UNIVERSITY OF SIERRA LEONE
FOURAH BAY COLLEGE
FREETOWN, SIERRA LEONE

# THE BIOCHEMICAL TAXONOMY OF PHLEBOTOMINE SANDFLIES (DIPTERA: PSYCHODIDAE) IN KENYA

BY

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THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY (PH.D.) DEGREE

AUGUST 1990

POUR MON ONCLE ABDERAHMAN MOUSSA

" Good methods can teach us to develop and use the faculties with which nature has endowed us, while poor methods may prevent us from turning them to good account. Thus the genius of inventiveness, so precious in the sciences, may be diminished or even smothered by a poor method, while a good method may increase and develop it.
...In biological sciences, the role of the method is even more important than in the other sciences because of the complexity of the phenomena and countless sources of error."

Claude Bernard (1865),
French physiologist

## DECLARATIONS

I certify that none of the material offered in my thesis has previously been submitted by me for a degree of this or any other university and that it represents work performed by me alone.

Hassane Mahamat

Candidate

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This thesis work has been submitted for examination with our approval as supervisors.

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# ACKNOWLEDGMENTS

My sincere thanks and deep gratitude go to Professor Hector Morgan, my university supervisor, who with his comments, advice and suggestions helped me to shape this work.

I also express my gratitude to Dr. Mutuku J. Mutinga, Programme leader of the Medical Vectors Research Programme and a member of the supervisory committee for assisting me to choose this challenging subject, for his advice and guidance during the period of the study and for reading the manuscript.

The assistance of Dr. Ngangu N. Massamba and Professor Ahmed Hassanali, members of the supervisory committee for their suggestions during the study and for their advice, assistance, comments, and for reading and reviewing the manuscript is highly appreciated.

Professor T.R. Odhiambo, Director of ICIPE gave me permission to carry out the study at ICIPE and provided through the African Regional Postgraduate Program in Insect Sciences (ARPPIS) logistic and material support, for which I am indeed very grateful.

I am indebted to the German Academic Exchange Services (DAAD) for its financial support throughout the study.

My gratitude goes to Dr. M.S. Smalley, former ARPPIS Academic Coordinator, a friend, for his advice, and encouragement throughout the study and also to Professor Z. Dabrowski, the new academic coordinator, Dr. Y.O. Olaniran, Mr. J. Omange, Professor R. Kumar and Professor R. Assoku.

I am also grateful to Drs W. Lwande, S. Mihok and Ms Nafisa Darji for their valuable suggestions and discussion throughout the study.

I thank Mrs F. Kyai and D. Omogo for their taxonomic assistance. My thanks are extended to Mr. Ndambuki, Ms Esther Mwangi, Rose Odenbo and the Medical Vectors Research Programme team in and outside Nairobi for their help in getting the required flies.

Last but not least, I would like to thank my dear wife Rosemary Wanjiru and our son Abderahman, who put up with so much, and for their moral support, patience and understanding throughout the period of the study.

## ABSTRACT

Isoenzyme analysis and pattern analysis of cuticular components were used to characterize sandfly species collected from the field and reared in the laboratory. The following sandfly species were used during the investigation: Phlebotomus duboscqi, P. elgonensis, P. martini, P. pedifer, Sergentomyia africanus, S. antennatus, S. bedfordi, S. garnhami, S. graingeri, S. ingrami, and S. schwetzi.

Eighteen isoenzymes systems were assayed to determine which could be of practical use in the identification of sandflies. Identification of the sandfly species was shown to be possible by examining three enzymes, glucose phosphate isomerase (GPI), malate dehydrogenase (MDH) and phosphoglucomutase (PGM) on thin layer starch gel electrophoresis. Phenetic relationships between the species based on the isoenzyme banding patterns gave two groups which corresponded to the genera Phlebotomus and Sergentomyia. Only S. garnhami was grouped in the genus Phlebotomus, a peculiarity that need to be further investigated. Phast system™ IEF was also carried out for various enzymes, but only three isoenzymes, GPI, ME and PGM, were found to give banding patterns capable of distinguishing between sandfly species.

Cuticular components of sandflies showed both quantitative and qualitative differences among species. Each species of sandfly had a unique pattern of peaks, which was discernable visually. Classification based on different characteristics of the peaks (e.g. area, width and area percent) was carried out and found equally useful in differentiating species. Peaks of females and males of the same species showed quantitative differences. These results showed that cuticular component analysis could be used as a rapid alternative technique for the identification of sandflies, where morphological problems exist.

# CHAPTER ONE

## GENERAL INTRODUCTION

Phlebotomine sandflies are dipterans of the family Psychodidae and subfamily Phlebotominae. They are very small, hairy flies, brownish in colour and are found mostly in the tropics (Quate, 1964). They can often be distinguished at first sight by their nearly erect, rather narrow wings and slender bodies. According to various authors (Theodor, 1958; Abonnenc, 1972; Lewis, Minter and Ashford, 1977 and Lewis, 1982) the subfamily Phlebotominae consists of the genera; Phlebotomus Franca and Parrot, Sergentomyia Franca and Parrot, Warilia Hertig, Lutzomyia Franca, Brumptomyia Franca and Parrot. The first two are found in the Afro-Tropical region, south of Europe and Asia (Kirk and Lewis, 1951) and the rest are found in the new world i.e. Central and South America.

Sandfly larvae, which live in soil, are difficult to find (Quate, 1964; Dedet, Desjeux and Derouin, 1980; Mutinga, Kyai and Omogo, 1986). The adult sandflies are usually found in animal burrows, termite hills, caves, and cracks in walls

of houses (Heisch, Guggisberg and Teedale, 1956; Minter, 1962, 1963; Lewis, Mutinga and Ashford, 1972; Mutinga, 1971, 1975, 1975a, 1980, 1986a, 1986b; Mutinga and Ngoka 1978a, 1981, 1983; Mutinga, Ngoka and Odhiambo, 1984; Mutinga and Odhiambo, 1982, 1986a, 1986b; Mutinga and Kamau, 1986; Mutinga, Kamau and Kyai, 1986; Basimike, 1988). They remain in various shelters during the day and are active at dusk, the whole night and also at dawn. Their movements vary from short hops to flights of a few hundred metres (Heisch, 1954; Heisch et al., 1956; Mutinga and Ngoka, 1978a; Basimike, 1988). Adults of both sexes feed on sugars, but females must take one or more vertebrate blood meals to be able to oviposit viable eggs (Lainson and Shaw, 1978; Mutinga and Odhiambo, 1982; Beach, Young and Mutinga, 1982; Mutinga, Kamau and Mwandandu, 1987).

Of the 40 Phlebotomine species identified in Kenya to date, the following have been incriminated as vectors of Leishmaniasis in man: Phlebotomus duboscqi Neveux-Lemaire (Beach, Young and Mutinga, 1982; Mutinga and Kaddu 1982; Mutinga, Kamau and Kyai, 1986; Mutinga, Kyai and Omogo, 1986; Mutinga, Kamau and Mwandandu, 1987), Phlebotomus martini Parrot (Heisch, Guggisberg and Teedale, 1956; Minter, 1962, 1963, 1964, 1964a; Minter et al., 1962; Mutinga, 1971; Mutinga and

Ngoka, 1978a, 1981; Mutinga, Ngoka and Odhiambo, 1984; Mutinga and Kaddu, 1983; Mutinga and Kamau, 1986), Phlebotomus vansomeranae Heish, Guggisberg and Teedale (Heisch, Guggisberg and Teedale, 1956; Mutinga, 1975a), Phlebotomus celiae Minter (Minter, 1962, 1963; Wijers and Minter, 1962; Wijers, 1963; Ayele and Mutinga, 1989), Phlebotomus pedifer Lewis, Mutinga and Ashford (Mutinga, 1971, 1975a; Lewis, Mutinga Ashford, 1972; Kaddu and Mutinga, 1981; Mutinga and Odhiambo, 1986a, 1986b), Sergentomyia garnhami Heisch, Guggisberg and Teedale (Mutinga and Odhiambo, 1982; Mutinga and Kyai, 1985) and Sergentomyia ingrami Newstead (Mutinga and Kamau, 1986; Mutinga, Kyai, Kamau and Omogo, 1986b).

The disease called Leishmaniasis is caused by species of a blood parasitic protozoon discovered in 1903 by Leishman and Donovan (Lewis, 1976; Garnham, 1977). The parasitic protozoon belongs to the Genus Leishmania Ross, Family Trypanomastidae Doflein (emend. Grobben) and Order Kinetoplastida Honnigberg (emend. Vickerman).

The parasite exists in two different forms: as flagellates known as promastigotes in the sandflies and in artificial media and, as round forms known as amastigotes in human and other

mammalian hosts. In sandflies, Leishmania parasites are mainly found in the form of promastigotes.

In general the body of promastigotes measures about 10 - 20 X 1.5 - 3.0 um, with the flagellum often longer than the body, and the nucleus lying in the centre of the body (Molyneux and Ashford, 1983). Molyneux and Ashford (1983) have pointed out that, in some species of Leishmania, two forms of promastigotes are found in the midgut of the sandfly: these are the nectomonad promastigotes, which are elongated, and the haptomonad promastigotes, which are short and fat.

Amastigotes of various species of Leishmania in mammalian hosts are frequently reported to be morphologically identical (Gardener, 1977). Amastigotes appear as round or oval bodies (1.0 - 1.2 um in diameter) within the cell or lying free from a ruptured cell. Their nuclei occupy a central position and the kinetoplast, which may be round, rod shaped, oval or curved, lies adjacent to it (Molyneux and Ashford, 1983).

Leishmaniasis in man is considered to be the second most important communicable disease after malaria in the tropics and subtropics (Lainson and Shaw, 1978; Lee, Hunter and Bovee, 1985). According to Molyneux and Ashford (1983), six

species of Leishmania parasites cause the disease in man. They are morphologically similar but their isoenzyme profiles and clinical symptoms they produce, are different. Three variations of the disease are known:

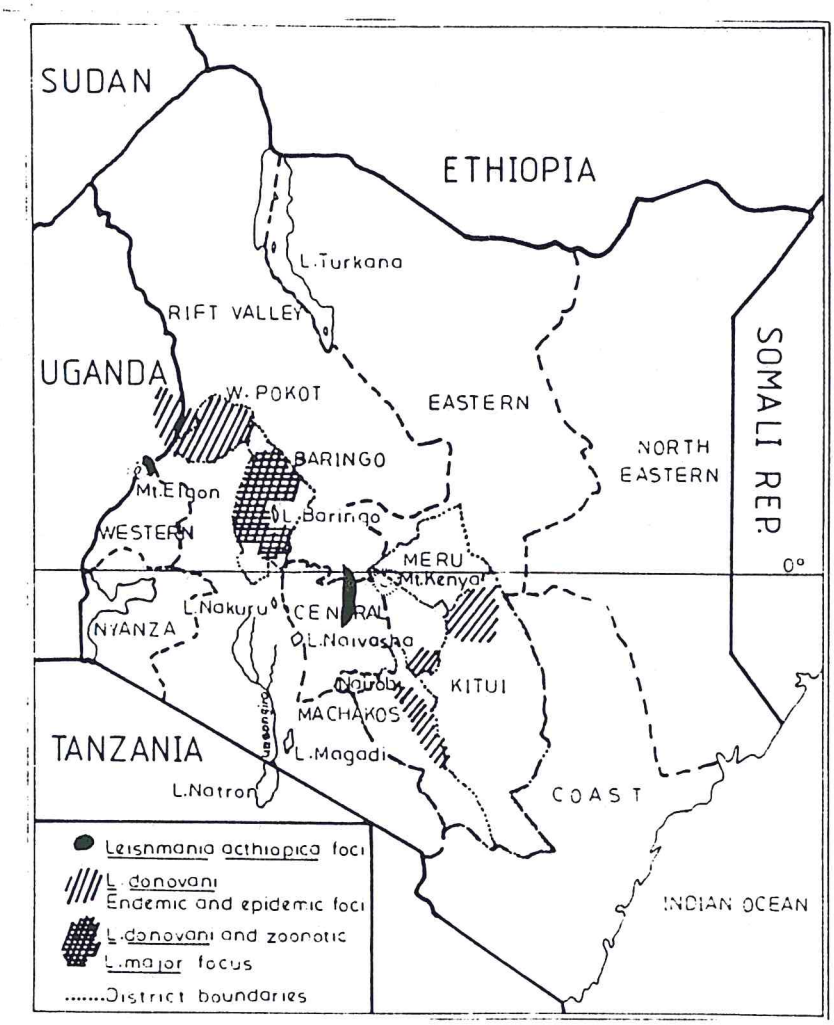
(i) Visceral leishmaniasis or Kala-azar is a deadly disease if left untreated. Its main symptoms are fever, splenomegaly, anemia, cachexia and eventually death. The disease is caused by L. donovani, transmitted by the sandfly species; Phlebotomus papatasi and P. martini. Visceral leishmaniasis is found in the Mediterranean region, Middle-East, Asiatic part of the Soviet-Union, India, South of China, Africa (mainly in Sudan and East-Africa) and South America.

(ii) Cutaneous leishmaniasis is characterized by the presence of skin lesions, which are small initially but often gradually increase in diameter until they involve large areas of the skin (ear region, legs, etc). L. tropica, L. aethiopica and L. major are, so far, known to be the causative agents of the various forms of cutaneous leishmaniasis. The disease occurs in the Middle East, India, Africa and the Mediterranean region.

(iii) Mucocutaneous leishmaniasis or "la Lepra blanca" is characterized by lesions of the mucocutaneous parts of the mouth, nose, the anus, and to some extent the external genitalia. These lesions can be quite disfiguring and lethal. Phlebotomine sandfly species belonging to the genus Lutzomyia are involved in the transmission of the Leishmania parasites in the New World e.g. Leishmania braziliensis braziliensis Vianna. This disease is found in Central and South America.

In the Afro-tropical Region, visceral leishmaniasis is caused by Leishmania donovani Laveran and Mensil, while cutaneous leishmaniasis is caused by Leishmania aethiopica Bray, Ashford and Bray, Leishmania major Yakimoff and Schokhor and Leishmania tropica Wright. Visceral leishmaniasis and cutaneous leishmaniasis are found in Kenya in Kitui, Baringo, West-Pokot, Meru and Machakos districts (Figure 1).

Visceral leishmaniasis outbreak was first reported in Kenya in the army barracks from Machakos district during the World War II (Anderson, 1943; Fendhall, 1952b; Mutinga, 1980). Since then, cases of visceral leishmaniasis have been reported from Kitui by Fendall (1952b),


SUDAN
ETHIOPIA
L.Turkana
RIFT VALLEY
UGANDA
W. POKOT
EASTERN
SOMALI REP.
NORTH EASTERN
BARINGO
Mt.Elgon
L.Baringo
WESTERN
MERU
Mt.Kenya
0°
L.Nakuru
CENTRAL
NYANZA
L.Naivasha
Nairobi
KITUI
MACHAKOS
TANZANIA
L.Magadi
L.Natron
COAST
INDIAN OCEAN
Leishmania aethiopica foci
L. donovani
Endemic and epidemic foci
L.donovani and zoonotic
L.major focus
.....District boundaries

Heisch (1954, 1955, 1963), Mckinnon and Fendall (1956).

