *et al.*, 2014), and South Ethiopia (Kebede *et al.*, 2016) also reported the use of traditional herbs as topical application for treating the skin lesions. The finding that poverty and ignorance was the leading reason for not seeking orthodox treatment indicated that the consciousness on the usage of orthodox treatment from health centers is low and the study participants attributed this to ignorance.

In relation to reservoirs, several species of wild and domestic mammals have been recorded as hosts and/or reservoirs of *Leishmania* species in different parts of the world (Alemayehu and Alemayehu, 2017). Among domesticated animals, dogs are the most important animal reservoir in the epidemiology of this disease (Eshetu *et al.*, 2016). Particularly, in the case of zoonotic cutaneous leishmaniasis, Ahuja, Bumb, and Mehta, (2012) reported that infected dogs serve as parasite reservoirs and contribute to human transmission. Moreover, dog ownership had the greatest correlation for cutaneous leishmaniasis in Turkey (Votypka, Kasap, Volfa, and Kodym, 2012). However, the findings of this study where study participants were asked whether they had any association with dogs, revealed that most of the participants (87.39%) did not have any interactions or so ever with dogs in the communities. This, probably sends information across that dogs could not possibly be the reservoirs for the disease in these communities since no report of reservoir host has been reported in Ghana yet (Kweku *et al.*, 2011).

4.2.2 Morphological Identification and Distribution of Sand flies

Leishmaniasis is being reported in hitherto non-endemic areas of the world where new species of the vectors are emerging. This necessitates the identification of the vectors collected in the endemic area (Alemayehu and

Alemayehu, 2017; Dvorak *et al.*, 2014). The sand flies, which are documented as the vectors of leishmaniasis, has received extensive attention in recent years. The identification of any species of sand fly is therefore crucial for leishmaniasis control intervention (Baharshahi *et al.*, 2016). In this study, CDC light and sticky paper traps which are standard surveillance techniques (Orshan *et al.*, 2010) for capturing sand flies was used. Only 3.31% of *Phlebotomus species* and 96.69% of *Sergentomyia* species of sand flies was identified in the cutaneous leishmaniasis endemic areas of Lume Atsiame, Dodome Dogblome and Dodome Awuiasu. Similar studies conducted in Algeria (Bennai *et al.*, 2018), Mali (Berdjane-Brouk *et al.*, 2012; Coulibaly *et al.*, 2016), Morocco (Ajaoud *et al.*, 2013) and Sudan (Adam *et al.*, 2017), identified species of these two sand fly genera. The *Sergentomyia* species were mainly prevalent and this was consistent with previous studies carried out by Boakye *et al.*, (2005), Fryauff *et al.*, (2006), Mosore, (2016) and Nzelu *et al.*, (2014) in the Ho Municipality of the Volta Region of Ghana. Boakye *et al.*, (2005) earlier reported that sand flies collected and identified in 1997 and 2002 from Navrongo in the Upper East Region also showed the prevalence of *Sergentomyia* species.

The identification of *Sergentomyia* species in both the semi-deciduous forest ecosystem and the arid sahel-savannah zone shows that this species has adapted to these climatic conditions. Additionally, the *Sergentomyia* species identified then included species that had been documented to feed on humans (Boakye *et al.*, 2005). Nonetheless, *Sergentomyia* species have been reported as dominant species in tropical areas where *Phlebotomus* species are scarce or absent (Akhoundi et al., 2016) and it is in fact not clearly delimited (Maia and Depaquit, 2016).

In this study, *Sergentomyia africana* was the most abundant sand fly and it was found in all the months that sand fly was captured. This was consistent with studies carried out by Mosore, (2016) and Nzelu et al., (2014) in the same and surrounding communities. However, *Phlebotomus duboscqi* which has been documented as the proven vector of leishmaniasis in West Africa (Anderson *et al.*, 2011; Boakye *et al.*, 2005; Kone, Thera, Faye, and Doumbo, 2018; Kweku *et al.*, 2011) was not identified. This could be attributed to the fact that *Sergentomyia* species appear to be able to colonise different environmental conditions and biotypes as reported by Jaouadi *et al.*, (2015) in their study. Only, small numbers of *Phlebotomus rodhaini* was identified yet its relevance has not been determined due to the low numbers that had been collected in previous surveys (Boakye *et al.*, 2005; Kweku *et al.*, 2011). Nevertheless. *Phlebotomus rodhaini* has been documented as a possible vector in eastern Sudan and other parts of Africa (Elnaiem *et al.*, 2011).

Another findings that was consistent with the report of Mosore, (2016) - Dodome Dogblome recorded the highest collection of the sand flies. representing 69.98% followed by Lume Atsiame (23.69%) and Dodome Awuiasu (6.34%). The high sand fly collection at Dodome Dogblome could be attributed to the many closely planted plantain and banana trees sited around the residence. This created sufficient moisture and organic detritus which are the main requirements that provide suitable breeding sites in the communities (Adam *et al.*, 2017; Kasap and Alten, 2006; Service, 2008). Also, the presence of organic debris in proximity of houses play an important role in facilitating sand fly breeding.

During the sampling, it was observed that due to the dry weather season in November and December, no sand fly was trapped by either the CDC light

traps or the sticky paper traps. However, fewer sand flies were captured in January to February. As the wet season began, the number of sand flies captured increased gradually. Moreover, in May it was observed that in the days without rainfall during the wet season, a large number of sand flies were captured. This could be attributed to the fact that sand flies are highly prevalent in the wet season (Ikpeama and Obiajuru, 2018; Silva *et al.*, 2010; Tiwary *et al.*, 2013). Noteworthy, the highest sand fly peak was most abundant in the early wet season – March, April and May. A similar finding was reported by Panthawong, Chareonviriyaphap, and Phasuk, (2015) in Thailand where the most abundant sand fly was captured in the early rainy season from April to June. Nevertheless, the extent to which sand fly population will increase in the course of the year relies on the local climate with significant seasonal changes in temperature and precipitation (Ikpeama and Obiajuru, 2018).

4.2.3 Molecular Identification of Sand flies

The DNA barcoding was employed as an efficient tool for the identification of species of sand flies. The ultimate aim of using the DNA barcoding as a tool was to employ a molecular approach that is used in complementing the morphological identification methods (Azpurua *et al.*, 2010; Gutiérrez *et al.*, 2014). The mitochondrial cytochrome *c* oxidase subunit I gene was able to molecularly confirm the randomly selected sand flies that had been morphologically identified and DNA sequenced. This was consistent with results obtained from other studies carried out by Adeniran *et al.*, (2019) in South Eastern Mexico and Nzelu *et al.*, (2015) in Peru. Where for instance, in the study by Adeniran *et al.*, 2019, DNA barcoding was employed as a tool for identifying the sand flies collected in localized cutaneous leishmaniasis endemic areas of

Quintana Roo, Mexico. Overall, the study reported that molecular taxonomic resolution using the 658 bp fragment of the mitochondrial cytochrome *c* oxidase subunit 1 gene was 100% congruent with the morphological identification. Therefore, the study indicated the utility of DNA barcoding for sand flies species identification. On the other hand, in the study by Nzelu *et al.*, 2015, it was reported that DNA barcoding was useful in revealing population differentiation, and therefore promises to be a valuable tool for epidemiological studies of leishmaniasis

To determine the genetic variability of the collected sand fly an attempt was made using the 18S rRNA gene for identification. The high conservation of the 18S rRNA gene and its flanking regions made it appropriate for structuring phylogenetic relationships (Akhoundi *et al.*, 2017). This approach targeted the genotype of the species. Following the DNA sequencing of the PCR amplified product of the 18S rRNA gene, the genetic variability was significant only among two species of the the genus *Sergentomyia*. However, among the genus *Phlebotomus*, the variability was not obtained because the consensus obtained from the sequencing could not form a contiq. This result was similar to the study carried out by that by Al-Ajmi, Ayaad, Al-Enazi, and Al-Qahtani, (2015) in which sand fies species of the genus *Phlebotomus* showed very little specific variability while that of genus *Sergentomyia* showed significant variability. It can therefore be inferred from this observation that, the sequence analysis of the 18S region of rRNA gene was an effective tool that aided in studying inter-species variability in organisms related to species of the genus *Sergentomyia*.

The digestion of the 18S rRNA gene PCR products by *RsaI* restriction enzyme was not successful. This could be attributed firstly to the quality and

quantity of DNA. Secondly, it could be attributed but not limited to the proximity of the recognition site to the end of the DNA fragment. Where it can be speculated that the enzyme did not locate the recognition site in the product. Thus, the enzyme used required additional flanking bases for efficient DNA binding and cleavage. On the other hand, in the study carried out by Al-Dakhil *et al.*, (2017), *Sergentomyia schwetzi* species of the 18S rRNA gene PCR products cleaved to produce two fragments with the enzyme *RsaI* while there was no digestion in 18S rRNA gene PCR products from the other species of sand fly. This therefore, suggests that the utility of PCR–RFLP profiles depend on the type of restriction enzymes that is used and also the restriction sites of these enzymes (Cortes *et al.*, 2006).

4.2.4 Identification of Source of Blood Meal

The identification of the source of blood meal in sand flies gives information about feeding preferences and a resultant control strategies (Abbate *et al.*, 2020; Alcover *et al.*, 2012; González *et al.*, 2015). The identification is of great ecological and epidemiological relevance since it will provide understanding in relation to the blood feeding patterns of the sand fly. Thus, in the effort to find the probable host(s) involved in the transmission of leishmaniasis by blood meal analysis, a molecular approach was employed. It has been reported that blood meal identification by molecular analysis depended on the amount of blood ingested and the period of blood digestion in the midgut of the insect (Kent and Norris, 2005). In the current study, although 34.43% (125/363) of the sand flies were blood fed, only eighty-four of these ampilflied at 359bp. The fact that PCR could not detect the presence of blood meal in the remaining forty one (41) sand fly species could be attributed to the display of

interspecific variation in sand flies target sizes (Abbasi *et al.*, 2009). As a result, the volume of blood the sand fly ingest during blood feeding could be low, ranging from 0.1 to 1.0 L, thus escaping detectable threshold by this method (Abbasi *et al.*, 2009; Bongiorno *et al.*, 2003; Cotteaux-Lautard *et al.*, 2016; Daba, Daba, Shehata, and El Sawaf, 2004). Elsewhere in Central Tunisia, Slama *et al.*, (2015), reported from their study on blood meal analysis of *Culicoides*, that of the one hundred and eighty two (182) species of *Culiciodes* midges that was tested by amplification at 359 bp, only one hundred and twenty five (125) tested postive at the expected 359 bp. Slama *et al.*, 2015 attributed this discrepancy to the fact that blood meal volume found in biting midges varies from 0.1 to 1.0 µL. Also, the study carried out by Carvalho *et al.*, (2017) in Brazil also reported that of the three hundred and sixty three (363) sand flies species that was engorged, only one hundred and twenty five (125) amplified at the expected 359 bp. They attributed the low percentage of successful identifications of meal sources due to the small volume of blood and the time elapsed after the feeding.

In order to zero-in on the particular hosts preference in the endemic communities of the sand fly species, it was important to obtain DNA sequence data. Unfortunately, the qualities of most of the DNA sequences of the amplified product were not good enough for further analysis. This could be because there was the presence of ethanol or EDTA in the DNA and as such the DNA was of poor quality. Additionally, it was possible that the purification did not remove properly all the PCR primers and excess dNTPs. Nonetheless, with the four sequence data obtained, the BLAST analysis provided a narrow range of potential hosts. The phylogenetic tree (Figure 4.9) showed close relatedness of the sequences to *Mus musculus* (house mouse) and *Homo sapiens* (human). Desewu,

unpublished observation from 2008 (as cited by Kweku *et al.*, 2011) had reported that the blood meal analysis showed that sand flies species had mixed blood meals including human but no rodent blood. Studies carried out by Berdjane-Brouk *et al.*, (2012) in Mali, Bennai *et al.*, (2018) in Algeria, Tateng *et al.*, (2018) in Cameroun and Yared *et al.*, (2019) in Ethipia reported source of blood meal in *Sergentomyia darlingi*, *Sergentomyia minuta*, *Sergentomyia similima* and *Sergentomyia schwetzi* respectively to be *Homo sapiens*. Thus implying that some of these species of sand flies feed on human blood. The preference for human blood by the *Sergentomyia* species of sand flies is not surprising given that *Sergentomyia* species are highly anthropophilic (Bravo-Barriga *et al.*, 2016).

The sequences B4, B5, B6 and B7 (Figure 4.9) are closely related to both house mouse and human. Striking, the B4 is blood meal from *Phlebotomus rhodaini* (blood fed) while B5, B6 and B7 are blood meals from *Sergentomyia similima* (blood fed). Despite the wide distribution of *Phlebotomus rodhaini* in most leishmaniasis endemic foci, it is considered a rare species and therefore it is counted as a possible vector of leishmaniasis parasites (Mosore, 2016). *Phlebotomus rodhaini* was collected in small numbers in this study but, it is a known vector for *Leishmania major* elsewhere in West Africa, specifically Mali (Coulibaly *et al.*, 2016).

The use of the cytochrome *b* gene for identification of blood meal is a worthy research path to take and the molecular approach as indicated by (Ernieenor Faraliana *et al.*, 2013), is the most straightforward and specific method to identify blood meals. Furthermore, this approach is ideal since primers may be improved based on initial data and later employed to amplify conserved homologous DNA fragments within the endemic communities (Kent, 2009). Also, the cytochrome

b gene has been commonly used for the blood meal identification due to their high copy numbers and sufficient genetic variation at the primary sequence level among vertebrate taxa (Boakye *et al.*, 1999; Lah, Ahamad, Haron, and Ming, 2012).

4.2.5 Detection of *Leishmania* DNA in Sand fly

It is well documented that cutaneous leishmaniasis is endemic in some communities in the Ho Municipality of the Volta region of Ghana (Boakye *et al.*, 2005; Kwakye-Nuako, 2016; Kweku *et al.*, 2011; Mosore, 2016; Nzelu *et al.*, 2014). The Ho District Directorate of Health Services (Ghana Health Services, 2013) had reported such and studies carried out by (Boakye *et al.*, 2005; Fryauff *et al.*, 2006; Kwakye-Nuako, 2016; Kwakye-Nuako *et al.*, 2015; Kweku *et al.*, 2011; Mosore, 2016; Nzelu *et al.*, 2014 and Villinski *et al.*, 2008) over the years affirms this. The role of sand fly species as vectors in causing leishmaniasis is suspected epidemiologically when the species is predominant in an endemic focus (Nzelu *et al.*, 2014). Studies carried out in this endemic area over the years have consistently reported the prevalence of *Sergentomyia* species. However, the belief and view is that *Sergentomyia* species do not feed on humans, and as such cannot transmit *Leishmania* to human (Berdjane-Brouk *et al.*, 2012; Kwakye-Nuako *et al.*, 2015; Maia and Depaquit, 2016; Sadlova *et al.*, 2013). Nonetheless, studies carried out in various parts of the world have questioned the belief and view where *Sergentomyia schwetzi, Sergentomyia garnhami, Sergentomyia babu* and *Sergentomyia sintoni* have been documented to feed on humans (Campino *et al.*, 2013; Jaouadi *et al.*, 2015; Parvizi and Amirkhani, 2008).

From this study *Leishmania* DNA was detected in only five (5) out of the five hundred and fifty three (553) sand fly species screened. This five

comprised two (2) *Phlebotomus rhodaini* and three (3) *Sergentomyia similima*. This result was similar to a study conducted in Iran (Azizi *et al.*, 2016) where out of a total of one hundred and fifty (150) female sand flies screened, seven (7) were infected with *Leishmania major*. Another study carried out in Mali (Berdjane-Brouk *et al.*, 2012) reported that *Leishmania major* DNA was detected by PCR in seven (7) of the four hundred and forty six (446) females specifically two (2) of *Phlebotomus duboscqi* and five (5) *Sergentomyia darlingi*. Furthermore, Bravo-Barriga *et al.*, (2016), reported from Spain that *Leishmania* DNA was detected in three (3) out of four hundred and thirty five (435) female sand flies – one (1) *Phlebotomus pernicious* and two (2) *Sergentomyia minuta*. Additionally, in Saudi Arabia, Haouas *et al.*, (2017), reported from their studies that of the seventy five (75) sand flies screened only five (5) had *Leishmania* DNA. Maia *et al.*, (2015) in Portugal – only three (3) out of the two thousand three hundred and eighty seven (2387) had *Leishmania* DNA and Vahabi *et al.*, (2016) in Iran also reported that out of two hundred and eighty (280) sand fly screened only five (5) had *Leishmania* DNA. In these studies, less than ten parasite species was detected in the many sand flies species collected. This emphasizes the fact that the rate of sand fly infection with *Leishmania* is generally extremely low (0.01–1%) or insignificant even in endemic areas (Kato *et al.*, 2007; Sharma and Singh, 2008). González *et al.*, (2017), Sant'Anna *et al.*, (2012) Tiwary *et al.*, (2013) also reported that, in epidemic leishmaniasis communities, the prevalence of *Leishmania* in female sand fly populations ranges from 0.7 to 2.0% and rarely exceeds 2%. The number and availability of mammals with infective leishmaniasis can influence the prevalence of sand fly species with transmissible *Leishmania* parasites (González *et al.*, 2017; Sant'Anna *et al.*,

2012). The infection of rate of the sand flies was very prevalent in the Dodome Dogblome community. A community typically characterised by closely planted plantain and banana trees providing warmth and moisture for sand fly breeding.