Cases of visceral leishmaniasis from Baringo and West Pokot have been reported by Heisch (1954), Mckinnon and Fendall (1956) and Mutinga and Ngoka (1983). An outbreak of visceral leishmania in parts of Meru district was reported by Wijers and Minter (1966). In 1978, Mutinga and Ngoka also reported an epidemic of visceral leishmaniasis in Machakos. Many authors including Heisch and Guggisberg (1952), Heisch, Guggisberg and Teedale (1956), Minter (1962; 1963), Minter and Wijers (1962), Minter et al. (1962) have incriminated P. martini, P. vansomeranae and P. celiae as vectors of visceral leishmaniasis in Kenya. In addition, Mutinga and Odhiambo (1982); and Mutinga and Kyai (1985) have also reported S. garnhami as a possible vector of visceral leishmaniasis in Kenya.

Cases of cutaneous leishmaniasis caused by L. aethiopica were described by Mutinga and Ngoka (1970), Kungu, Mutinga and Ngoka (1972). Leishmania-like parasites which cause a mild form of cutaneous leishmaniasis in man were isolated from rodents by Heisch in 1963 and were later confirmed to be L. major by Chance, Schnur, Thomas and Peters (1978), Mutinga and Ngoka (1983) and Githure et al. (1984).

According to several groups of workers (Mutinga 1971; Chance, Schnur, Thomas and Peters 1978; Beach, Young and Mutinga 1982; and Beach, Kiilu, Hendricks and Leeuwenberg 1984) L. aethiopica and L. major, which cause cutaneous leishmaniasis, can be transmitted by P. pedifer and P. duboscqi. Recently, P. duboscqi was incriminated as a possible vector of the zoonotic L. major, Mutinga and Kaddu (1981); Mutinga and Ngoka (1983); Beach, Kiilu, Hendricks and Leeuwenberg (1984); and Mutinga, Kyai and Omogo (1986c). Furthermore, Mutinga et al. (1986) showed that S. ingrami could also be a possible vector of L. major parasites through the isolation of the disease causing parasites from this species.

Recently, L. major parasites were also discovered in dogs, goats, and sheep in Machakos and in the Rift Valley (Mutinga, Ngoka, Schnur and Chance, 1980; Mutinga and Odhiambo, 1982; Mutinga and Kyai, 1985; Mutinga, Kihara, Ngindu, Loghing and Mutero, 1988).

Other diseases:

The next important disease transmitted by sandflies to man is Bartonellosis or Corrion's

disease, which is found in South-America. This disease is caused by a microorganism known as Bartonella bacilliformis Strong, Tyzzer and Sellards. It is a small, motile, aerobic, gram-negative Bacillus, that was observed by Barton in 1905 (Byrne, 1976; Freeman, 1979). Bartonellosis occurs in two forms:

(a) The systemic form which is severe and often fatal. Febrile anemia may result due to the direct action of the microorganisms on the erythrocytes. The resulting haemolysis and tissue hemorrhages can lead to a high number of red cell deterioration (Freeman, 1979).

(b) The histoid cutaneous or miliary form which is commonly found on the face and extremities, appearing as a macule that becomes nodular and eventually disappearing without leaving any scar.

Freeman (1979) has reported that Bartonella bacilliformis infection can become complicated with Salmonella to produce high mortality.

The identification of Phlebotomine sandflies in Kenya has been based to date on morphological characteristics. Separation of genera is based on the disposition of hairs on the abdominal tergites, the disposition of the buccal armature, the presence or absence of a pigmented area, and the shape of the spermatheca and male terminalia

(Kirk and Lewis, 1951). However, using these characteristics, Heisch, Guggisberg and Teedale (1956); Minter (1962; 1963) and Rogo (1985) were unable to convincingly separate females of three phlebotomine species: P. martini, P. vansomeranaeΩ and P. ciliae, which belong to the Martini species complex. Also according to Lewis, Minter and Ashford (1974) males of P. pedifer and P. elgonensis are also morphologicaly indistinguishable. This two species also belong to a species complex. Separation of these sibling species and some times of subspecies such as the group of S. africanus, S. antennatus and S. bedfordi by use of morphological dissimilarities remains unresolved or ist very difficult to date.

In this regard, it is important to note that morphological taxonomy is of very little use in distinguishing sibling species. Other techniques (such as cytotaxonomy), used to separate sibling species, are very often restricted to only larval stages or to only adults in a precise physiological state or age. There is therefore an urgent need for the development of new methods for sibling species separation which are not so restrictive in their requirements.

Objectives of the present study.

The present studies were undertaken with the following objectives:

a) To differentiate phlebotomine sandfly species by isoenzyme analysis based on thin layer starch gel electrophoresis and isoelectric focusing

b) To develop histograms of specific enzyme systems which can be used to identify different species;

c) To analyse the cuticular components of these species with the aim of identifying specific taxonomical patterns for each sandfly species;

d) To compare the reliability of the above mentioned techniques (isoenzyme and pattern cuticular components analysis) with morphological taxonomy; and

e) to evaluate the usefulness of the isoenzyme and cuticular components analysis techniques for routine taxonomic work.

It is hoped that isoenzyme and cuticular components analysis techniques can be used to accurately understand the ecogeographical distribution of medically important phlebotomine

sandfly species, particularly sibling species of complexes in Kenya. This is expected to lead to a better understanding of the distribution and dynamics of the leishmaniasis disease complex in Kenya as well as providing the ground work for comparable studies elsewhere.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Morphology of Phlebotomine sandfly species

Kirk and Lewis (1951) have described important morphological parameters used in the taxonomy of the genus Phlebotomus. These parameters were refined successively by Heisch, Guggisberg and Teedale (1956), Quate (1964), Theodor (1958), Abonnenc (1972), Lewis (1973), Lewis, Young, Fairchild and Minter (1977), Lewis, Beach, Young and Mutinga (1982), and Lewis (1982), among others.

The features currently in use for taxonomic purposes include the dimension, pigmentation and shape of the mouth parts, antennae, palps, wings and the genitalia of the adult flies. The main morphological characters of these organs are summarized below:

Mouth parts

Taxonomically useful features of the mouth parts include:

a) the number of teeth found in the inner ventral surface of the buccal cavity and the presence or absence of preteeth. When present,

the preteeth are pointed upward,and they look like nodules when viewed from below;

b) the pattern of pigmentation of the thick cuticle on the dorsal wall of the cibarium opposite the teeth;

c) the shape of the pharynx;

d) the appearance of the posterior teeth; and

e) the length of the labrum from the tip of the clypeus seen in ventral view to the tips of the labral papillae.

Antennae

These consist of sixteen segments. The lengths of segments 3, 4 and 5 are usually measured. The relative lengths of segments 3 and 5 are often expressed as ratios of the length of the labrum. Segments 3 to 15 bear one or two sensory ascoids each.

Palps

Each palp comprises five segments, the first of which is sometimes regarded as the palpifer. The first two are difficult to measure accurately, however, these are the least important. Relative lengths may be expressed in relation to the first one which is given a value of 10. Some segments slightly overlap with each other so that the sum of segment lengths exceeds that of the palp. Segment 3 and sometimes others have small sensillae known as the Newstead sensilla or bulb organ.

Wings

Wing lengths are usually measured from the proximal end of the hairy basal costal node. The length of the vein $R_2$ is divided by that of the tip of $R_{2+3}$ to give a figure which, though variable, is often useful. $R_1$ apex indicates either the part of $R_1$ distal to the tips of $R_{2+3}$ or, when shown with a minus sign, the distance by which $R_1$ ends proximal to the tip of $R_{2+3}$. Upper and lower pleural setae are present in some groups. On the hind margins of the abdominal tergites 2 to 6, the presence or absence of erect hairs (usually lost during mounting but indicated by conspicuous round sockets) is an important generic character in the Old world.

The genitalia

In the female, the furca or genital fork may be used to locate the outlet of the spermathecal ducts which indicate the position of the two spermathecae. These take the form of tubes or of smooth, wrinkled or annulated capsules. At the tips (the distal end of the ducts) of each spermatheca are hair-like ductules leading from glands which vary in size, shape and texture in the different species. The accessory glands are relatively large but are of no taxonomic value.

On the abdomen of the male, segment 7 is partly and segment 8 wholly rotated dextrally

through 180 °C as reported by Davies (1967). Segment 9 is not clearly discernible. Behind segment 8 are the aedeagus and parameres (fused together),the claspers (comprising coxites and styles) and the surstyles. The inconspicuous segment 10 bears the cerci.

Between the two halves of the aedeagus lie the tips of the genital filaments, which lead from the genital pump. For taxonomic purposes, the relative length of a filament is expressed by dividing it by the length of the pump.

The coxite bears sensory hairs, which may be concentrated on a setiferous lobe. The style varies in shape and often bears five sensory spines, one or more of which may be reduced or absent. Lastly, the surstyles which are also of taxonomic importance are lateral lobes of tergum 10.

In addition to adult morphological features, the surface sculpture of the eggs has been used to differentiate between sandfly species (Rogo, 1985).

## 2.2. Phlebotomine sandflies in Kenya

The first positive identification of Phlebotomine sandflies from Kenya was by J.A.Sinton in India of flies sent to him by

G.B.Dyme in 1930 and 1932 (Heisch and Guggisberg, 1952).

According to Heisch and Guggisberg (1952), the importance of Phlebotomine sandflies in Kenya was not generally recognised until two decades after they were first reported by Mansteufel in 1912. Following the outbreak of a serious epidemic of Kala-azar in Kitui district towards the end of 1952, and the discovery of the disease in Baringo district in 1954, more importance was given to the study of the vectorial capacities of Phlebotomine sandflies. Heisch and Guggisberg (1956) were the first to list twenty local Phlebotomine sandfly species, which included the collections made by P.C.C.Garnham, R.B.Heisch and V.D. van Someren from 1930 to 1945. Heisch et al., (1956) and Minter (1962; 1963) described a total of nine new species in Kitui district. In 1964, Minter reported the distribution of 34 species of Phlebotomine sandflies in Kenya. Also since 1965, many workers including Lewis, Mutinga and Ashford (1972), Ngoka,Madel and Mutinga (1975), Beach, Young and Mutinga (1982), Mutinga, Kaddu, Olobo, Kyai and Omogo (1983), Rogo (1985) have described new Phlebotomine sandfly species in Kenya. There are 40 Phlebotomine sandfly species known to date in Kenya (Table 1).

Table 1: Sandfly species found in Kenya

| SANDFLY GENUS | SPECIES | FIRST RECORDED IN | REFERENCE |
|---|---|---|---|
| Phlebotomus | celiae Minter 1963 | Kitui | Minter; 1962 |
| | martini Parrot 1936 | Kitui | Heisch et al.; 1956 |
| | vansomeranae Heisch, Guggisberg & Teedale 1956 | Kitui | Heisch et al.; 1956 |
| | duboscqi Neveu-Lemaire 1906 | Baringo | Mutinga and Ngoka; 1983 |
| | pedifer Lewis, Mutinga & Ashford 1972 | Mount Elgon | Lewis et al.; 1972 |
| | orientalis Parrot 1936 | Sericho | Heisch & Guggisberg; 1952 |
| | guggisbergi Kirk & Lewis 1952 | Kiambu | Kirk & Lewis; 1952 |

Table 1: continued

| SANDFLY GENUS | SPECIES | FIRST RECORDED IN | REFERENCE |
|---|---|---|---|
| | aculeatus Lewis, Minter & Ashford 1974 | Naivasha | Lewis et al.; 1974 |
| | rhodaini Parrot 1930 | Kitui | Heisch & Guggisberg: 1952 |
| | saevus Parrot & Martin 1939 | Kerio vallye | Heisch & Guggisberg; 1952 |
| | elgonensis Ngoka, Madel & Mutinga | Kerio vallye | Ngoka et al.; 1975 |
| Sergentomyia | antennatus Newstead 1912 | Mombassa | Kirk & Lewis; 1952 |
| | bedfordi Newstead 1912 | Mombassa | Kirk & Lewis; 9152 |
| | schwetzi Adler, Theodor & Parrot 1929 | Mombassa | Sinton; 1930 |

Table 1: continued

| SANDFLY GENUS | SPECIES | FIRST RECORDED IN | REFERENCE |
|---|---|---|---|
| | alderi Theodor 1933 | Kerio valley | Heisch & Guggisberg 1952 |
| | affinis Theodor 1933 | Kerio valley | Heisch & Guggisberg 1952 |
| | clydei Sinton 1928 | Sericho | Kirk & Lewis 1952 |
| | graingeri Heisch, Guggisberg & Teedale 1956 | Kitui | Heisch et al.; 1956 |
| | meilloni Sinton 1932 | Kwale | Heisch et al.; 1956 |
| | decipiens Theodor 1931 | Western Kenya | Minter; 1964 |
| | dureni Parrot 1934 | Kitui | Heisch & Guggisberg; 1952 |
| | harveyi Heisch, Guggisberg & Teedale 1956 | Kitui | Heisch et al.; 1956 |

Table 1: continued

| SANDFLY GENUS | SPECIES | FIRST RECORDED IN | REFERENCE |
|---|---|---|---|
| | ingrami Newstead 1914 | Kerio Valley | Heisch & Guggisberg 1953 |
| | kirki Parrot 1918 | Kitui | Kirk & Lewis; 1952 |
| | heischi Kirk & Lewis 1950 | Machakos/Wajir | Kirk & Lewis; 1950 |
| | africanus Newstead 1912 | Western Kenya | Minter; 1964 |
| | squamipleuris Newstead 1912 | Sericho | Kirk & Lewis; 1952 |
| | serratus Parrot & Malbrand 1945 | Rift valley | Heisch and Guggisberg; 1953 |
| | teesdalei Minter 1963 | Kitui | Minter; 1964 |
| | kitonyii Minter 1963 | Kitui | Minter; 1964 |
| | wynnae Watson 1951 | South East | Mutinga et al. (1986) |
| | christophersi Sinton 1927 | Kitui | Minter; 1964 |

Table 1: continued

| SANDFLY GENUS | SPECIES | FIRST RECORDED IN | REFERENCE |
|---|---|---|---|
| | yusafi Sinton 1930 | Mombassa | Sinton; 1930 |
| | gracillis Kirk & Lewis 1952 | Kwale | Kirk & Lewis; 1952 |
| | blossi Kirk & Lewis 1952 | Kwale | Kirk & Lewis; 1952 |
| | garnhami Heisch Guggisberg & Teedale 1956 | Kitui | Heisch et al.; 1956 |
| | suberectus Sinton 1932 | Mombassa | Sinton; 1932 |
| | multidens Heisch, Guggisberg & Teedale 1956 | Kitui | Heisch et al.; 1956 |
| | rosanae Heisch, Guggisberg & Teedale 1956 | Kitui | Heish et al.; 1956 |
| | adami Abonnenc 1960 | Machakos | Rogo; 1985 |

## 2.3. Vectors of Leishmaniases in Kenya

To date, only 7 out of the 40 known sandfly species in Kenya have been incriminated as vectors of leishmaniasis (Table 2).