Characterization of the obtained DNA sequences upon sequencing by using the neighbour joining method for phylogenetic analysis revealed L2 and L3 showed close relatedness of the parasite with *Leishmania major* in both *Phlebotomus rodhaini* and *Sergentomyia similima* with L4 and L5 clustering around *Leishmania amazonensis*. The generated pairwise heat map of the obtained DNA sequences from L2 and L3 also showed close relatedness to *Leishmania major* (present in both species of vectors) whereas that from L4 and L5 showed close relatedness to *Leishmania donovani* and *amazonensis* respectively (present only in the *Sergentomyia* species). Given that previous studies had reported the occurrence of both *Leishmania major* (Fryauff *et al.*, 2006) and an uncharacterized species (Villinski *et al.*, 2008) as the parasites causing the disease in this endemic area in Ghana, the possibility that other *Leishmania* species may be present in this endemic focus cannot be ignored. Furthermore, Nzelu *et al.*, (2014) in their study also revealed infection of *Leishmania major* DNA in *Sergentomyia ingrami* pools, and *Leishmania tropica* DNA in *Sergentomyia ingrami* and *Sergentomyia hamoni* pools by ITS1 PCR-RFLP and sequencing analysis. This indicates that there are emerging parasites in this endemic area. This was evident when Kwakye-Nuako *et al.*, (2015) identified the parasites in individuals with active cutaneous leishmaniasis in the endemic area to be new members of the *Leishmania enriettii* complex.

Typically then, only one species of sand fly was found in a particular ecological niche and landscape (Ready, 2013). Over the years, it has been

demonstrated that most sand fly species can support the development of multiple *Leishmania* species (Volf and Myskova, 2007). Also, a global map drawn using information by the WHO, (2016b) showed that more than half of the endemic countries, have had at least two or more *Leishmania* species present.

One interesting finding of this study was the detection of *Leishmania* DNA in *Sergentomyia similima*. Mosore, (2016), in a study carried out in the same area detected *Leishmania DNA* in *Sergentomyia africana*, thus, further questioning the vectorial capacity of sand flies from the genus *Sergentomyia* and their role in leishmaniasis transmission. No study carried out in the endemic area had identified this vector as carrying *Leishmania* DNA. This assumption needs to be more thoroughly evaluated in further studies. This finding further challenges the belief and view that cutaneous leishmaniasis is exclusively transmitted by species belonging to the *Phlebotomus* genus. This is because there have been reports of the potential role of *Sergentomyia* species as a vector of leishmaniasis (Maia and Depaquit, 2016). For instance, *Leishmania major* DNA has been detected in *Sergentomyia minuta* from Portugal (Campino *et al.*, 2013), and Tunisia (Jaouadi *et al.*, 2015). It has also been detected from *Sergentomyia clydei* (Ayari *et al.*, 2016) also in Tunisia. In addition, *Leishmania infantum* DNA has been detected in *Sergentomyia dubia*, *Sergentomyia magna*, and *Sergentomyia schwetzi* from Senegal (Senghor *et al.*, 2016). Therefore, the finding and identification of these *Leishmania* parasites in the *Sergentomyia* sand flies species in endemic areas of the Ho Municipality are fundamental factors in assessing the disease transmission risk over time. This will help in designing prevention and control measures, and also help predict disease epidemics in the endemic areas.

CHAPTER FIVE

SUMMARY, CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

5.1 Summary

This study was aimed at assessing the relationship of species of sand fly, *Leishmania* parasite and host in some endemic communities in the Ho Municipality of the Volta Region. It briefly delved into the geographic distribution of leishmaniasis focusing on Ghana with regards to the vectors and parasites that have been reported in the endemic areas over the years. The justification for the study was made known with its significance. Relevant literature was reviewed to help in discussing the results that will be obtained. Structured questionnaires were administered to study participants with the aim of assessing the knowledge, attitude and practice regarding leishmaniasis in order to assist in the control strategies that will be adapted in the endemic communities. Collected sand flies was morphologically identified using taxonomical keys and the DNA barcode approach was used to molecularly confirm the identified the sand fly species collected. Additionally, the amplification of the cytochrome *b* (*cyt b*) gene and subsequent DNA sequencing was used for identification of source of blood meal. Mincr1/Mincr2 primers were explored in the determination of *Leishmania* DNA in sand flies and subsequent DNA sequencing was conducted.

5.2 Conclusion

From the KAP study, majority of the individuals living in the endemic coommunities were informed about the disease and identified it with a local

name. Additionally, only a few percentage of individuals knew about the mode of transmission of the disease. Overall, only a hand full of the individuals were informed about how to protect themselves from the bite of the sand fly species.

Regarding the vector, the captured sand fly species were identified morphologically using taxonomical keys. These identified sand fly species was confirmed by the molecular based techniques employed.

Of the eleven different sand fly species identified within the endemic communities only two carried *Leishmania* DNA. These were *Phlebotomus rhodaini* and *Sergentomyia similima.*

The analysis of the molecular data suggested that the sand flies species carried *Leishmania* DNA. These sand fly species fed on blood from human (*Homo sapiens)* and house mouse (*Mus musculus).*

5.3 Limitations

The findings of this study were limited by the low quality of PCR products such that although the concordance between morphological and molecular methods were high it was not determined for all the samples of sand fly captured. Additionally, this also affected the quality of the sequence data and the phylogenetic analysis.

5.4 Recommendations

5.4.1 Policy

It is recommended that the Ho Municipal Health Directorate under the Ghana Health Service provide education on leishmaniasis emphasizing the mode of transmission and control strategies such as the use of insecticide treated net.

5.4.2 Practice

The inhabitants of the endemic commmunities should be educated to sleep under bed nets, wear protective clothing particularly from dusk to dawn and seek orthodox treatment in case of bites from the sand flies.

5.4.3 Further Studies

Further studies would be aimed at exploring the activity of *Hyptis suaveolens (L) Poit* extracts against isolates of *Leishmania* species. Additionally, investigate the vector competence of *Sergentomyia similima* if it plays a role in disease transmission. Finally, conduct studies with the aim to isolate promastigotes of the *Leishmania* parasite in the digestive tracts of *Sergentomyia similima.*

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APPENDICES

APPENDIX A

Informed Consent Form

UNIVERSITY OF CAPE COAST

INSTITUTIONAL REVIEW BOARD

INFORMED CONSENT FORM

Title: Identification of the vector and *Leishmania* species in endemic cutaneous leishmaniasis communities in the Ho Municipality.

Principal Investigator: Edna Dzifa Doe

Address: University of Cape Coast-Department of Biomedical Sciences. Mobile: 024 4297762.

General Information about Research

You are being invited to take part in this research so as to contribute to the knowledge, attitude and practices with regards to Leishmaniasis in the endemic areas. This study, will seek to assess the relationship of species of sand fly, *Leishmania* parasites and human host in the endemic communities of the Ho Municipality in the Volta Region of Ghana. The study will utilize molecular methods to identify the sand flies and also the infection rate of the sand flies.

Procedures

To find answers to some of the questions raised with regards to this research, we invite you to take part in this study. If you accept:

You will be asked to complete a questionnaire or you will be assisted to do so in the local language (or in English, whichever is appropriate for you). The assistance to complete the questionnaire will be by a one-to-one interview. You

will be asked to provide some demographic information and provide answers to questions seeking information on the knowledge. attitude and practices in relation to the disease. No one will be identified by name.

You are being invited to take part in this study because the community in which you reside is one of the endemic sites for the leishmaniasis disease and as such, your answers will contribute towards control strategies for the disease.

The decision to join, or not to join, is up to you. If you do not wish to answer any of the questions included in the questionnaire, you may skip them and move on to the next question. It is estimated that your part in this study will last for not more than 15 minutes.

I have obtained information on related cultural norms and have been respecting them, during the community engagement, please draw my attention to any more as I may not have obtained all that may apply and I will respect them.

Possible Risks and Discomforts

No physical, social and psychological risks are anticipated for participating in this study, however if any are identified during the study please let me know and I will respond appropriately so as to protect your interest.

Possible Benefits

There are no direct benefits/compensation to you as a participant during this study, nonetheless, based on the results of the study, your community will benefits from the improvement in the control strategies that will be adapted.

Confidentiality

Your collected information will be coded and recorded in electronic database which will be password protected and any other means of confidentiality that may be needed will be applied to protect your data.

Voluntary Participation and Right to Leave the Research

The participation in this study is voluntary. You have the right not to participate at all or to leave the study at any time. Deciding not to participate or choosing to leave the study will not result in any penalty or loss.

Contacts for Additional Information

You can contact my supervisors (Dr. Godwin Kwakye-Nuako and Dr. Alexander Yawson) on mobile numbers 024 413 0451 or 024 296 6341, respectively, if you have questions about the study, any problems, unexpected physical or psychological discomforts, any injuries, or think that something unusual or unexpected is happening.

Further, if you have any issues on your rights as a participant you can contact the address below:

Administrator, University of Cape Coast-Institutional Review Board Office, Cape Coast. irb@ucc.edu.gh and on phones lines 0332133172 and 0244207814

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of University of Cape Coast with identification number (UCCIRB/CHAS/2017/32).

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title **Identification of the vector and *Leishmania* species in endemic cutaneous leishmaniasis communities in the Ho Municipality** has been read and explained to me. I have been given an opportunity to ask questions about the research and received answers to my satisfaction. I agree to participate as a volunteer.

_____

Date Name and signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

_____

Date Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____

Date Signature of Person Who Obtained Consent

APPENDIX B

Study Questionnaire

DEPARTMENT OF BIOMEDICAL SCIENCES, SCHOOL OF ALLIED HEALTH SCIENCES

UNIVERSITY OF CAPE COAST

Title: Study of cutaneous Leishmaniasis in an endemic focus in the Volta Region of Ghana

Date questionnaire was taken.....

A. DEMOGRAPHIC INFORMATION

1. Identification No._____

2. Age: _____

3. Gender: _____

4. Religion: _____

5. Marital status:

[ ] Married [ ] Never married [ ] Cohabiting

[ ] Divorced

6. Occupation

_____

7. Educational status:

[ ] No Formal Education [ ] Primary [ ] Junior Secondary

[ ] Senior Secondary [ ] Tertiary

B. KNOWLEDGE

8. Have you ever heard of leishmaniasis?

[ ] Yes [ ] No

9. What is it local name?

_____

10. How or from where did you get to hear of leishmaniasis?

_____

11. Do you know the mode of transmission of leishmaniasis?

_____

12. What are the symptoms of leishmaniasis?

_____

13. How can leishmaniasis be prevented? (Multiple selections may be made)

[ ] Use repellent [ ] Use bed [ ] I Do not know [ ]

others (specify) _____

14. Have you had any education on leishmaniasis?

[ ] Yes [ ] No

15. Have you seen someone with the disease/symptoms before?

[ ] Yes [ ] No

C EXPERIENCE WITH INFECTION/DISEASE

16. i. Do you or have you ever had this infection/disease in the past?

[ ] Yes [ ] No

ii. Does or has any member of your family ever had this disease?

[ ] Yes [ ] No

iii. Do you know anybody who has or have ever had this disease?

[ ] Yes [ ] No

D. ATTITUDE

17. i. What are the problems associated with getting the infection/disease?

_____

ii. Do you think this disease only affects some category of people?

[ ] Yes [ ] No [ ] can't tell

iii. Which group of people do you think mostly get the infection?

_____

iv. Do you think this disease is worth worrying about?

[ ] Yes [ ] No [ ] Can't tell

v. Do you think this disease has some spiritualties to it?

[ ] Yes [ ] No [ ] Can't tell

E PRACTICES

18. i. Do you sleep under bed net?

[ ] Always [ ] Often [ ] Sometimes/rarely [ ] Not at all

ii. Do you often have direct interactions with dogs?

[ ] Always [ ] Often [ ] Sometimes/rarely [ ] Not at all

iii. How do you often prevent bite from the sand fly?

_____ you or a family member has or have ever had this infection/disease, respond to questions 19-i-iii

19.i. At what stage of infection did you or your family member seek treatment?

[ ] When symptoms appear [ ] Sometime [ ] I Do not know

ii. How long did it take you or your family member to seek treatment?

[ ] Within days [ ] Within weeks [ ] Within months [ ] They often do not [ ] I Do not know

iii. Did you or your family member follow through to the end of the treatment process?

[ ] Yes [ ] No [ ] Can't remember

Thank you very much for your participation

APPENDIX C
Result Tables

Table A: Demographic information of participants

Variable		Frequency	Percentage
Age group			
	<21	17	15.32
	>50	24	21.62
	21-30	21	18.92
	31-40	26	23.42
	41-50	23	20.72
Occupation			
	Artisan	9	8.11
	Farmer	33	29.73
	Formal employment	10	9.01
	Student	22	19.82
	Trading	23	20.72
	Unemployed	14	12.61
Formal education			
	No Formal Education	10	9.01
	Primary	24	21.62
	Junior Secondary	31	27.93
	Senior Secondary	37	33.33
	Tertiary	9	8.11

Table B: Knowledge of cutaneous leishmaniasis by participants

Variable		**Frequency**	**Percentage**
Local Name			
	Agbamekanu	98	88.29
Mode of Transmission			
	Bite from fly	22	19.82
Leishmaniasis Education			
	Yes	3	2.70
	No	108	97.30
Seen disease before			
	Yes	91	82.0
Past experience			
	Have had	49	44.14
Family experience			
	Have had	66	59.46

Table C: Attitude related to leishmaniasis among participants

Variable	Categories	Frequency	Percentage
Associated problems			
	Low productivity, stigmatization	19	17.12
	Severe pain, big sore, swelling	62	55.86
	Do not know	28	25.22
Those infected			
	Elderly	2	1.80
	Children	3	2.70
	Everybody	52	46.85
	Some people	1	0.90
	Cannot tell	53	47.75
Dog Interaction			
	Always	3	2.70
	Sometimes	7	6.31
	Not at all	97	87.39
	Often	4	3.60

Table D: Practices towards leishmaniasis among participants

Variable	Categories	Frequency	Percentage
Bite Prevention			
	Protective clothing/Repellent	20	18.02
	Spraying and Weeding	26	23.42
	Sleeping under bed net	10	9.01
	Do not know actions	55	49.55
Bed net usage			
	Always	44	39.64
	Sometimes	36	32.43
	Often	5	4.50
	Not at all	26	23.42
Sought Herbal Treatment			
	When symptoms appear	41	49.40
	Sometimes	4	4.82

APPENDIX D

Raw Data of Sand Fly Collection and PCR Results

Tube Number	Location	Species	PCR results
N1	Dodome Awuiasu	*Phlebotomus rodhaini*	Negative
N2	Dodome Awuiasu	*Sergentomyia africana*	Negative
N3	Dodome Awuiasu	*Sergentomyia schwetzi*	Negative
N4	Dodome Awuiasu	*Sergentomyia schwetzi*	Negative
N5	Dodome Awuiasu	*Sergentomyia schwetzi*	Negative
N6	Dodome Awuiasu	*Phlebotomus rodhaini*	Positive
N7	Lume Atsiame	*Sergentomyia africana*	Negative
N8	Lume Atsiame	*Phlebotomus rodhaini*	Negative
N9	Lume Atsiame	*Sergentomyia africana*	Negative
N10	Lume Atsiame	*not good*	Negative
N11	Lume Atsiame	*Sergentomyia dureni*	Negative
N12	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N13	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N14	Lume Atsiame	*Sergentomyia africana*	Negative
N15	Lume Atsiame	*Sergentomyia ingrami*	Negative
N16	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N17	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N18	Lume Atsiame	*Sergentomyia africana*	Negative
N19	Lume Atsiame	*Sergentomyia africana*	Negative
N20	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N21	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N22	Lume Atsiame	*Phlebotomus rodhaini*	Negative
N23	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N24	Lume Atsiame	*Sergentomyia africana*	Negative
N25	Lume Atsiame	*Sergentomyia ingrami*	Negative
N26	Lume Atsiame	*Sergentomyia africana*	Negative
N27	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N28	Lume Atsiame	*Sergentomyia ingrami*	Negative
N29	Lume Atsiame	*Sergentomyia collarti*	Negative
N30	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N31	Lume Atsiame	*Sergentomyia ghesquierei*	Negative

N32	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N33	Lume Atsiame	*Sergentomyia africana*	Negative
N34	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N35	Lume Atsiame	*Sergentomyia africana*	Negative
N36	Lume Atsiame	*Sergentomyia africana*	Negative
N37	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N38	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N39	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N40	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N41	Dodome Dogblome	*Sergentomyia africana*	Negative
N42	Dodome Dogblome	*Sergentomyia similima*	Positive
N43	Dodome Dogblome	*Sergentomyia schwetzi*	Negative
N44	Dodome Dogblome	*Sergentomyia africana*	Negative
N45	Dodome Dogblome	*Sergentomyia africana*	Negative
N46	Dodome Dogblome	*not good*	Negative
N47	Dodome Dogblome	*Sergentomyia schwetzi*	Negative
N48	Dodome Dogblome	*Sergentomyia africana*	Negative
N49	Dodome Dogblome	*Sergentomyia ghesquierei*	Negative
N50	Dodome Dogblome	*Sergentomyia africana*	Negative
N51	Dodome Dogblome	*Sergentomyia similima*	Positive
N52	Dodome Dogblome	*not good*	Negative
N53	Dodome Dogblome	*Sergentomyia similima*	Negative
N54	Dodome Dogblome	*Sergentomyia similima*	Negative
N55	Dodome Dogblome	*Sergentomyia africana*	Negative
N56	Dodome Dogblome	*Sergentomyia africana*	Negative
N57	Dodome Dogblome	*Sergentomyia schwetzi*	Negative
N58	Dodome Awuiasu	*Sergentomyia similima*	Negative