### 2.3.1. Kala azar or visceral leishmaniasis

Heisch et al. (1956) found P. martini biting man in Marigat in the Rift Valley. These authors were the first to report the occurence of P. vansomeranae, a species similar to P. martini, in Kenya. Minter (1962) working in the Kitui Kala-azar area found P. celiae, a third new anthropophilic species which is morphologically indistinguishable from P. martini and P. vansomeranae. Minter (1963, 1964, 1964a) pointed out the importance of P. martini, P. vansomeranae, and P. celiae, all members of the Synphlebotomus complex (or P. martini group) in transmitting Kala-azar in Kenya. More recently P. martini has been incriminated as the major vector of Kala-azar in Kenya (Mutinga and Ngoka, 1978). In addition to the Synphlebotomus complex, S. garnhami, which normally feeds on lizards and transmits Leishmania adleri Heisch in these animals, has been incriminated as a possible vector of human Kala-

Table 2: Sandfly species found to be carrying Leishmania parasites and Leishmania-like organisms in Kenya.

| Genus | Species | Parasites | References |
|---|---|---|---|
| Phlebotomus | duboscqi | L. major | Mutinga (1978), Mutinga et al. (1982), Beach et al. (1984) |
| | martini | L. donovani<br>L. major | Heisch et al. (1956), Wijers and Minter (1962), Mutinga and Ngoka (1981), Mutinga and Odhiambo (1982) |
| | pedifer | L. aethiopica | Mutinga (1971), Peters et al. (1977), Cance et al. (1978) Mutinga and Odhiambo (1986a, b) |
| Sergentomiya | antennatus | L. species | Mutinga and Ngoka (1978) Mutinga and Odhiambo (1982) |
| | bedfordi | L. adleri<br>L. species | Heisch et al. (1956), Mutinga and Ngoka (1978), Mutinga and Odhiambo (1982) |
| | garnhami | L. adleri<br>L. species | Heisch et al. (1956) Mutinga and Odhiambo (1982) |

Table 2: continued

| Genus | Species | Parasites | References |
|---|---|---|---|
| | ingrami | L. adleri<br>L. species | Heisch et al. (1956)<br>Mutinga et al. (1983) |
| | clydei | L. adleri | Heisch et al. (1956), Mutinga et al. (1982) |
| | africanus | L. species | Mutinga and odhiambo (1982) |
| | squamipleuris | L. adleri | Heisch et al. (1956) |

Note: P. celiae and P. vansomeranae females are undistinguishable morphologically from P. martini females

azar in Machakos (Mutinga and Odhiambo, 1982; Mutinga and Kayi, 1985). The evidence is based on the ability of experimentally infected S. garnhami to transmit L. donovani to mice (Kaddu and Mutinga, 1983) as well as on field observations of the flies which have been found to bite man. Isolation of L. donovani parasites from P. martini has been reported by Heisch et al. (1956), Minter (1962, 1963, 1964), Minter et al. (1962), Wijers and Minter (1962), among others. In 1977, Schnur and Zuckerman, followed by Chance et al. (1978), using new techniques, confirmed that parasites, isolated from P. martini were the causative agents of Kala-azar. Furthermore, Mutinga and Ngoka (1976), and Mutinga and Odhiambo (1982) isolated Leishmania parasites from Phlebotomine sandflies, including P. martini during an epidemic outbreak, thus comfirming the vectorial importance of this species in the transmission of Kala-azar. Heisch et al. (1956) isolated L. alderi and other uncharacterised Leishmania spp. from Phlebotomine sandflies including S. garnhami. This has been confirmed by other workers (Minter, 1962; Mutinga and Odhiambo, 1982). Experimentally-infected S. garnhami were able to transmit L. donovani to mice (Mutinga et al., 1982; Kaddu and Mutinga, 1984). Recently Mutinga and Kyai (1985) incriminated S. garnhami as a possible vector of Kala-azar.

### 2.3.2. Cutaneous leishmaniasis

P. pedifer the vector of L. aethiopica was first reported from Mount-Elgon by Mutinga (1971) and later confirmed by Lewis et al. (1972). Peters, Chance, Mutinga, Ngoka and Schnur (1977) were able to confirm the parasite isolated from P. pedifer, as L. aethiopica. In 1978 Mutinga and Ngoka reported the existence of P. duboscqi in Baringo. This was later confirmed by Beach, Young and Mutinga (1982) in the Rift Valley province. P. duboscqi was later shown to be a vector of another form of cutaneous leishmaniasis normally associated with rodents and caused by L. major. In 1983, Mutinga, Kaddu and Kyai (1983) incriminated P. duboscqi as the vector of zoonotic Leishmania in Marigat. Beach et al. (1984) used isoenzyme characterization techniques to confirm this. Also, Mutinga et al. (1986) found S. ingrami, and to some extent, P. martini to be vectors of L. major.

## 2.4 The Use of Biochemical Techniques in the Taxonomic Analysis of Insects.

### 2.4.1. Isoenzyme Analysis

When an electric field is applied to a protein mixture in solution it results in different proteins migrating at different rates towards one of the electrodes. A modification of this procedure called zone electrophoresis of proteins is performed in a solution stabilized within a supporting medium, which allows the investigator to fix the separated proteins at the final positions immediately after electrophoresis. This avoids the loss of resolution which results from post-electrophoretic diffusion. The supporting media currently in use are: cellulose, cellulose acetate, silica gel, allumina, agarose, starch or polyacrylamide gels.

The successful use of electrophoresis to analyze isoenzyme patterns for the identification of mosquitoes, blackflies and sandfly species has been reported by many workers including Ayala and Jeffrey (1972), Bullini and Colluzi (1973), Mahon, Green and Hunt (1976), Saul, Grimstad and Graig (1977a), Saul, Sinsko, Grimstad and Graig (1977b), Townson, Meredith and Thomas (1978), Miles and Ward (1978), Ward, Pasteur and Rioux (1981), Petersen (1982), Agustuma, Uemoto and Onofre Ochoa (1986), Rogo (1985) and Rogo, Khamala and Mutinga (1988).

Isoenzyme studies on Phlebotomine sandflies were first reported by Miles and Ward (1978).

They used larvae and adults of two different populations of Lutzomiya flavisculata Manya Beira. Ten enzymes; aldolase dehydrogenase (ADH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G-6PD), malic enzyme (ME), asparate amino transferase (ASAT), alanine aminotransferase (ALAT), esterase (ES), phosphoglucomutase (PGM) and glucose phosphate isomerase(GPI) were tested using thin layer starch gel electrophoresis. Of these, only five namely MDH, GPI, ME, ES, PGM, were detected. The authors concluded that MDH, GPI and PGM were suitable for population genetic studies.

In 1981 Ward, Pasteur and Rioux conducted electrophoretic studies on the genetic polymorphism and on the differentiation of Phlebotomine sandflies from southern France and Tunisia. From a panel of 21 enzymes, they found that the most useful diagnostic enzymes were hexokinase (HK), MDH, PGM, PGI(GPI), glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT). They also demonstrated clear enzymatic differences between Phlebotomus perniciosus Newstead and Phlebotomus perfiliewi Parrot. They concluded that biochemical methods may have a useful role to play in both the analysis of population structure and differentiation of the Phlebotominae.

Petersen (1982) used cellulose acetate electrophoresis to identify sandfly species in Panama. Out of 20 enzymes assayed, he found that 6, namely PGM, PGI(GPI), ME, MDH, inositol dehydrogenase (IDH) and HK were detectable in single sandflies. This allowed biochemical identification of the most common anthropophylic species collected from the field. Rogo (1985) and Rogo et al. (1988) also used thin layer starch gel electrophoresis and isoelectric focusing to study 20 enzymes. She could demonstrate only that GPI showed detectable banding patterns, which could distinguish between P. pedifer Lewis, Mutinga and Ashford, and P. elgonensis Ngoka, Madel and Mutinga.

The practical potential of isoenzyme electrophoresis for the identification of sibling species is illustrated by examples reported below and taken from work in mosquitoes and blackflies. Thus isoenzymes of PGM have been used to separate members of the Simulium damnosum complex Theobald (Meredith, 1982). Similarly, adult Simulium pernobescontensis, Simulium nyssa, and Simulium jennegsi, which are indistinguishable morphologicaly, were separated by isoenzyme analysis (May, Bauer, Vadas and Granett, 1977). Blackflies of the S. damnosum complex, consists of 6 morphologically indistinguishable species.

They are the only known vectors of human onchocerciasis (River blindness) in West-Africa. Until recently the only method used to distinguish these species was by analysis of their polytene chromosome banding patterns prepared from larval salivary glands. A number of authors including May et al. (1977) and Meredith (1982), have shown that PGM and trehalase banding patterns could be used to distinguish Simulium yahense Vajime and Dunbar and Simulium squamosum Enderlein from a collection of S. damnosum s. l.. Agatsuma, Uemoto and Onofre Ochoa (1986) could distinguish isoenzyme variations between the Guatemalan blackflies, Simulium ochraceum, Simulium metallicum and Simulium horacioi using starch gel electrophoresis. Out of the seven enzymes (acid phosphatase (AP), GPI, HK, PGM, leucine amino peptidase (LAP), GPT and GOT) examined, they found that AP, GPT and LAP showed differences in the electrophoretic patterns for each species. Furthermore, GPI and PGM were both highly polymorphic. Using Nei's genetic distance (D) and allelic distance ($A_d$), Agustuma et al. (1986) found that S. horacioi and S. metallicum are not: closely related.

A review of the literature on the use of isoenzymes to distinguish species of mosquitoes shows a wide application of polyacrylamide and

thin layer starch gel electrophoresis before 1973 (Bullini and Coluzzi 1973). Cellulose acetate electrophoresis was tried first by Kreutzer, Posey and Brown (1977) to study mosquitoes spp.

Townson et al. (1977) reported practically no polymorphism in their studies of enzymes in the Aedes sentillaris Walker group from laboratory colonies. Nevertheless, they concluded that there is already evidence of considerable biochemical divergence in this morphologically rather uniform group.

In 1980 Kreutzer and Galindo compared: isoenzyme profiles of mosquitoes from laboratory colonies of Culex ocossa Dyar and Culex panocossa Dyar and Knab. Of 18 enzymes, they found that acid phosphatase, glycerol-3-phosphate dehydrogenase (Gly-3-PDH), PGI(GPI) and XDH were useful for analysis. An interesting new application was the analysis of one leg of an etherised mosquito Haemogogus equinus Theobald, the vector of the Sylvan yellow fever, using PGM, PyGI and ME (Petersen, 1982).

### 2.4.2. Cuticular Components Analysis

The technique is based on extracting a cuticular wax mixture followed by quantitative and

qualitative analysis of the wax components. Ecclinton and Hamilton (1963) used this technique to compare insect and plant waxes (Blomquist and Jackson, 1979).

Cuticular lipids of insects play a major part in the regulation of water loss. They also protect them from insecticides and micro-organisms (Blomquist and Jackson, 1979). The value of cuticular components in chemotaxonomic differentiation of insects and plants has been recognised for a decade (Blomquist and Jackson, 1979; Jackson, 1981). The insect cuticular wax consists of a large proportion of hydrocarbons with varying amounts of alcohols, aldehydes, fatty acids, esters, and triglycerides. Some of the hydrocarbon components have been shown to act as pheromones in some species (Carlson, Langley and Huyton, 1978 and Jallon 1985). Analysis of the cuticular waxes is usually carried out by gas chromatography. The technique is potentially useful for field workers because most cuticular components are chemically stable. Thus, one can utilize live adults from trap collections, dead adults, and even, old pinned specimens.

One of the major uses of cuticular component pattern analyses has been in the differentiation of individuals of morphologically similar insect species. Two major approaches have been used:

(1) where there are qualitative differences in the cuticular component patterns, species differentiation is made by visual examination; (2) where only quantitative difference are seen, then multivariate analysis is used to differentiate the individual insects.

Some of the early demonstrations of the value of cuticular component pattern analyses were reported by Lok, Cupp and Blomquist (1975) and Lockey (1978, 1982).

Lok et al. (1975) studied the cuticular lipids of imported fire ants, Solenopsis invicta and Solenopsis richteri. They found that hydrocarbons comprise 65 to 75% of the cuticular lipids from adults and that the two species had distinguishable hydrocarbon patterns. Significant differences were also found between the hydrocarbon compositions of larvae, pharate pupae, and pupae. Lockey (1978) was able to separate two closely related tenebrinoid beetle species by analyzing their cuticular hydrocarbons.

Surface lipids of grasshoppers Melanoplus bivittatus, Melanoplus femurrubrum and Melanoplus dawsoni have also been analysed (Jackson, 1981). These were found to be similar to one another, and to species other than Melanoplus. The major components of the cuticular lipids of the Melanoplus species were found to be secondary

alcohols, waxes, and esters. It was concluded that the grasshoppers had chromatograms that were similar qualitatively, but which differ quantitatively.

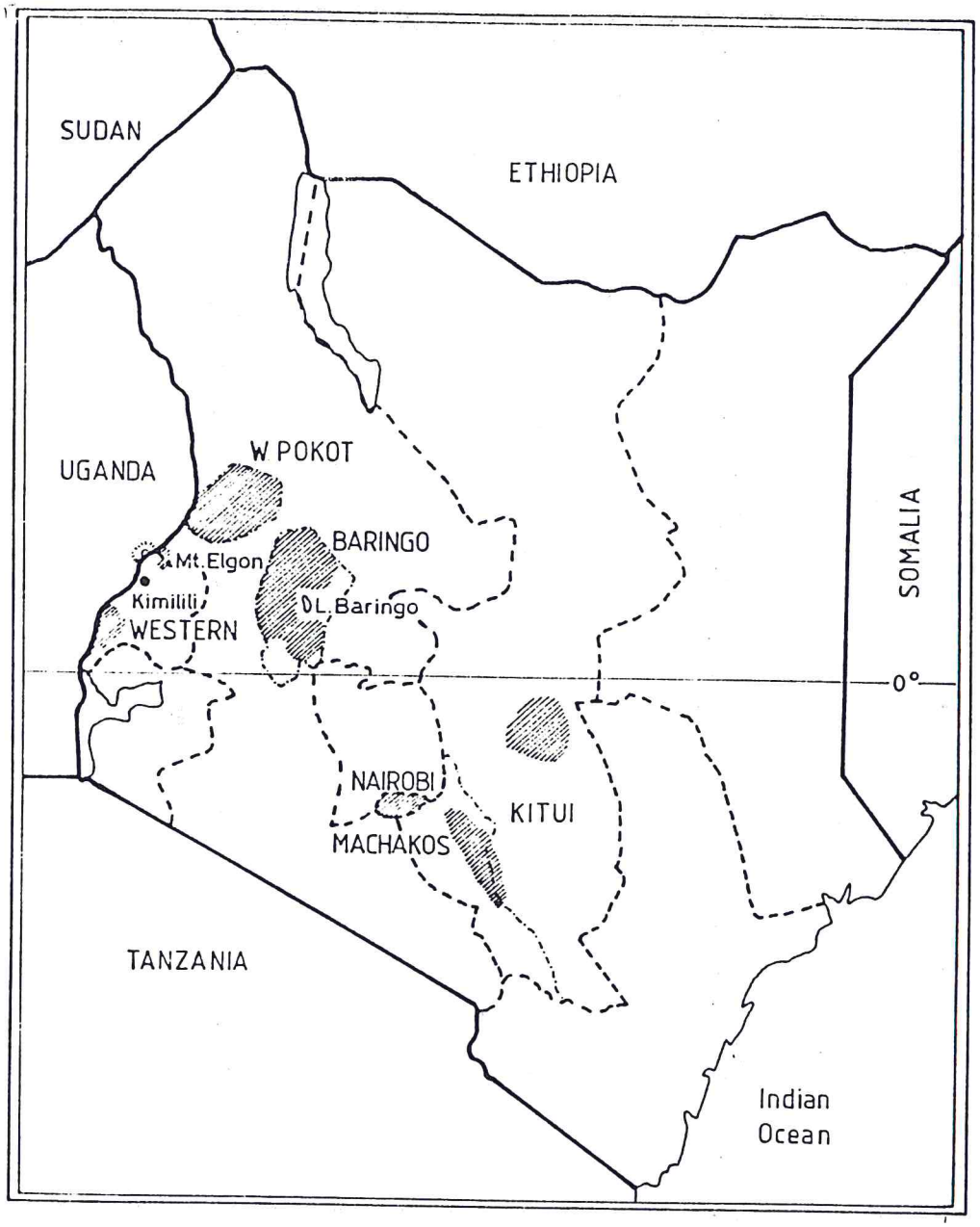
Nelson, Dillwith and Blomquist (1981) identified cuticular hydrocarbons which serve as components of sex pheromones of the housefly Musca domestica, by using gas chromatography-mass spectrometry (GC-MS). They also found that the male housefly has a much simpler hydrocarbon composition as well as a smaller amount of hydrocarbon than the female.

Differentiation between members of the Anopheles gambiae:s. l. Giles complex has been based on the banding pattern of the polytene chromosomes of either the 4th instar larval salivary glands or the adult ovarian nurse cells of half gravid females (Colluzzi and Sabatini, 1967; Colluzzi, 1968). The methods of fluorochrome bisbenzimid staining of meiotic karyotypes assayed by Galti, Santini, Pimpelli and Colluzzi (1977) as well as isoenzyme analysis as assayed by Miles and Ward (1978) are of limited applicability. Carlson and Service (1979) investigated the possibility of identifying adults of both sexes of A. gambiae s. s. Giles and A. arabiensis Patton by extracting and analyzing cuticular hydrocarbons. They suggested a more

detailed appraisal of the non-volatile and chemically inert cuticular hydrocarbons for the separation of A. gambiae and A. arabiensis and other species within the gambiae complex. Further study showed that identification of laboratory-reared adult male or female individuals of either species of the gambiae complex was possible by the analysis of cuticular components using gas chromatography (Carlson and Service, 1980).

West-African female blackflies (Diptera Simuliidae) of the Simulium damnosum species complex (S. sirbanum and S. squamosum) are important vectors of onchocerciasis (River Blindness). These species have been separated by the analysis of their cuticular hydrocarbon components using gas chromatography and comparing five sets of consistently appearing peaks (Carlson and Walsh, 1981). Phillips, Walsh, Garms, Molyneux, Milligan and Ibrahim (1985) used advanced gas liquid chromatography and multivariate statistical analysis to show that both adult male and female flies of S. damnosum, S. sirbanum, S. yahense and S. sanctipauli could also be separated by quantitative analysis of the cuticular hydrocarbon peaks.

Individual females of Psychodopygus wellcomei Fraiha, Shaw and Lainson, are the


SUDAN
ETHIOPIA
UGANDA
W POKOT
BARINGO
Mt. Elgon
Kimilili
WESTERN
L. Baringo
SOMALIA
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NAIROBI
KITUI
MACHAKOS
TANZANIA
Indian
Ocean

vectors of Leishmania braziliensis braziliensis Vianna, which causes cutaneous leishmaniasis in Brazil. P. wellcomei cannot be distinguished from sympatric Psychodopygus complexes either by isoenzyme or morphometric profiles (Ready and da Silva, 1984; Lane and Ready, 1985).

However Ryan, Phillips, Milligan, Lainson and Shaw (1986) used gas liquid chromatography and discriminant function analysis to successfully separate individual females of P. wellcomei Fraiha, Shaw and Lainson from other Psychdopygus females.

Cuticular hydrocarbons extracted from individual dried females of two populations of Phlebotomus ariasi Tonnoir in the Gevennes focus: of leishmaniasis in the South of France have also been analysed by Kamhawi, Molyneux, Killick-Kendrick, Phillips, Wilkes and Killick-Kendrick (1987). They found that five of the samples collected from sylvatic and domestic sites separated by a distance of 900m formed two distinct groups, whereas a sixth was identified as a mixture of both groups. Using principal components analysis method these authors reported different patterns of cuticular hydrocarbons for larvae and adults of P. ariasi.

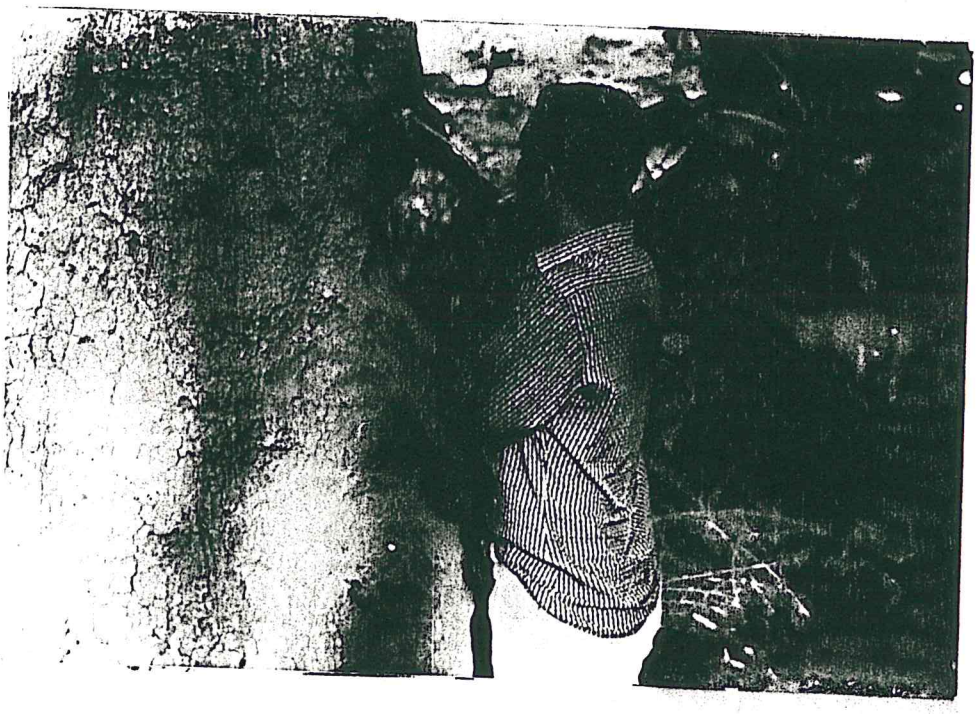
# CHAPTER THREE

# COLLECTION, HANDLING AND REARING OF SANDFLIES

## 3.1. Source of Phlebotomine sandflies

Phlebotomine sandfly species were collected from animal burrows, caves and termite hills in a number of locations known to be endemic foci of leishmaniases in Kenya. The following phlebotomine sandflies species were collected from their respective breeding areas: Phlebotomus elgonensis from caves in Mount Elgon National Park, P. duboscqi from animal burrow in Marigat (Baringo District), P. pedifer from caves near Kimilili (Bungoma District), Sergentomyia antennatus, and S. ingrami near termites hills in Marigat, and S. bedfordi and S. garnhami near termite hills in Tseikuru (Kitui District) (Figure 2). Furthermore P. martini (provided by the Walter Reed Army Institut based in the Kenya Medical Research Institut) and S. africanus and S. schwetzi from the I.C.I.P.E Medical Vectors Research Programme colonies were also used in the study.

## 3.2. Collection of Phlebotomine sandflies



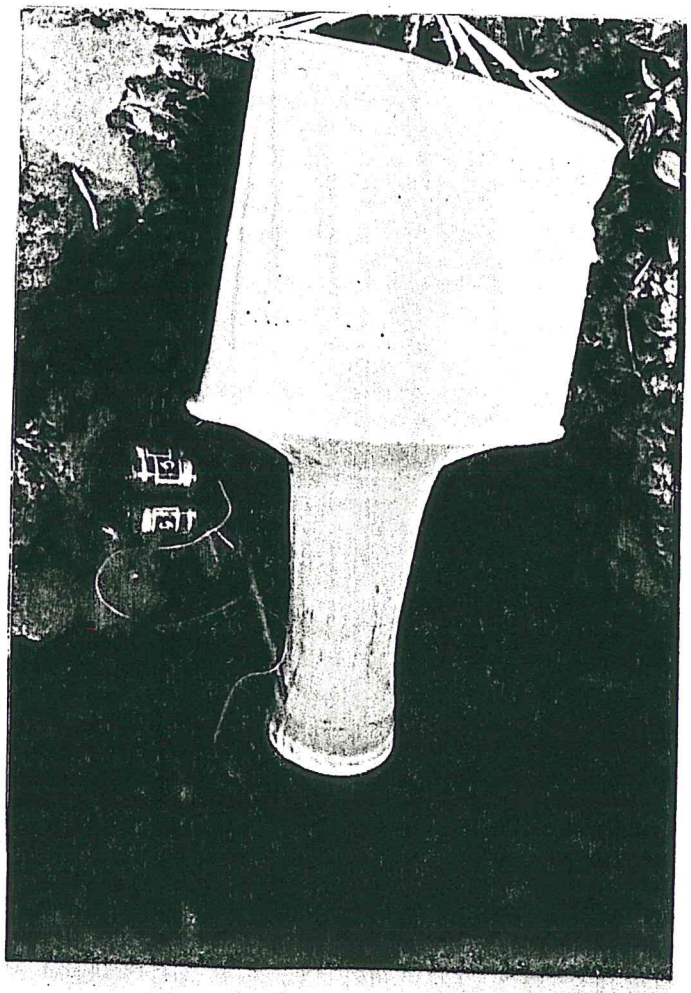
Adult sandflies were collected using either: aspirators (suction tubes) or a fan suction trap.

### 3.2.1. Aspirators (suction tubes)

This method was used to collect incoming and outgoing sandflies near ventillation shafts of termite hills (Plate 1), and those resting on tree barks, tree holes, rock crevices and in caves. The sandflies were sucked into the aspirator and then blown into a sandfly rearing cage for further treatment. A torch was used to locate the Phlebotomine sandflies in caves.

#### 3.2.1.3. Fan suction trap

This was used to collect incoming and outgoing sandflies at the openings of animal burrows (Plate 2). The trap was made from a fan rotor soldered inside a tin open at both ends. The tin was 15 cm long and 10 cm in diameter. The fan sucked all flying insects passing near the tin into a collecting chamber. The collecting chamber was made of nylon gauze attached to the other end opposite to the fan. The fan used four 1.5 watt batteries and was very convenient for field use.



## 3.2. Handling of collected sandflies

The sandflies collected from the field were grouped according to the following requirements:

Group I

Male and female sandflies required for isoenzyme analysis were kept in screw-capped glass vials and stored in liquid nitrogen.

Group II.

Sandflies of both sexes required for pattern analysis of cuticular components were kept in glass vials with screw caps at room temperature.

Group III.

Blood fed, gravid sandflies were kept individually in glass lined with plaster of Paris, on which they could lay eggs. Each vial was covered with nylon gauze which allow flow of air into the vial. A piece of cloth soaked in a sugar solution was placed on the nylon gauze, enabling the flies to take some sugar.

Group IV.

Unfed male and female flies were kept in rearing cages and fed on either a lizard or gerbil kept in the cage. Mated females were transferred singly to glass vials containing plaster of Paris and treated as those of Group III. The males were removed and kept in glass vials either at room temperature or in liquid nitrogen and used for either cuticular components or isoenzyme analyses studies.

Female flies from Group III and IV were removed after eggs had been laid. Dead females were mounted on Gum Chloral de Faure (a solution containing 50 ml of distilled water, 30 mg of gum arabicum, 20 ml of glycerin and 50 gm of chloral hydrate). The severed head was mounted ventrally upwards and placed next to the rest of the body, so as to reveal morphologically important taxonomic features. The flies were examined under a dissecting microscope, and identified species were then kept as voucher specimens. This made it possible to keep a record of the identity of the progeny of all field-collected flies.

### 3.2.1. Rearing of sandfliesa

The sandflies were reared according to methods used by Ward (1977); Beach, Young and Mutinga (1983); and Mutinga, Kamau and Mwandandu (1987). After the removal of all blood-fed females, the eggs were washed into a petri dish containing plaster of Paris. The petri dishes were then kept in an incubator at 25 - 27 °C and 80 - 90% humidity to enable the larvae to hatch from the developing eggs. After hatching the larvae were retained in the same petri dish in the same oven incubator under the same conditions. Sandfly diet, consisting of rabbit faeces is sprinkled on the plaster of Paris, thus enabling the larvae of all stages from first instar to the fourth to feed. The pupae developed from the fourth larval instar and adult sandflies emerged from the pupae after 5 days. Flies were removed as they emerged and, were then kept in either liquid nitrogen or were dried at room temperature in glass vials until required for isoenzyme or cuticular components analysis.

# CHAPTER FOUR

# ISOENZYME CHARACTERIZATION OF PHLEBOTOMINE SANDFLIES

## 4.1. Introduction

Available information records Miles and Ward (1978) as the first to establish isoenzyme analysis as a useful method for distinguishing between species of sandflies. Subsequently, Ward et al. (1981), Petersen (1982), Rogo (1985) and Rogo, Khamala and Mutinga (1988) successfully employed this technique to separate sandfly species in France, Tunisia, Latin America (Panama) and Kenya and they also suggested isoenzymes that are potentially diagnostic.