	Dodome		
N59	Awuiasu Dodome	*Sergentomyia africana*	Negative
N60	Awuiasu Dodome	*Sergentomyia similima*	Negative
N61	Awuiasu Dodome	*Sergentomyia africana*	Negative
N62	Dogblome Dodome	*Sergentomyia similima*	Negative
N63	Dogblome Dodome	*Sergentomyia ingrami*	Negative
N64	Dogblome Dodome	*Sergentomyia similima*	Negative
N65	Dogblome Dodome	*Sergentomyia antennata*	Negative
N66	Dogblome Dodome	*Sergentomyia africana*	Negative
N67	Dogblome Dodome	*Sergentomyia africana*	Negative
N68	Dogblome Dodome	*Sergentomyia africana*	Negative
N69	Dogblome Dodome	*Sergentomyia africana*	Negative
N70	Dogblome Dodome	*Sergentomyia dureni*	Negative
N71	Dogblome Dodome	*Sergentomyia africana*	Negative
N72	Dogblome Dodome	*Sergentomyia africana*	Negative
N73	Dogblome Dodome	*Sergentomyia africana*	Negative
N74	Dogblome Dodome	*Sergentomyia africana*	Negative
N75	Dogblome Dodome	*Sergentomyia similima*	Negative
N76	Dogblome Dodome	*Sergentomyia africana*	Negative
N77	Dogblome Dodome	*Sergentomyia africana*	Negative
N78	Dogblome Dodome	*Sergentomyia africana*	Negative
N79	Dogblome Dodome	*Sergentomyia similima*	Negative
N80	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N81	Dogblome Dodome	*Sergentomyia africana*	Negative
N82	Dogblome Dodome	*Sergentomyia africana*	Negative
N83	Dogblome	*Sergentomyia africana*	Negative

	Dodome		
N84	Dogblome Dodome	*Sergentomyia hamoni*	Negative
N85	Dogblome Dodome	*Sergentomyia africana*	Negative
N86	Dogblome Dodome	*Sergentomyia africana*	Negative
N87	Dogblome Dodome	*Sergentomyia similima*	Negative
N88	Dogblome Dodome	*Sergentomyia similima*	Negative
N89	Dogblome Dodome	*Sergentomyia africana*	Negative
N90	Dogblome Dodome	*Sergentomyia africana*	Negative
N91	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N92	Dogblome Dodome	*Sergentomyia similima*	Negative
N93	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N94	Dogblome Dodome	*Sergentomyia africana*	Negative
N95	Dogblome Dodome	*Sergentomyia similima*	Negative
N96	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N97	Dogblome Dodome	*not good*	Negative
N98	Dogblome Dodome	*not good*	Negative
N99	Dogblome Dodome	*Sergentomyia similima*	Negative
N100	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N101	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N102	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N103	Dogblome Dodome	*Sergentomyia africana*	Negative
N104	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N105	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N106	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N107	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N108	Dogblome	*Sergentomyia buxtoni*	Negative

	Dodome	*Sergentomyia*	
N109	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N110	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N111	Dogblome	*ghesquierei*	Negative
	Dodome		
N112	Dogblome	*Sergentomyia ingrami*	Negative
	Dodome	*Sergentomyia*	
N113	Dogblome	*ghesquierei*	Negative
	Dodome		
N114	Dogblome	*Sergentomyia africana*	Negative
	Dodome	*Sergentomyia*	
N115	Dogblome	*ghesquierei*	Negative
	Dodome		
N116	Dogblome	*Sergentomyia schwetzi*	Negative
	Dodome		
N117	Dogblome	*Sergentomyia africana*	Negative
	Dodome	*Sergentomyia*	
N118	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N119	Dogblome	*ghesquierei*	Negative
	Dodome		
N120	Dogblome	*Sergentomyia antennata*	Negative
	Dodome		
N121	Dogblome	*Sergentomyia antennata*	Negative
	Dodome	*Sergentomyia*	
N122	Dogblome	*ghesquierei*	Negative
	Dodome		
N123	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N124	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N125	Dogblome	*Sergentomyia hamoni*	Negative
	Dodome		
N126	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N127	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N128	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N129	Dogblome	*Sergentomyia ingrami*	Negative
	Dodome		
N130	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N131	Dogblome	*Sergentomyia africana*	Negative
	Dodome	*Sergentomyia*	
N132	Dogblome	*ghesquierei*	Negative
	Dodome		
N133	Dogblome	*Sergentomyia africana*	Negative

	Dodome	*Sergentomyia*	
N134	Dogblome	*ghesquierei*	Negative
	Dodome		
N135	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N136	Dogblome	*not good*	Negative
	Dodome		
N137	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N138	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N139	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N140	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N141	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N142	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N143	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N144	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N145	Dogblome	*not good*	Negative
	Dodome		
N146	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N147	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N148	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N149	Dogblome	*Phlebotomus rodhaini*	Negative
	Dodome		
N150	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N151	Dogblome	*not good*	Negative
	Dodome		
N152	Dogblome	*Sergentomyia schwetzi*	Negative
	Dodome		
N153	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N154	Dogblome	*Sergentomyia dureni*	Negative
	Dodome		
N155	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N156	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N157	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N158	Dogblome	*Sergentomyia africana*	Negative

	Dodome		
N159	Dogblome Dodome	*Sergentomyia africana*	Negative
N160	Dogblome Dodome	*Sergentomyia africana*	Negative
N161	Dogblome Dodome	*not good*	Negative
N162	Dogblome Dodome	*Sergentomyia similima*	Negative
N163	Dogblome Dodome	*Sergentomyia similima*	Negative
N164	Dogblome Dodome	*Sergentomyia similima*	Negative
N165	Dogblome Dodome	*Sergentomyia similima*	Negative
N166	Dogblome Dodome	*Sergentomyia similima*	Negative
N167	Dogblome Dodome	*Sergentomyia similima*	Negative
N168	Dogblome Dodome	*Sergentomyia similima*	Negative
N169	Dogblome Dodome	*Sergentomyia similima*	Negative
N170	Dogblome Dodome	*Sergentomyia similima*	Negative
N171	Dogblome Dodome	*Sergentomyia similima*	Negative
N172	Dogblome Dodome	*not good*	Negative
N173	Dogblome Dodome	*not good*	Negative
N174	Dogblome Dodome	*Sergentomyia similima*	Negative
N175	Dogblome Dodome	*Sergentomyia similima*	Negative
N176	Dogblome Dodome	*Sergentomyia similima*	Negative
N177	Dogblome Dodome	*Sergentomyia similima*	Negative
N178	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N179	Dogblome Dodome	*Sergentomyia similima*	Negative
N180	Dogblome Dodome	*Sergentomyia africana*	Negative
N181	Dogblome Dodome	*Sergentomyia similima*	Negative
N182	Dogblome Dodome	*Sergentomyia similima*	Negative
N183	Dogblome	*Sergentomyia similima*	Negative

	Dodome		
N184	Dogblome Dodome	*Sergentomyia similima*	Negative
N185	Dogblome Dodome	*Sergentomyia antennata*	Negative
N186	Dogblome Dodome	*Sergentomyia similima*	Negative
N187	Dogblome Dodome	*Sergentomyia africana*	Negative
N188	Dogblome Dodome	*Sergentomyia africana*	Negative
N189	Dogblome Dodome	*Sergentomyia similima*	Negative
N190	Dogblome Dodome	*Sergentomyia similima*	Negative
N191	Dogblome	*Phlebotomus rodhaini*	Positive
N192	Lume Atsiame	*Sergentomyia hamoni*	Negative
N193	Lume Atsiame	*Sergentomyia similima*	Negative
N194	Lume Atsiame	*Sergentomyia similima*	Negative
N195	Lume Atsiame	*Sergentomyia similima*	Negative
N196	Lume Atsiame	*Sergentomyia similima*	Negative
N197	Lume Atsiame Dodome	*Sergentomyia similima*	Negative
N198	Awuiasu	*Sergentomyia similima*	Negative
N199	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N200	Lume Atsiame	*not good*	Negative
N201	Lume Atsiame Dodome	*Sergentomyia similima*	Negative
N202	Dogblome Dodome	*Sergentomyia ingrami*	Negative
N203	Dogblome Dodome	*Sergentomyia africana*	Negative
N204	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N205	Dogblome Dodome	*Sergentomyia ingrami*	Negative
N206	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N207	Dogblome Dodome	*Sergentomyia hamoni*	Negative
N208	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N209	Dogblome Dodome	*Sergentomyia similima*	Negative
N210	Dogblome Dodome	*Sergentomyia buxtoni*	Negative
N211	Dogblome	*Sergentomyia similima*	Negative

	Dodome		
N212	Dogblome	*Sergentomyia schwetzi*	Negative
	Dodome		
N213	Dogblome	*Sergentomyia similima*	Negative
	Dodome	*Sergentomyia*	
N214	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N215	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N216	Dogblome	*ghesquierei*	Negative
	Dodome		
N217	Dogblome	*Sergentomyia schwetzi*	Negative
	Dodome	*Sergentomyia*	
N218	Dogblome	*ghesquierei*	Negative
	Dodome		
N219	Dogblome	*Sergentomyia similima*	Negative
	Dodome	*Sergentomyia*	
N220	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N221	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N222	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N223	Awuiasu	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N224	Awuiasu	*ghesquierei*	Negative
	Dodome		
N225	Awuiasu	*Sergentomyia africana*	Negative
	Dodome		
N226	Awuiasu	*Sergentomyia ingrami*	Negative
	Dodome		
N227	Awuiasu	*Sergentomyia africana*	Negative
	Dodome	*Sergentomyia*	
N228	Awuiasu	*ghesquierei*	Negative
	Dodome		
N229	Awuiasu	*Sergentomyia similima*	Negative
	Dodome		
N230	Awuiasu	*Sergentomyia africana*	Negative
N231	Lume Atsiame	*Sergentomyia hamoni*	Negative
		Sergentomyia	
N232	Lume Atsiame	*ghesquierei*	Negative
N233	Lume Atsiame	*Sergentomyia similima*	Negative
		Sergentomyia	
N234	Lume Atsiame	*ghesquierei*	Negative
		Sergentomyia	
N235	Lume Atsiame	*ghesquierei*	Negative
		Sergentomyia	
N236	Lume Atsiame	*ghesquierei*	Negative
		Sergentomyia	
N237	Lume Atsiame	*ghesquierei*	Negative

N238	Lume Atsiame	*Sergentomyia similima*	Negative
N239	Lume Atsiame Dodome	*Sergentomyia ghesquierei*	Negative
N240	Dogblome Dodome	*Sergentomyia africana*	Negative
N241	Dogblome Dodome	*Phlebotomus rodhaini*	Negative
N242	Dogblome Dodome	*Sergentomyia similima*	Negative
N243	Dogblome Dodome	*Sergentomyia similima*	Negative
N244	Dogblome Dodome	*Sergentomyia africana*	Negative
N245	Dogblome Dodome	*Sergentomyia similima*	Negative
N246	Dogblome Dodome	*Sergentomyia similima*	Negative
N247	Dogblome Dodome	*Sergentomyia similima*	Negative
N248	Dogblome Dodome	*Sergentomyia ingrami*	Negative
N249	Dogblome	*Sergentomyia ghesquierei*	Negative
N250	Lume Atsiame	*Sergentomyia ingrami*	Negative
N251	Lume Atsiame	*Sergentomyia africana*	Negative
N252	Lume Atsiame	*Sergentomyia africana*	Negative
N253	Lume Atsiame	*Sergentomyia similima*	Negative
N254	Lume Atsiame	*Sergentomyia ingrami*	Negative
N255	Lume Atsiame	*Sergentomyia buxtoni*	Negative
N256	Lume Atsiame	*Sergentomyia similima*	Negative
N257	Lume Atsiame	*Sergentomyia africana*	Negative
N258	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N259	Lume Atsiame Dodome	*Sergentomyia antennata*	Negative
N260	Dogblome Dodome	*Sergentomyia africana*	Negative
N261	Dogblome Dodome	*Sergentomyia similima*	Negative
N262	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N263	Dogblome Dodome	*Sergentomyia similima*	Negative
N264	Dogblome Dodome	*Sergentomyia africana*	Negative
N265	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N266	Dogblome	*Sergentomyia ghesquierei*	Negative

	Dodome		
N267	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N268	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N269	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N270	Dogblome	*Sergentomyia similima*	Negative
	Dodome	*Sergentomyia*	
N271	Dogblome	*ghesquierei*	Negative
	Dodome		
N272	Dogblome	*Phlebotomus rodhaini*	Negative
	Dodome		
N273	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N274	Dogblome	*Sergentomyia similima*	Negative
	Dodome	*Sergentomyia*	
N275	Dogblome	*ghesquierei*	Negative
	Dodome		
N276	Dogblome	*Sergentomyia hamoni*	Negative
	Dodome		
N277	Dogblome	*Sergentomyia similima*	Negative
	Dodome	*Sergentomyia*	
N278	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N279	Dogblome	*ghesquierei*	Negative
	Dodome		
N280	Dogblome	*Phlebotomus rodhaini*	Negative
	Dodome		
N281	Dogblome	*Sergentomyia similima*	Negative
	Dodome		
N282	Dogblome	*Sergentomyia antennata*	Negative
	Dodome		
N283	Dogblome	*Sergentomyia africana*	Negative
	Dodome		
N284	Dogblome	*Sergentomyia ingrami*	Negative
	Dodome		
N285	Dogblome	*Sergentomyia schwetzi*	Negative
	Dodome	*Sergentomyia*	
N286	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N287	Dogblome	*ghesquierei*	Negative
	Dodome		
N288	Dogblome	*Sergentomyia ingrami*	Negative
	Dodome	*Sergentomyia*	
N289	Dogblome	*ghesquierei*	Negative
	Dodome	*Sergentomyia*	
N290	Dogblome	*ghesquierei*	Negative
	Dodome		
N291	Dogblome	*Sergentomyia africana*	Negative

N292	Dodome Dogblome	*Sergentomyia ghesquierei*	Negative
N293	Dodome Dogblome	*Sergentomyia similima*	Negative
N294	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N295	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N296	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N297	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N298	Lume Atsiame	*Sergentomyia similima*	Negative
N299	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N300	Lume Atsiame	*Sergentomyia africana*	Negative
N301	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N302	Lume Atsiame	*Sergentomyia ingrami*	Negative
N303	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N304	Lume Atsiame	*Sergentomyia schwetzi*	Negative
N305	Lume Atsiame	*Sergentomyia africana*	Negative
N306	Lume Atsiame	*Sergentomyia similima*	Negative
N307	Lume Atsiame	*Sergentomyia similima*	Negative
N308	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N309	Lume Atsiame	*Sergentomyia similima*	Negative
N310	Lume Atsiame	*Sergentomyia similima*	Negative
N311	Lume Atsiame	*Sergentomyia similima*	Negative
N312	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N313	Lume Atsiame	*Sergentomyia similima*	Negative
N314	Lume Atsiame	*Sergentomyia ghesquierei*	Negative
N315	Lume Atsiame	*Sergentomyia similima*	Negative
N316	Lume Atsiame	*Sergentomyia antennata*	Negative
N317	Lume Atsiame	*Sergentomyia similima*	Negative
N318	Lume Atsiame	*Sergentomyia africana*	Negative
N319	Lume Atsiame	*Sergentomyia similima*	Negative
N320	Dodome Dogblome	*Sergentomyia antennata*	Negative
N321	Dodome Dogblome	*Sergentomyia africana*	Negative
N322	Dodome Dogblome	*Sergentomyia similima*	Negative
N323	Dodome Dogblome	*Sergentomyia similima*	Negative

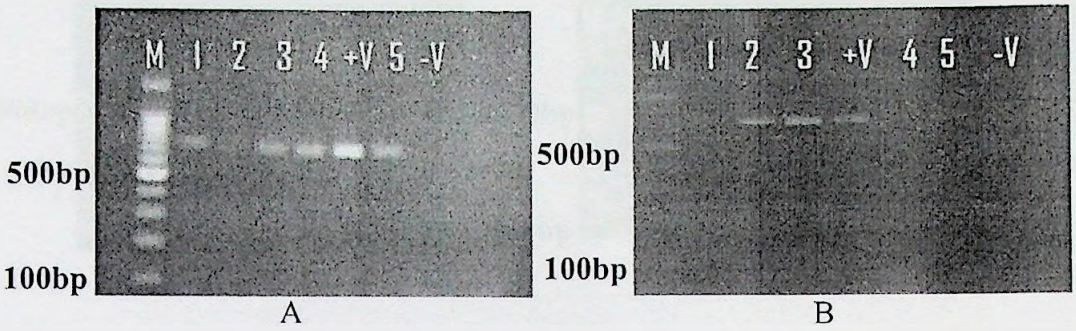
	Dodome		
N324	Dogblome Dodome	*Sergentomyia africana*	Negative
N325	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N326	Dogblome Dodome	*Sergentomyia hamoni*	Negative
N327	Dogblome Dodome	*Sergentomyia africana*	Negative
N328	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N329	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N330	Dogblome Dodome	*Sergentomyia similima*	Negative
N331	Dogblome Dodome	*Sergentomyia similima*	Negative
N332	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N333	Dogblome Dodome	*Sergentomyia africana*	Negative
N334	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N335	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N336	Dogblome Dodome	*Sergentomyia antennata*	Negative
N337	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N338	Dogblome Dodome	*Sergentomyia ingrami*	Negative
N339	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N340	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N341	Dogblome Dodome	*Sergentomyia africana*	Negative
N342	Dogblome Dodome	*Sergentomyia africana*	Negative
N343	Dogblome Dodome	*Sergentomyia antennata*	Negative
N344	Dogblome Dodome	*Sergentomyia ingrami*	Negative
N345	Dogblome Dodome	*Sergentomyia ingrami*	Negative
N346	Dogblome Dodome	*Sergentomyia similima*	Positive
N347	Dogblome Dodome	*Sergentomyia africana*	Negative
N348	Dogblome	*Sergentomyia ghesquierei*	Negative