The study reported here was designed (a) to select a group of enzymes that could be used to discriminate all known sandfly species in Kenya, and (b) establish the phenetic relationships between the sandflies studied.

## 4.2. Thin Layer Starch Gel Electrophoresis

### 4.2.1. Materials and Methods

#### 4.2.1.1. Biological materials

The following Phlebotomine sandfly species were used in the study: P. elgonensis from Mount Elgon National Parc ,P. pedifer from Kimilili (Bungoma District), P. duboscqi, S. ingrami from Marigat (Baringo District), P. martini (from the Walter Reed Army Institut, Nairobi), S. garnhami and S. bedfordi from Tseikuru (Kitui District),and S. schwetzi from the Medical Vectors Research Programme of I.C.I.P.E. Trypanosoma congolense homogenat was run at the begenning to confirm the presence or absence of the enzymes assayed in the sandflies.

#### 4.2.1.2. Preparation of insect homogenates for isoenzyme studies

Insect homogenates for the studies were prepared just before use. The head and terminalia of the adults flies collected in the field and kept in liquid nitrogen were retained as voucher specimen for morphological taxonomic purposes. The thorax and abdomen were homogenized on ice in 20 µl of

TABLE 3: Tank and gel buffers solutions used during thin layer starch gel electrophoresis

| ENZYME | | TANKBUFFER | GEL BUFFER | DILUTION |
|---|---|---|---|---|
| 1. MDH | E.C.1.1.1.37 | 0.05 M Na2HPO4 | | |
| ICD | E.C.1.1.1.42 | 0.007M CITRATE | PH 7.0 H | 1:4 |
| 2. ME | E.C.1.1.1.40 | 0.081M Na2HPO4 | | |
| GPI | E.C.5.3.19 | 0.019M NaH2PO4 | PH 7.4 | 1:9 |
| 3. PGM | E.C.2.7.5.1 | 0.1 M TRIS<br>0.1 M MALEATE<br>0.01 M EDTA<br>O.O1 MgCl2 | PH 7.4 | 1:9 |
| 4. GAPDH | E.C.1.2.1.12 | 0.1 M KH2PO4-KOH | PH 7.0 | 1:9 |
| 5. TDH | E.C.1.1.1.103 | 0.15 M TRIS<br>O.OO7M CITRATE | PH 9.0 | 1:9 |
| 6. NH | E.C.3.2.2.1 | 0.04M TRIS<br>0.44M BORATE PH 6.5 | 0.013M TRIS PH 7.2<br>0.004M CITRATE | |
| 7. 6-PGD | E.C.1.1.1.44 | 0.1 M Na2HPO4 | | |
| SOD | E.C.1.15.1.1 | 0.1 M NaH2PO4 | PH 7.0 | 1:9 |
| 8. G-6PD | E.C.1.1.1.49 | 0.1 M Na2PO4<br>0.1 M TRIS | PH 7.4 | 1:9 |
| 9. PEP A, B & D | E.C.1.4.11/13 | 0.18M TRIS | | |
| ALAT | E.C.2.6.1.2 | 0.02M KH2PO4 | PH 9.3 | 1:9 |
| 10.ASAT | E.C.2.6.1.1. | 0.I5M GLYCINE-NaOH | PH 9.5 | 1:9 |
| 11.HK | E.C.2.7.5.1 | 0.135M TRIS<br>0.042M CITRIC ACID | PH 7.0 | 1:9 |
| 12.LDH | E.C.1.1.1.27 | 0.2 M Na2HPO4<br>0.2 M NaH2PO4 | PH 7.0 | 1:9 |

deionised water and 5 µl of enzyme stabilizer in a glass haemagglutination plate using a homogenizing stick. The homogenate was kept for a minimum of 5 minutes on ice and then a 0.5 cm long piece of cotton thread (ANCHOR 1 2X) previously boiled for 5 minutes and dried in an oven, was placed in it. The 0.5 cm cotton thread was then inserted into a 0.5 cm slot already made on the gel by pressing a perspex comb.

It is important to note that during this study, the homogenates used to determine the isoenzyme bands were from single individuals. The plates 4, 5 and 6 and Figure 3 are representative of similar ones obtained from ten replicates for each member of the species studied.

4.2.1.3. Chemicals

Fructose-6-phosphate, fructose-1,6-diphosphate (trisodium salt), glucose-1-phosphate with 1% (w/w) glucose-1,6-diphosphate were obtained from British Drug House, Poole (England). Lactate dehydrogenase (hog muscle, 50% glycerol), and malate dehydrogenase (pig heart, 50% glycerol solution) were from Boehringer, Manheim (West Germany). Aldolase, 3-amino-9-ethylcarbazole, DL-isocitrate (trisodium salt), DL -malic acid, L-alanine, L-amino oxidase (crude Crotalus

adamanteus venom), glucose-6-phosphate dehydrogenase, L-asparate, L-leucyl-glycyl-alanine, L-leucyl-glycyl glycine, L-phenyl-alanyl-L-proline, L-threonine, methyl-thiazolyl-tetrazolium (MTT), nicotine adenine dinucleotide (NAD), nicotine adenine dinucleotide reduced form (NADH) (tetrasodium salt), 2-oxoglutarate, peroxidase (horseradisch), phenazine methosulfate (PMS), xanthine oxidase (milk) were from Sigma, Poole (England). All the other chemicals were bought locally through dealers or from some pharmaceutical compagnies and were of the highest analytical grade.

The eighteen isoenzymes assayed, were: Lactate dehydrogenase (LDH E.C. 1.1.1.27), malate dehydrogenase (MDH E.C. 1.1.1.37), malic enzyme (ME E.C. 1.1.1.40), isocitrate dehydrogenase (ICD E.C. 1.1.1.42), phosphogluconate dehydrogenase decarboxylating (6-PGD E.C. 1.1.1.44), glucose-6-phosphate dehydrogenase (G-6PD E.C. 1.1.1.49), L-threonine 3 dehydrogenase (TDH E.C. 1.1.1.103), glyceraldehyde phosphate dehydrogenase (GAPDH E.C. 1.2.1.12), superoxide dismutase (SOD E.C.1.15.1.1), asparate aminotransferase (ASAT E.C.2.6.1.1), alanine aminotransferase (ALAT E.C.2.6.1.2), hexokinase (HK E.C. 2.7.1.1), phosphoglucomutase (PGM E.C. 2.7.5.1), peptidase

(Pep A, B and D E.C. 3.4.11 or 3.4.13), nucleosidase (NH E.C. 3.2.2.1) and glucose phosphate isomerase (GPI E.C. 5.3.1.9). These enzymes were assayed because they gave different results for different insect species.

4.2.1.4. Thin Layer Starch Gels

Gels were prepared by using 60 ml of 10% Electrostarch (Electrostarch Co., Madison, Wisconsin USA) in gel buffer solution. Six (6) mg of the starch was dissolved in 6 ml gel buffer (Table 3) and 54 ml deionised water by heating to boiling in a conical glass flask, on a magnetic stirring plate. The solution was then degassed under vacuum and poured into one millimeter thick horizontally-leveled 22 X 15 cm glass plates, spread evenly with a perplex spreader and then covered to prevent any contamination. After solidification the plate was stored at 4° C overnight before use.

4.2.1.5. Running condition of samples on starch gel

The samples were electrophoresed essentially as described by Wraxall and Culliford (1968). The glass plate containing the gel was placed on the

Table 4: Buffers used for staining mixtures after IEF and thin layer starch gel electrophoresis

| | | |
|---|---|---|
| A | 0.081 m $Na_2HPO_4$ | |
| | 0.019 m $NaH_2PO_4$ | PH 7.4 |
| B | 0.3 M Tris HCl | PH 7.4 |
| B* | 0.3 M Tris HCl | PH 8.0 |
| C | 0.011 M $Na_2HPO_4$ | |
| | 0.003 M $NaH_2PO_4$ | |

flat bed apparatus (FBE 3000 Pharmacia, Sweden, Plate 3), which was previously cooled at 4°C using a hetofrig cooler (Heto Bikerod, Denmark). Spontex wicks were placed on the gel so that current could flow straight from the anode to cathode. The wicks were held in place with two glass plates which covered the gel plate. Current was supplied with either a Pharmacia, ECPS 3000/150 an LKB, 3371 ED or a 2103 Power pack (Plate 3). Combinations of durations of 100 to 180 minutes with constant voltages of 250, 300 or 580 were used, depending on the enzyme system being studied. After the electrophoretic runs the power was switched off and the plates containing the gels were removed and placed in a box containing staining mixture (Table 4 and Table 5). The isoenzyme bands were made visible using either the fluorogenic or the electron trasfer dye staining procedures:

The fluorogenic staining method depends on either the generation of highly fluorescent product by enzyme action of a non-fluorescent substrate derivative of 4-methylumbelliferone (i.e. a so called "positive" fluorescent stain) or the generation of a non-fluorescent product from a fluorescent substrate (i.e. a so called "negative" fluorescent stain). The fluorogenic staining methods give bands which are only visible to UV-

Table 5: Staining mixtures for each isoenzymes after IEF and thin layer starch gel electrophoresis

| Enzymes | Buffer | Substrates | Co-enzyme | Linking enzyme | Additional ions | Dye |
|---|---|---|---|---|---|---|
| LDH | C | | 3 mg NAD | 25 ul Calcium lactate | | 10 mg MTT<br>5 mg PMS |
| MDH | C | 1 M Malic acid | 3 mg NAD | | .1M $MgCl_2$ | 10 mg MTT<br>5 mg PMS |
| ME | C | 0.1M Malic acid | 3 mg NADP | | .1M $MgCl_2$ | 10 mg MTT<br>5 mg PMS |
| ICD | C | 10 mg Isocitric acid | 3 mg NADP | | .1M $MgCl_2$ | 10 mg MTT<br>5 mg PMS |
| 6-PGD | B | 10 mg 6-Phospho-gluconate | 5 mg NADP | | .1M $MgCl_2$ | 10 mg MTT<br>5 mg PMS |
| G-6-PD | B | 5 mg D-Glucose-6-phosphate | 3 mg NADP | 10 mg ATP | .1M $MgCl_2$ | 10 mg MTT<br>5 mg PMS |
| TDH | C | 70 mg L-Threonine | 3 mg NAD | | .1M $MgCl_2$ | 10 mg MTT<br>5 mg PMS |
| GAPDH | B*<br><br>C | 40 mg Fructose-1, 6-diphosphate<br>150 ul Aldolase (80 U) | 5 mg NAD | | 10 mg Sodium arsenate | 10 mg MTT<br>5 mg PMS |
| SOD | A | | | | | 10 mg MTT<br>5 mg PMS |

Table 5: continued

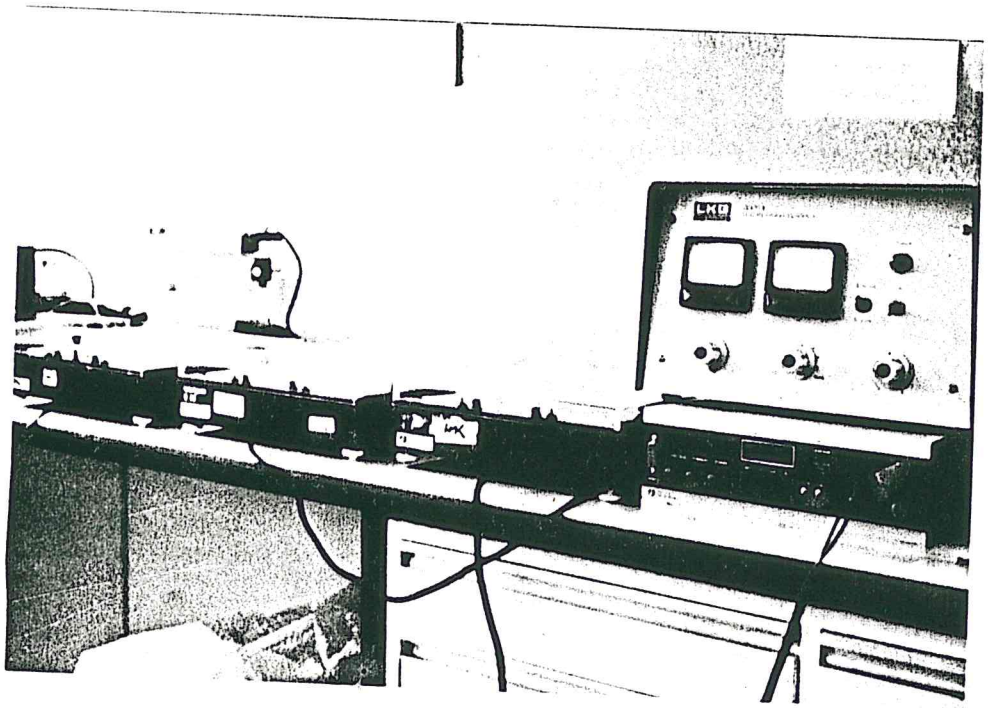
| | | | | | | |
|---|---|---|---|---|---|---|
| ASAT | A | 20 mg 2-Oxo-glutarate<br>46 mg Aspartate | 3 mg NADH | 15 ul MDH | | |
| ALAT | A | 20 mg 2-Oxo-glutarate<br>120 mg L-Alanine | 3 mg NADH | 15 ul LDH | | |
| HK | B | | | | | |
| PGM | B | 10 mg Glucose-1-phosphate with Glucose-1,6-di-phosphate | 3 mg NADP | 50 ul G-6PD | .1M $MgCl_2$ | 10 mg MTT<br>5 mg PMS |
| PEP | C | 10 mg L-Leucyl-glycyl-glycine | | 5 mg Peroxidase | .1M $MgCl_2$ | 2 mg 3-Amino-9-ethylcarbazole |
| GPI | C | 10 mg L-Leucyl-glycyl-alanine | | 5 mg Peroxidase | .1M $MgCl_2$ | 2 mg 3-Amino-9-ethylcarbazole |
| | | 10 mg L-Phenyl-alanine-L-proline | | 5 mg Peroxidase | .1M $MgCl_2$ | 2 mg 3-Amino-9-ethylcarbazo |
| NH | B | 5 mg Inosine | | 20 ul Xanthine oxidase (4 U) | | 10 mg MTT<br>5 mg PMS |
| GPI | C | 10 mg Fructose-6-phosphate | 3 mg NADP | 50 ul G-6PD | .1M $MgCl_2$ | 10 mg MTT<br>5 mg PMS |

light (Godfrey and Kilgour, 1976; Hopkinson and Harris, 1976).