	Dodome		
N349	Dogblome Dodome	*Sergentomyia africana*	Negative
N350	Dogblome Dodome	*Phlebotomus rodhaini*	Negative
N351	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N352	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N353	Dogblome Dodome	*Sergentomyia dureni*	Negative
N354	Dogblome Dodome	*Sergentomyia antennata*	Negative
N355	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N356	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N357	Dogblome Dodome	*Sergentomyia ingrami*	Negative
N358	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N359	Dogblome Dodome	*Sergentomyia africana*	Negative
N360	Dogblome Dodome	*Sergentomyia schwetzi*	Negative
N361	Dogblome Dodome	*Sergentomyia similima*	Negative
N362	Dogblome Dodome	*Phlebotomus rodhaini*	Negative
N363	Dogblome Dodome	*not good*	Negative
N364	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N365	Dogblome Dodome	*Sergentomyia hamoni*	Negative
N366	Dogblome Dodome	*Sergentomyia africana*	Negative
N367	Dogblome Dodome	*Sergentomyia ghesquierei*	Negative
N368	Dogblome Dodome	*Phlebotomus rodhaini*	Negative
N369	Dogblome Dodome	*Sergentomyia africana*	Negative
N370	Dogblome Dodome	*Sergentomyia dureni*	Negative
N371	Dogblome Dodome	*Sergentomyia buxtoni*	Negative
N372	Awuiasu Dodome	*not good*	Negative
N373	Awuiasu	*Sergentomyia similima*	Negative

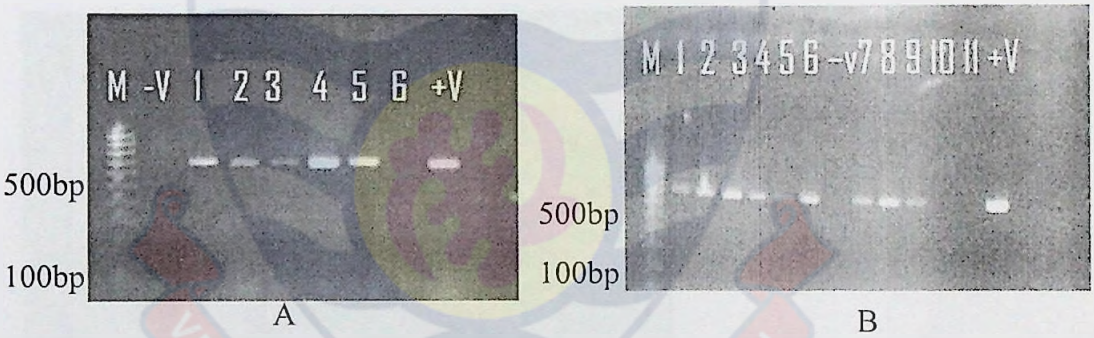
N374	Dodome Awuiasu	*Sergentomyia africana*	Negative
N375	Dodome Awuiasu	*Sergentomyia ghesquierei*	Negative
N376	Dodome Dogblome	*Sergentomyia ghesquierei*	Negative
N377	Dodome Awuiasu	*Sergentomyia africana*	Negative

APPENDIX E

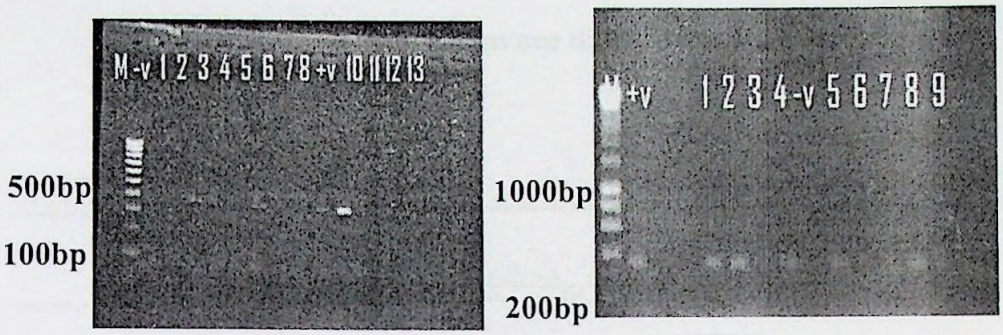
Agarose Gel photos and Photo of *Hyptis suaveolens* (L) Poit



Agarose gel electrophoresis of PCR products of *Sergentomyia antennata* [A] *Sergentomyia ingrami* [B] after amplification of COI gene (658bp). Lane M: Molecular weight marker (100bp). +V, positive control. -V, negative control



Agarose gel electrophoresis of PCR products of *Sergentomyia africana* [A]. *Sergentomyia ghesquierei* [B] after amplification of COI gene (658bp). Lane M: Molecular weight marker (100bp). +V, positive control. -V, negative control.