The electron transfer dyes are widely used as stains for detecting isoenzymes after electrophoresis. There are two dyes in general use which give visible bands: (i) methyl-thiazolyl-tetrazolium, usualy known as MTT, which is an acceptor of dehydrogenase reactions, and (ii) 3-amino-9-ethyl-carbazole which is an acceptor for oxidases and peroxidases.

MTT is reduced by an electron donor to form a dark-blue to purple insoluble formazan. The reaction proceeds rapidly in the presence of a catalyst. Both dyes are standard ingredients in stains used to detect the reduced form of isoenzyme NAD and NADP. The tetrazolium-phenazine methosulfate staining mixtures are light-sensitive and therefore incubation must be carried out in the dark (Bagster and Parr, 1973; Hopkinson and Harris, 1976; Miles, Toye, Oswald and Godfrey, 1977; Miles and Ward, 1978; Gibson, Mehlitz, Lanham and Godfrey, 1978). The yellow soluble 3-amino-9-ethyl-carbazole which can be oxidized to a dark brown insoluble compound is a very useful general purpose reagent for detecting oxidases and peroxidases (Hopkinson and Harris, 1976).

4.2.1.6. Data analysis


LKB

Data of this study were analysed using each species as the Operational Taxonomic Unit (OTU). From the electrophoretic results for the three enzymes the presence (1) or absence (0) of the bands on starch gel just after staining (Appendix 1). Similarities between OTUs were measured with the Jaccard's and simple matching coefficient. The Jaccard's matching is the proportion of positive matches of all the matches excluding negative matches (The Coefficient of Jaccard:

$$S_J = a : a + u = a: a+ b + c)$$

The Simple Matching is the proportion of positive and negative matches of all the present possible combinations (The Simple Matching Coefficient:

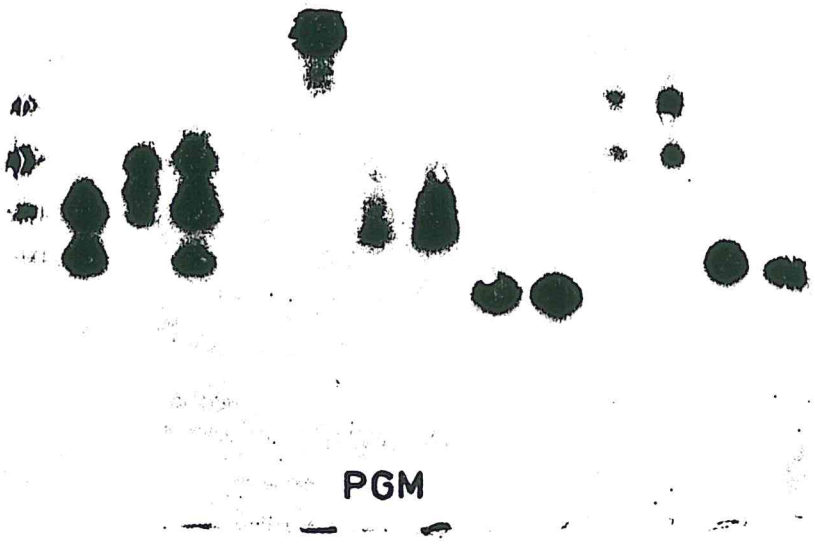
$$S_{SM} = m : m + u = a + d : a + b + c + d)$$

Note $m = a + d$ is the number of matches

$u = b + c$ is the number of mismatches

Clusters were defined by Unweigthed Pair-Group Method using arithmetic Averages (UPGMA, Sneath and Sokal).

UPGMA algorithm computes the average similarity or dissimilarity of a candidate OTU to an extant cluster, weighting each OTU in that cluster equally, regardless of its structural subdivision. Cluster analysis was performed with the NTSYS package (VERSION 1.40, 1988).




$\overline{1S}$ $\overline{2S}$ $\overline{1I}$ $\overline{2I}$ — $\overline{2G}$ $\overline{1D}$ $\overline{2D}$ $\overline{1E}$ $\overline{2E}$ $\overline{1B}$ $\overline{2B}$ $\overline{1P}$ $\overline{2P}$

S = S. schwetzi
I = S. ingrami
G = S. garnhami
D = P. duboscqi
E = P. elgonensis
B = S. bedfordi
P = P. pedifer

1 = male
2 = female

4.2.2. Results

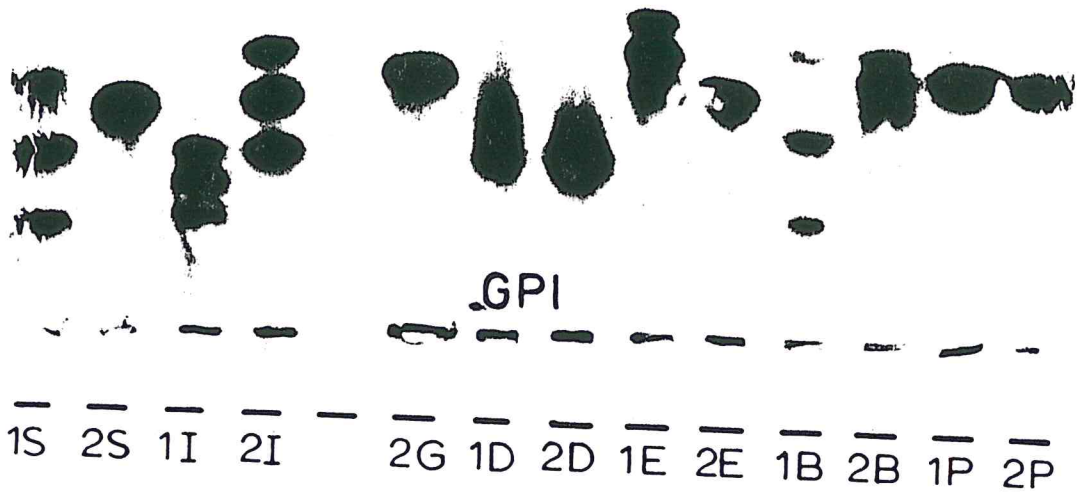
It is important to note that that enzyme bands may diffuse, spread or become faint few minutes after staining. And it is common that the presence or absence of a band is shown in a form of a schematic representation. The eighteen enzymes assayed by thin layer starch gel electrophoresis in this study fall into one of three categories vis:

a) those which produced no bands include LDH, TDH, ALAT and NH.

b) Peptidases and ME which produce faint bands that could not be used to distinguish between sandfly species represented isoenzymes that did not show any bands in the various experiments with sandflies.

c) The enzymes ICD, GAPDH, ASAT, GPI, G-6PD, HK, MDH, 6-PGD and PGM which gave visible banding patterns, that could be used for taxonomic purposes.

GAPDH, ASAT, MDH, G-6PD and HK showed no differences in the bandings patterns of males and females of the same species. However GPI, ICD, MDH, 6-GPD and PGM have shown a greater number of bands between sandflies species and between males and females of the same species compared to the other enzymes.

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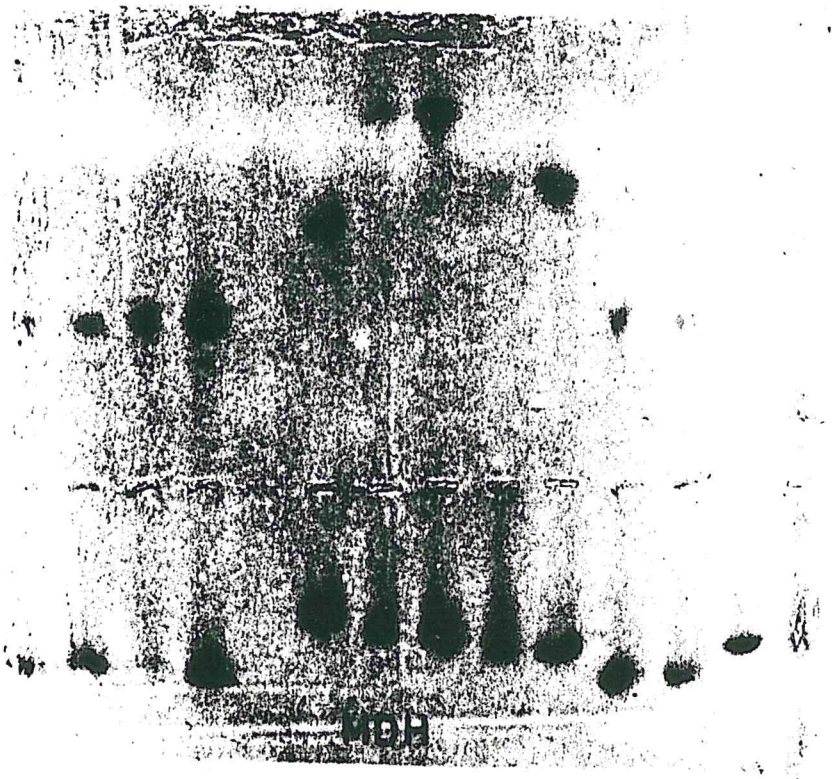
P = P. pedifer

1 = male
2 = female

GPI, MDH and PGM which gave visible banding pattern were selected for use in the identification of sandfly species and the development of a dendogram.

Examinations of phosphoglucomutase (PGM) banding patterns for the following species; S. bedfordi, S. garnhami, S. ingrami, S. schwetzi, P. duboscqi, P. elgonensis, P. martini and P. pedifer (Plate 4) shows male and female banding patterns that are. practically specific for each species. S. bedfordi, S. garnhami, S. ingrami and S. schwetzi belong to the genus Sergentomyia whereas P. duboscqi, P. elgonensis, P. martini and P. pedifer belong to the genus Phlebotomus. The PGM patterns of S. ingrami and S. schwetzi showed many bands suggesting the existence of a higher degree of polymorphism. Males of S. schwetzi showed 4 bands and the females 2 bands, whereas for S. ingrami the males and females showed the same numbers of bands (3). S. garnhami females banding pattern is unique compared to the other species. The pattern was characterized by one fast moving band. Patterns of S. bedfordi are represented by the same number of bands (4) for males and females. Males and females P. duboscqi showed the same banding patterns.

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P = P. pedifer

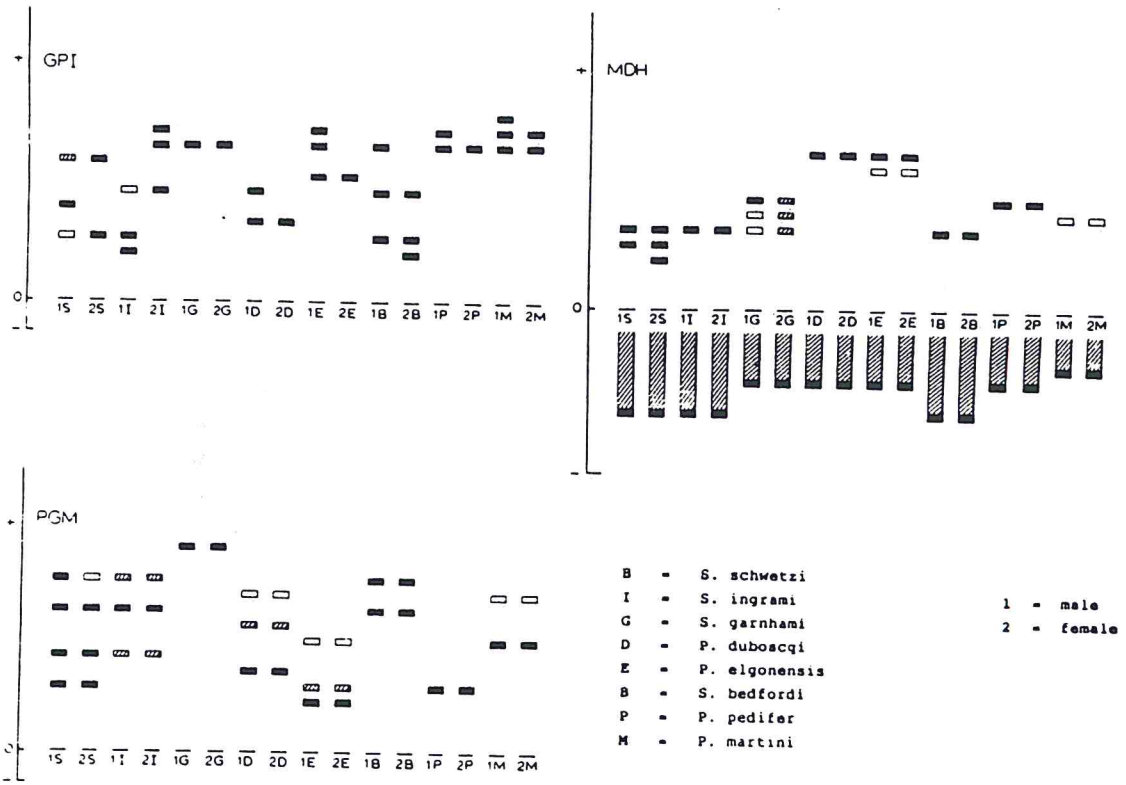
1 = male
2 = female

P. martini showed polymorphism in both male and female suggesting that the underlying population from which the sample was drawn is heterogenous.

P. elgonensis and P. pedifer which are morphologically similar, were readily distinguishable using their banding pattern.

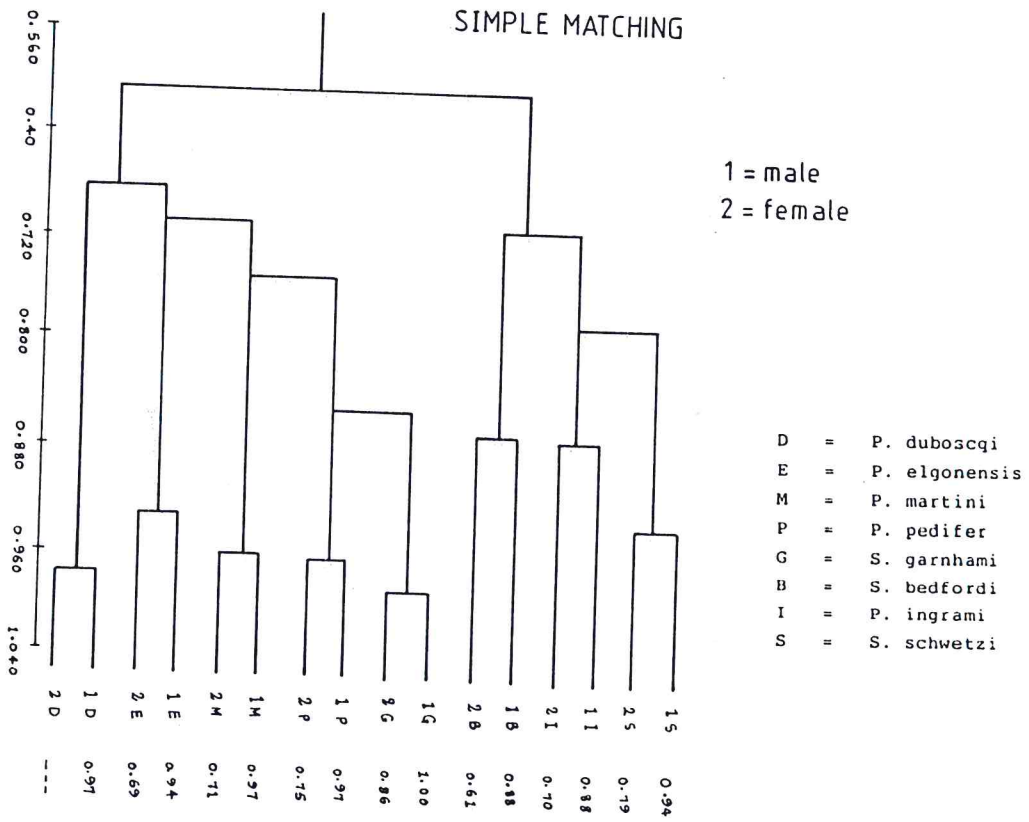
Glucosephosphate isomerase (GPI) banding patterns as shown in Plate 5 were polymorphic for S. bedfordi, S. garnhami, S. ingrami, S. schwetzi, P. duboscqi, P. elgonensis, P. martini and P. pedifer. As in the case of PGM, males and females of sandflies studied do share one or two bands. Males and females of P. elgonensis, S. schwetzi, S. garnhami showed only one major band with different mobility from the slots where the coton threads were placed. The males of P. pedifer showed 3 bands although the females of the species showed one band. P. elgonensis and P. pedifer banding patterns were different from each others. P. duboscqi, S. bedfordi and S. ingrami patterns for males and females showed the same number of bands.

Malate dehydrogenase (MDH) of sandflies was characterized by bands, which run in two directions ie positive and negative (Plate 6). Males and females of the same species have the same number of bands but different species have


GPI
MDH
PGM
+
0
-
1S 2S 1I 2I 1G 2G 1D 2D 1E 2E 1B 2B 1P 2P 1M 2M
B = S. schwetzi
I = S. ingrami
G = S. garnhami
D = P. duboscqi
E = P. elgonensis
B = S. bedfordi
P = P. pedifer
M = P. martini
1 = male
2 = female

bands which are positioned at different migration distances from the origin of the migration. Taken together, the three enzymes GPI, MDH and PGM could give schematically a diagnostic kit (Figure 3).

Dendograms (schematic representation of relationship among species) of phenetic similarity among sandfly species based on the three enzymes, GPI, MDH and PGM obtained using the simple matching and the Jaccard's coefficient gave very interesting results (Figure 4 and Figure 5). The simple matching coefficient is obtained by making a list of all characters found in the studied species, whereas the Jaccard's coefficient excludes negative matches. The individual species are then compared two by two and the species sharing the most characters in common are grouped together. This way it is possible to generate a tree showing the similarity between the species (dendogram). Correlation coefficients among the cophenetic matrices derived from the two clustering methods (Figure 4 and 5) ranged from poor (<0.6 single linkage versus UPGMA for the Simple and Jaccard's Matching) to very good (>1.0 complete linkage versus UPGMA for Simple and Jaccard's Matching). All the eight species arise from the same origin and their similarity can be seen in Figure 4 and Figure 5. There are two

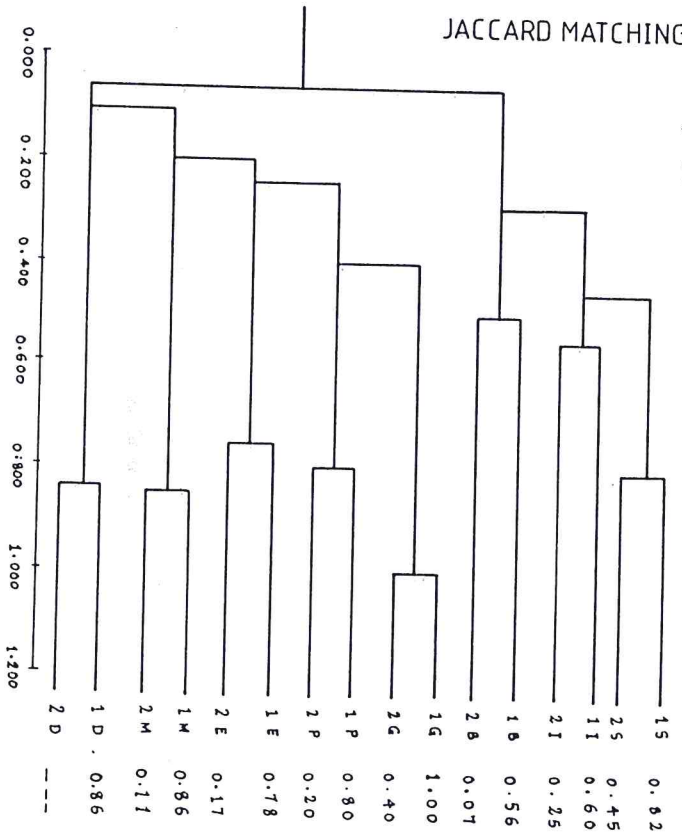

SIMPLE MATCHING
0.560
0.640
0.720
0.800
0.880
0.960
1.040
2 D
1 D
2 E
1 E
2 M
1 M
2 P
1 P
1 G
2 B
1 B
2 I
1 I
2 S
1 S
---
0.97
0.69
0.94
0.71
0.97
0.75
0.97
0.86
1.00
0.61
0.88
0.70
0.88
0.79
0.94
1 = male
2 = female
D = P. duboscqi
E = P. elgonensis
M = P. martini
P = P. pedifer
G = S. garnhami
B = S. bedfordi
I = P. ingrami
S = S. schwetzi

major groupings, the first group includes S. bedfordi, S. ingrami and S. schwetzi and the second group includes P. elgonensis, P. martini, P. pedifer and S. garnhami. P. duboscqi seem to belong to the second group but it is more or less on its own. It is important to note that the morphologically similar P. elgonensis and P. pedifer are seen to be very closely associated genetically when the Jaccard matching is used (Figure 5). The Jaccard and Simple matching dendograms show S. garhami as closely associated to the species known to be vectors of Leishmania species.

### 4.2.3. Discussion

Isoenzyme analysis by thin layer starch gel electrophoresis was used to identify P. elgonensis, P. duboscqi, P. martini, P. pedifer, S. bedfordi, S. garnhami, S. ingrami and S. schwetzi. Of the eighteen isoenzymes assayed, nine gave banding patterns which showed potential for use in typing sandflies species. These 9 enzymes - ASAT, GAPDH, GPI, G-6-PD, HK, ICD, MDH, 6-PGD and PGM, have also been demonstrated as taxonomic indicators for sandfly species from the New World by Miles and Ward (1978). These workers also suggested that MDH, GPI and PGM could be of use in studies of the population genetic of Phlebotomine sandflies.

Attempts at distinguishing Phlebotominae in France and Tunisia (Ward et al., 1981), Latin America (Petersen, 1982) and Kenya (Rogo, 1985 and Rogo et al., 1988) have also been described. The most useful enzymes found by these authors were MDH, PGM, GPI, HK, 6-PGD, ES, ME, IDH, ASAT, ICD and G-6-PD. In the study reported here ES and IDH were not investigated. With exception of the isoenzymes which did not show banding pattern and those which showed very faint banding patterns, the other enzymes ie ASAT, HK, GPI, PGM, ICD, MDH, 6-PGD, G-6-PD gave results that were consistent



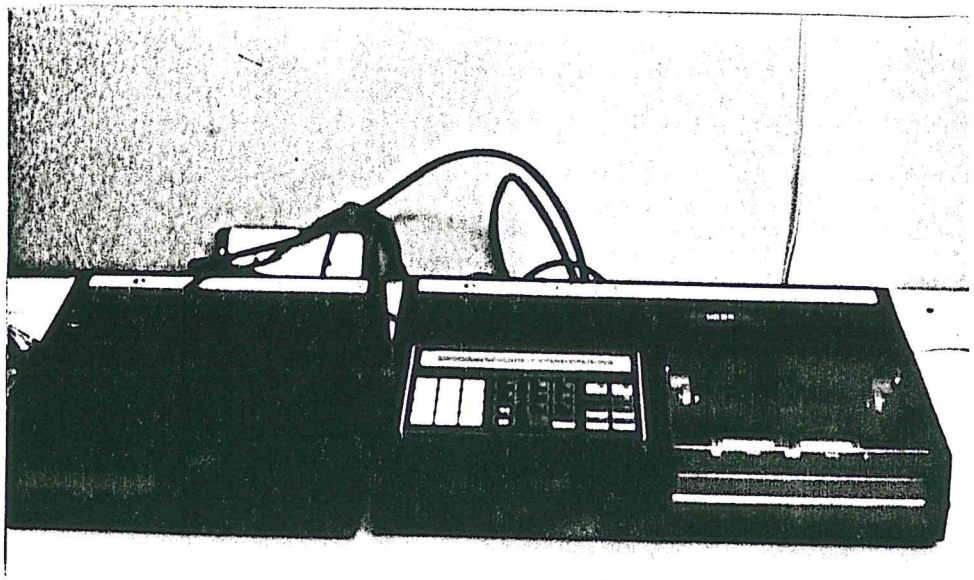

D = P. duboscqi
M = P. martini
E = P. elgonensis
P = P. pedifer
G = S. garnhami
B = S. bedfordi
I = P. ingrami
S = S. schwetzi

with the finding of these authors. The results show that GPI, MDH and PGM provide an effective set of enzymes for the identification of Phlebotomine sandflies in Kenya. The patterns produced by this set were similar to those of Agatsuma et al. (1986). While analysing Guatemalan blackflies PGM and GPI, these authors found a very high degree of polymorphism between the different species. The results show that on. analysis the GPI, MDH and PGM banding pattern of the morphologically similar species P. elgonensis and P. pedifer are different (Plate 4, 5, 6 and Figure 4). This result confirms those of Rogo (1985) and Rogo et al. (1988).

The results from the application of phenetic similarity segregated the sandfly species into the two genera Phlebotomus and Sergentomyia found in the Afro-tropicale region (Table 1). However, it was surprising to find that S. garnhami was associated with the genus Phlebotomus. P. elgonensis and P. pedifer are shown to be genetically close to each other and this could explain the problem faced by many taxonomists in trying to distinguish between them on the basis of morphological characters only.

In summary, isoenzyme analysis of sandflies has been shown to distinguish between the morphologically similar sandfly species examined.

However, this technique requires a high level of expertise and expensive chemicals. Specimens have to be partially destroyed (head and lower parts of the abdomen can be mounted on a slide) through homogenization. The flies also have to be kept in liquid nitrogen or brought alive in the laboratory for processing.

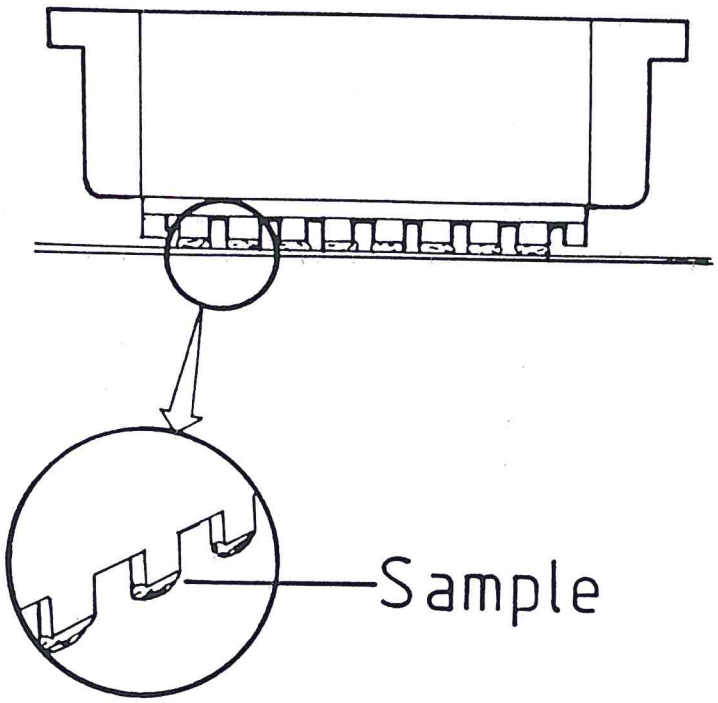


## 4.3. Isoelectric Focusing (IEF)

Isoelectric focusing is an electrophoretic method which separates proteins in a stable pH gradient established between two electrodes by a mixture of soluble carrier ampholytes. In this system, a protein migrates within the gradient to its isoelectric point (pI) and then stops to form a sharp concentrated band. Thus because the positive and negative charges of the molecule cancel out at the isoelectric point (pI) movement ceases in the electric field. This technique gives excellent resolution of proteins (Svensson, 1961). However, considerable time, effort and skill are required to achieve good results, especially when large horizontal gels are used.

With the development of horizontal ultra thin precast polyacrylamide gels for conventional IEF and mini ultra thin polyacrylamide gels for the Phast System™, it is possible to use very small amounts of proteins to run an IEF.

The Phast System™, is a horizontal electrophoresis system integrated with an automated development unit for staining and destaining (Plate 7). SDS-Page, IEF, agarose electrophoresis and two dimensional gel electrophoresis can be performed using the Phast System™. The Phast System™ has been used to


Sample

characterize protein and Isoenzyme patterns of parasites and Insects (Olsson, Fredriksson, Degerman and Olsson, 1988; and Kambona, 1989).

Isoenzyme analysis of insects has so far been carried out on agarose, cellulose acetate, and thin layer starch gels (Miles and Ward, 1978; Petersen, 1982; Rogo, 1985 and Rogo et al., 1988). Relatively few workers have used IEF to identify insect species (Kambona, 1989). In this study, the Phast System™ IEF polyacrylamide gels was used to evaluate its usefulness in differentiating sandfly species.

### 4.3.1. Methodology

#### 4.3.1.1. Biological sample preparation

Sandfly homogenates were prepared in the same way as those used for thin layer starch gel electrophoresis (4.2.1.) from females belonging to the following species P. elgonensis from mount Elgon, P. duboscqi S. antennatus and S. ingrami from Marigat, P. pedifer from Kimilili, S. bedfordi, S. garnhami, S. graingeri from Tseikuru, and S. schwetzi from the ICIPE medical Vectors research Programme.

#### 4.3.1.2. Phast system™ isoelectric focusing

The sample required for Phast System™ IEF was transferred with a sample applicator carrying 2 µl (Figure 6) into the gels. To ensure reproducible results all the conditions (temperature, voltage, duration of separation, current) necessary for separation were programmed by a microprocessor. The duration of each step was controlled by volt hours (Vh). The temperature of the horizontal separation bed was also regulated by the microprocessor, a temperature sensor and peltier element. The electrodes are made of platinized titanium rods mounted in a spring system to give good electrical contact with the gels. The interelectrode distance for IEF is 38 mm.