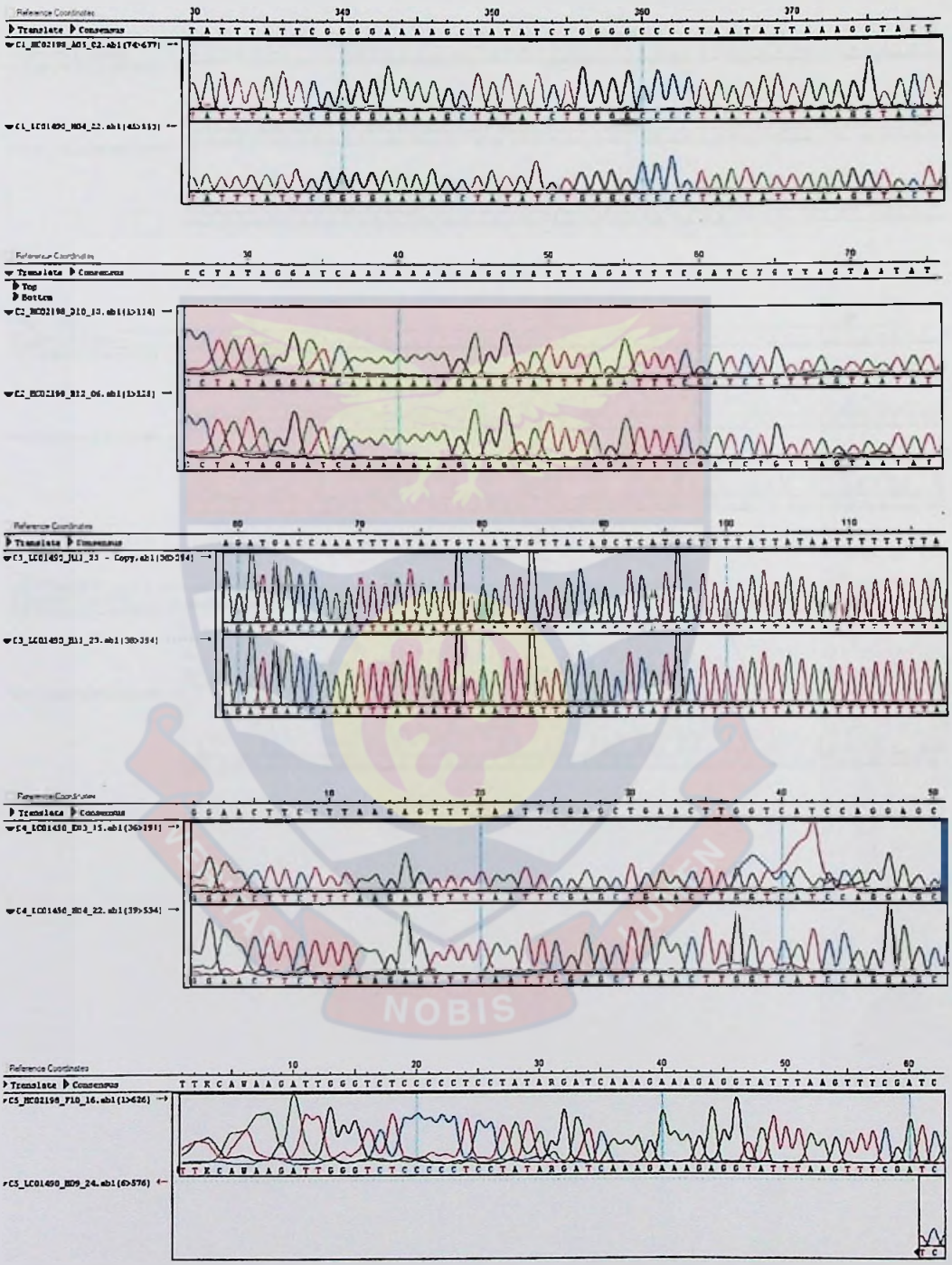


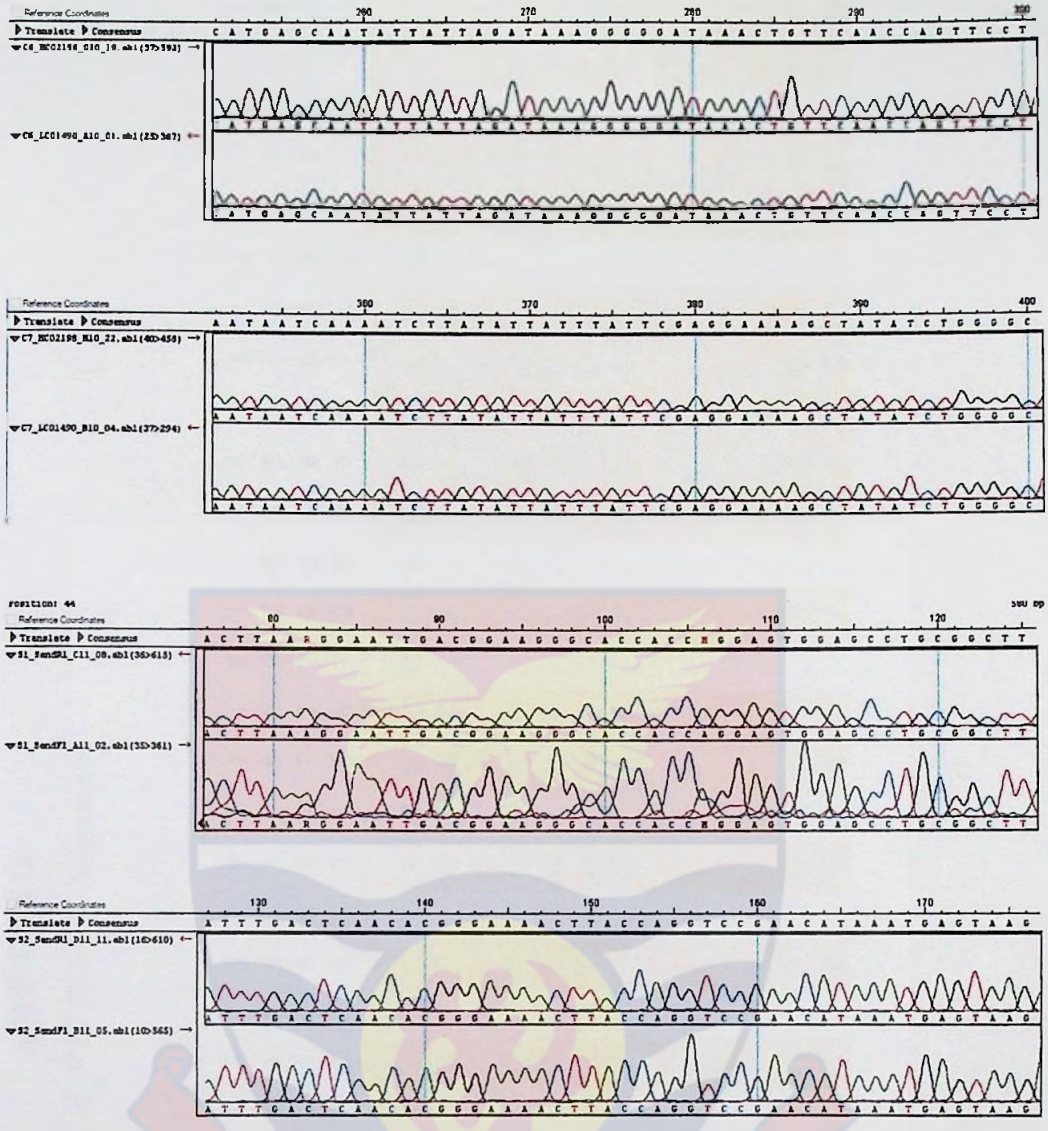
Agarose gel electrophoresis of PCR products of *Sergentomyia ghesquierei* [A] *Sergentomyia africana* [B] after amplification of cyt b gene (359bp). Lane M: Molecular weight marker (100bp). PC-positive control. NC-negative control.

Photo of Hyptis suaveolens (L) Poit - identified by the botanist.

APPENDIX F

Sequence data





APPENDIX G

Sand_F & Sand_R Alignment

	1292	1293	1294	1295	1296	1311	1312	1554	1555	1562	1563	1567	1568	1575	1576	1577	1582	1583	1584	1585	1586	1587	1588	1589	1590	1591	1592	1593	1594	1595
AJ244414.1 (*Phleobotomus papatasi*)	A	G	C	T	T	A	A	T	T	C	T	A	C	G	C	C	G	T	A	T	G	T	T	G	G	T	T	G	C	T
JQ929125.1 (*Sergentomyia clydei*)	C	.	A	C	.	G	.	C	.	T	.	G	.	A	T	.	A	.	.	.	C	.	G	.	A	.	.	-	-	.
AJ391739.1 (*Sergentomyia schwetzi*)	.	.	.	.	.	.	.	C	.	T	.	G	.	A	T	.	T	.	G	A	.	C	.	.	A	G	.	T	A	.
AJ391738.1 (*Sergentomyia dubia*)	.	.	.	.	.	.	.	C	.	T	.	G	.	A	T	.	T	.	G	.	.	C	.	.	A	G	.	T	A	.
AJ391737.1 (*Sergentomyia buxtoni*)	.	.	.	.	.	.	.	C	.	T	.	G	.	A	T	.	T	.	G	.	A	C	.	.	A	G	.	T	A	.
AJ244425.1 (*Sergentomyia dentata*)	.	.	.	.	.	.	.	C	.	T	.	G	.	A	T	.	T	.	G	.	.	C	.	.	A	G	.	T	A	.
AJ244411.1 (*Phlebotomus papatasi*)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AJ244397.1 (*Phlebotomus alexandri*)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	C	.	.	.	.
S2 (*Sergentomyia schwetzi*)	.	.	.	.	.	.	.	C	.	T	.	G	.	A	T	.	T	.	G	A	.	C	.	.	A	G	.	T	A	.
S1 (*Sergentomyia schwetzi*)	.	.	.	.	.	.	.	C	.	T	.	G	.	A	T	.	T	.	G	A	.	C	.	.	A	G	.	T	A	.

	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	7	7	8
	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	5	7	1	1	1	
	6	7	8	9	0	1	2	3	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	4	3	4	6	2
AJ244414.1 (*Phleobotomus papatasi*)	T	T	C	G	G	G	T	T	A	T	G	G	A	G	T	G	G	T	T	T	G	A	T	T	G	A	T	G
JQ929125.1 (*Sergentomyia clydei*)	.	A	.	.	C	A	C	.	-	.	A	T	.	.	.	T	T	.	.	C	A	G	.	.	.	.	.	.
AJ391739.1 (*Sergentomyia schwetzi*)	A	A	.	A	C	T	A	.	-	C	.	A	T	.	A	C	T	.	G	.	.	T	.	.	.	.	C	.
AJ391738.1 (*Sergentomyia dubia*)	A	A	.	A	C	T	G	.	-	C	.	A	T	.	G	C	T	.	A	.	.	T	.	.	.	.	C	A
AJ391737.1 (*Sergentomyia buxtoni*)	A	A	.	A	C	T	G	.	-	C	.	A	T	.	G	C	T	.	A	.	.	T	.	.	.	.	C	A
AJ244425.1 (*Sergentomyia dentata*)	A	A	.	A	C	T	G	.	-	C	.	A	T	.	G	C	T	.	A	.	.	T	.	.	.	.	C	A
AJ244411.1 (*Phlebotomus papatasi*)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
AJ244397.1 (*Phlebotomus alexandri*)	.	.	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S2 (*Sergentomyia schwetzi*)	A	A	.	A	C	T	A	.	-	.	.	A	T	.	A	C	T	.	G	.	.	T	.	.	A	.	C	.
S1 (*Sergentomyia schwetzi*)	A	A	.	A	C	T	A	.	-	.	.	A	T	.	A	C	T	.	G	.	.	T	.	.	A	.	C	.

Mincir1 & Mincir Algnment

	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	8	2	4	4	5	5	5	6	6	6	6	6	6	5	5	5	6	6	6	6	6	6	6	6	8
	9	0	8	9	0	1	2	0	1	2	3	4	5	7	8	9	0	1	2	3	4	5	6	7	8	6
AJ010074.2_*Leishmania donovani*	G	T	C	T	T	T	T	A	T	T	T	G	G	-	-	-	A	T	G	A	G	T	G	C	A	T
AJ010080.2_*Leishmania donovani*	.	.	G	.	.	.	.	G	.	-	-	-	.	A	A	C	.	.	A	T	A	A	A	.	G	G
AF308682.1_*Leishmania chagasi*	A	.	G	C	.	.	C	.	.	A	.	A	A	-	-	-	G	.	-	-	-	G	T	T	G	A
KC869685.1_*Leishmania major*	T	.	.	A	.	.	.	T	.	A	.	.	T	-	-	-	.	.	T	T	.	G	T	T	T	A
HQ600661.1_*Leishmania major*	T	A	G	A	.	.	.	C	.	A	A	.	.	A	A	C	C	.	A	.	.	G	.	A	.	G
KY698860.1.(*Leishmania amazonensis*)	T	G	T	.	C	.	C	.	.	G	A	.	T	A	A	T	T	.	A	T	.	.	C	T	G	.
L2	T	G	T	.	.	.	Y	T	.	.	-	-	Y	-	-	-	T	.	T	-	-	Y	S	.	.	A
L5	-	.	T	.	.	.	C	T	.	A	.	.	A	-	A	A	T	.	A	T	.	A	A	.	G	-
L3	-	G	T	.	.	.	.	T	.	.	-	-	Y	-	-	-	T	.	T	-	-	Y	.	.	M	A
L4	-	.	A	.	.	Y	C	.	W	A	.	.	A	-	A	A	.	W	A	T	.	A	A	.	G	