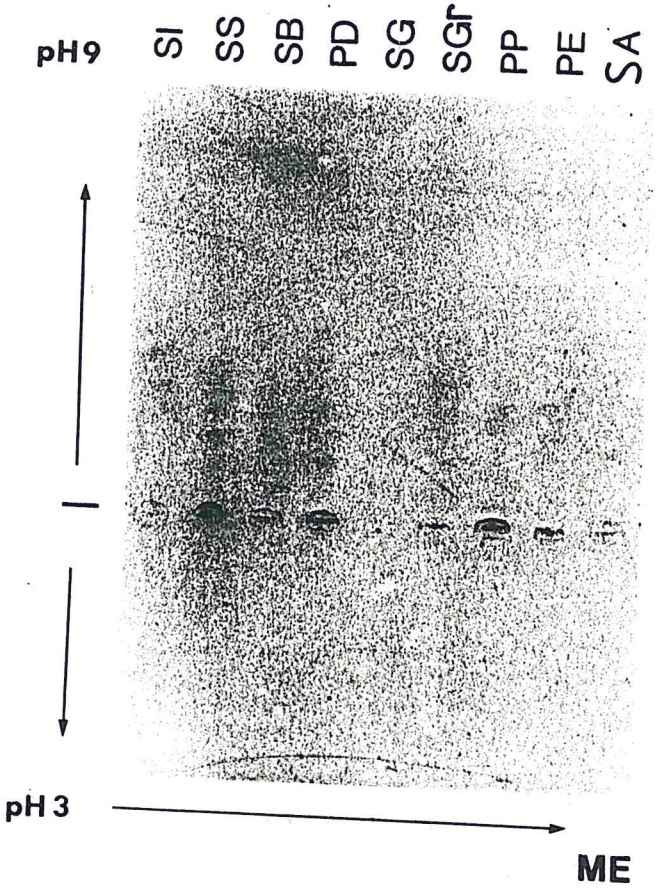
Phast IEF gel media used during the investigation had a pH range from 3 - 9 and were 50 mm long, 43 mm wide and 0.35 mm thick. The gel contained Pharmalyte® carrier ampholytes with a buffering capacity of 0.02 mmol/ml gel and pH unit with a concentration of polyacrylamide (PAA) T5C3. The gels were placed in the separation chamber and the system programmed. The temperature of the system was programmed for 15 °C. Homogenates of single individuals of the species studied were transferred with an Eppendorf micropipette to wells made in the Phast System sample applicator.

Table 6: Programme for Phast system™ isoelectric focusing

| Step | Stage | Voltage v | Current in mA | Power in watts | Temperature in °C | vh |
|---|---|---|---|---|---|---|
| 1 | Pre-focusing | 2000 | 2 | 3.5 | 15 | 75 |
| 2 | Sample application (a) | 200 | 2 | 3.5 | 15 | 15 |
| 3 | Focussing (b) | 2000 | 2 | 3.5 | 15 | 410 |

(a) Sample applicator down at Step 2 after 0 vh (accumulated 75 vh)

(b) Sample applicator up at Step 3 after 0 vh (accumulated 90 vh)




SI = S. ingrami
SS = S. schwetzi
SB = S. bedfordi
PD = P. duboscqi
SG = S. garnhami
SGr = S. graingeri
PP = P. pedifer
PE = P. elgonensis
SA = S. antennatus

The sample applicator was then placed in the middle of the system for separation. The platinized electrode rods were placed on the gels before the chamber was covered. After the prefocusing period the sample applicator was automatically pressed against the gels and the focusing was performed (Table 6). Twenty minutes later the current was switched off and the gels were placed into enzyme developer solutions for 20 minutes for visualization. The reaction was stopped with 7% acetic acid. A total of fifteen individuals of each species were analysed. Plates 8, 9 and 10 and Figure 7 are representative of the 15 replicates.

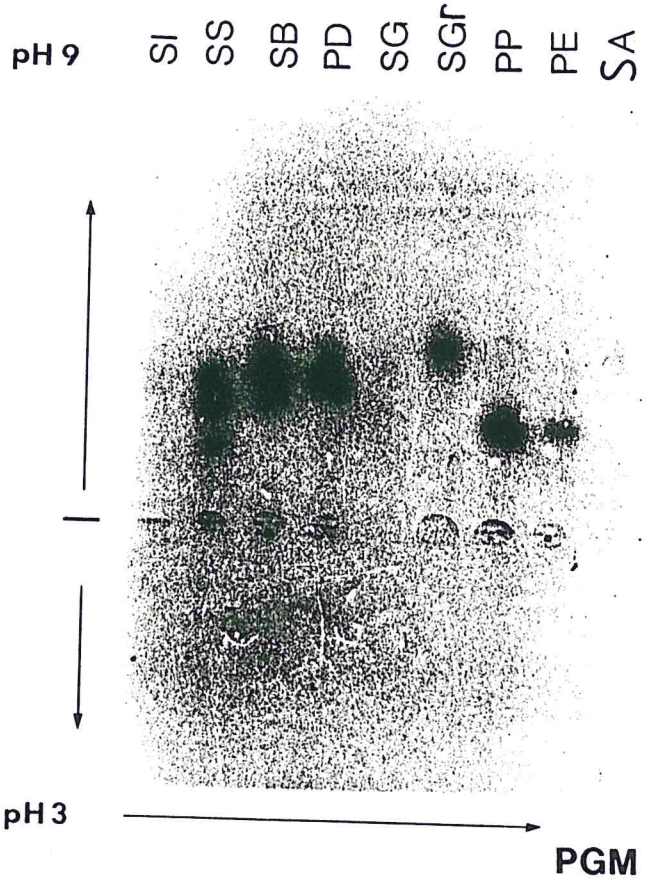
4.3.1.3. Staining procedure of Phast System$^{R}$ gels

The enzymes ICD, G-6PD, MDH, 6-PGD, HK, ME, GAPDH, GPI and PGM were assayed. After the run, the gels were removed with forceps, placed into an enzymes staining solution (Table 4 & Table 5) in a petri dish and allowed to develop. The method used for visualization was the electron transfer dye technique, which gives visible bands to the naked eye.

4.3.2. Results

The isoenzymes MDH, G-6-PD, PGM, ME, ICD, GAPDH, 6-PDG, HK and GPI were analyzed. These enzymes gave visible banding patterns after thin layer starch gel electrophoresis (Chapter IV). Out of the nine, only three, namely phosphogluconate (PGM), and glucose phosphate isomerase (GPI) gave sharp banding patterns of taxonomic utility. Malic enzyme (ME) gave banding patterns which were visible and useful for taxonomic purposes. It is important to note that enzyme bands spread, diffuse or become faint only few minutes after staining. For these raesons it is common practice to draw histograms of the analysed enzymes just after staining. Plate 6, 7 and 8 showed that bands can in fact be seen, however discussion of the result were based on Figure 7.

Malic enzyme (Plate 8) showed very faint bands, which could not be distinguished easily, even though it reacted within a few minutes of incubation. The bands for the various species were not similar in either number or position. Many bands could be seen on the gels. Phlebotomus species showed fewer bands (two) than the Sergentomyia species (plate 8). All species produced a kind of smear (plate 8).




SI = S. ingrami
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SB = S. bedfordi
PD = P. duboscqi
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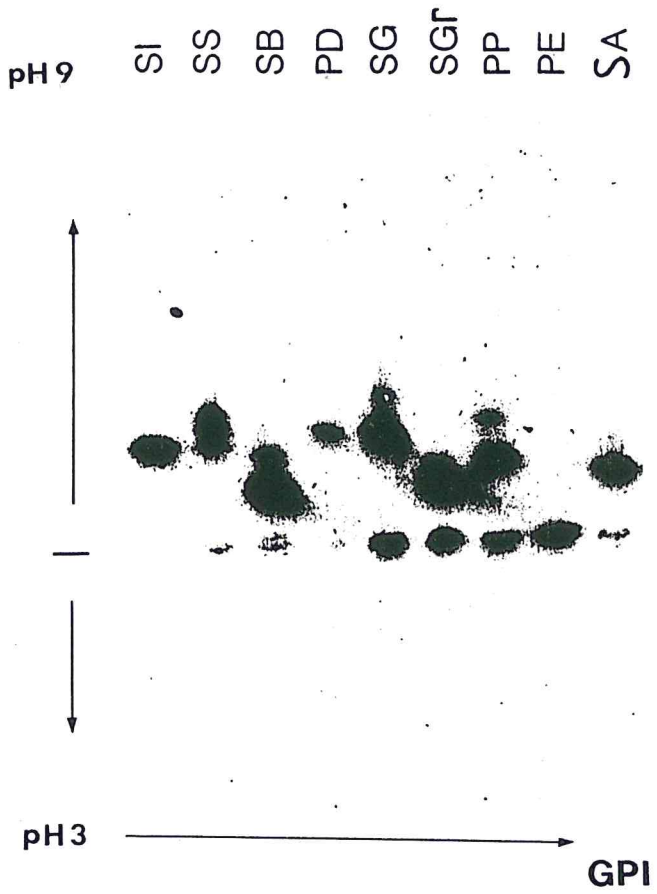
SGr = S. graingeri
PP = P. pedifer
PE = P. elgonensis
SA = S. antennatus

Phosphoglucomutase (PGM) in Plate 9 showed banding patterns which were not very polymorphic when compared to those obtained from thin layer starch gel electrophoresis. S. schwetzi gave three bands, P. duboscqi and S. bedfordi gave two bands each with one common to both sandfly species. While P. elgonensis, and P. pedifer showed only one band, which was more or less the same, the band for P. pedifer was more prominent.

Plate 10 shows that glucose phosphate isomerase (GPI) gave banding patterns specific for each sandfly species used in the study. It is important to note that P. duboscqi , P. elgonensis, S. antennatus, S. graingeri and S. ingrami showed only one band at different levels. S. schwetzi had three bands which were unique. P. pedifer, S. bedfordi and S. garnhami showed two bands each at different positions (Plate 10).

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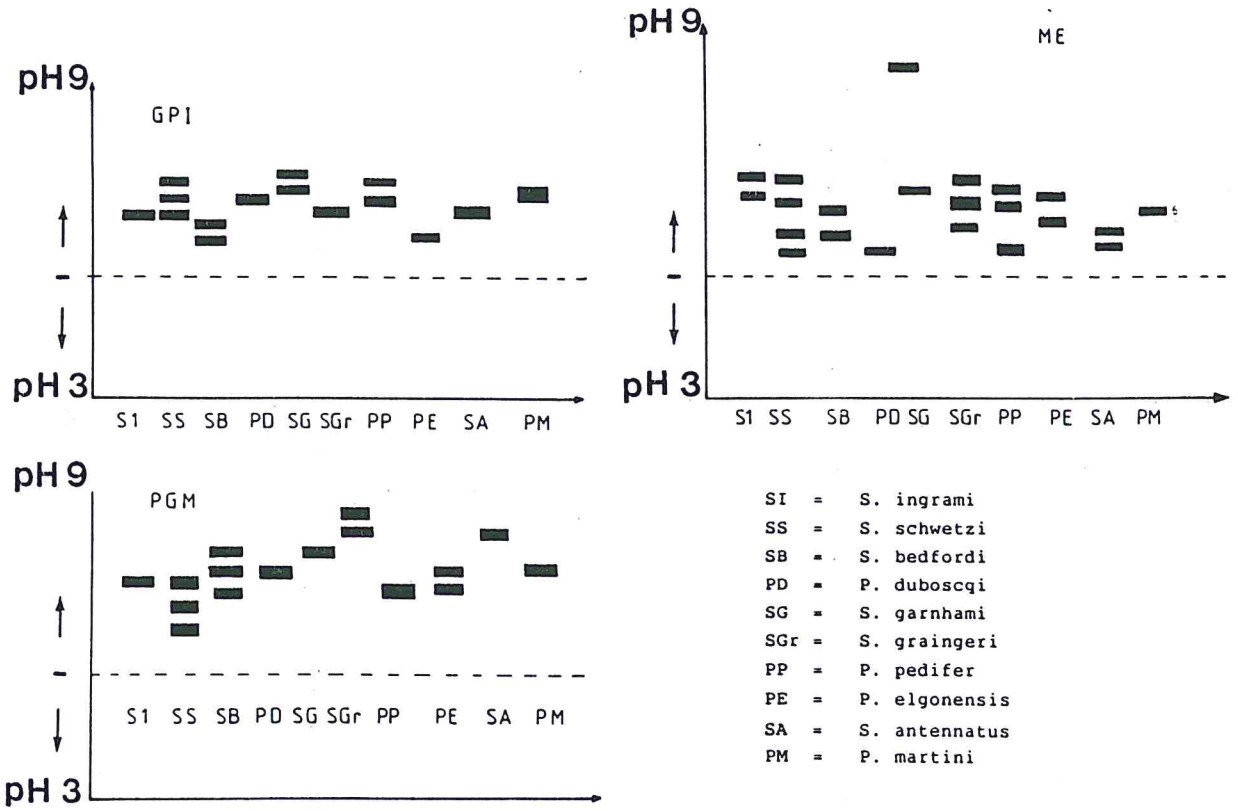
SA = S. antennatus

4.3.3. Discussion

The use of the isoelectric focusing technique in the study of sandfly species was first reported by Rogo (1985). However Rogo (1985) used conventional IEF. Allsopp and Gibson (1983) in their isoenzyme study of trypanosomes found IEF to be more discriminating when compared with other techniques, whereas Rogo (1985) found thin layer starch gel electrophoresis to be better compared with IEF, for analyzing sandfly species. This study represents the first time that IEF based on the Phast System™ and precast IEF mini gels was carried out to type sandflies. In this study nine isoenzymes MDH, G-6-PD, PGM, ME, ICD, GAPDH, 6-PDG, HK and GPI known to give good resolution in thin layer starch gel electrophoresis were assayed, to characterize the following sandfly species: P. duboscqi, P. elgonensis, P. martini, P. pedifer, S. antennatus, S. bedfordi, S. garnhami, S. graingeri, S. ingrami and S. schwetzi. Only three enzymes (GPI, ME and PGM) out of the nine showed banding patterns of taxonomic value (Figure 7). Rogo (1985) found GPI and PGM to be of practical use. Based on the finding of this work Kambona (1989) studying cassava green mites using Phast System™ found GPI, ME and PGM to be discriminatory. The fact




SI = S. ingrami
SS = S. schwetzi
SB = S. bedfordi
PD = P. duboscqi
SG = S. garnhami
SGr = S. graingeri
PP = P. pedifer
PE = P. elgonensis
SA = S. antennatus
PM = P. martini

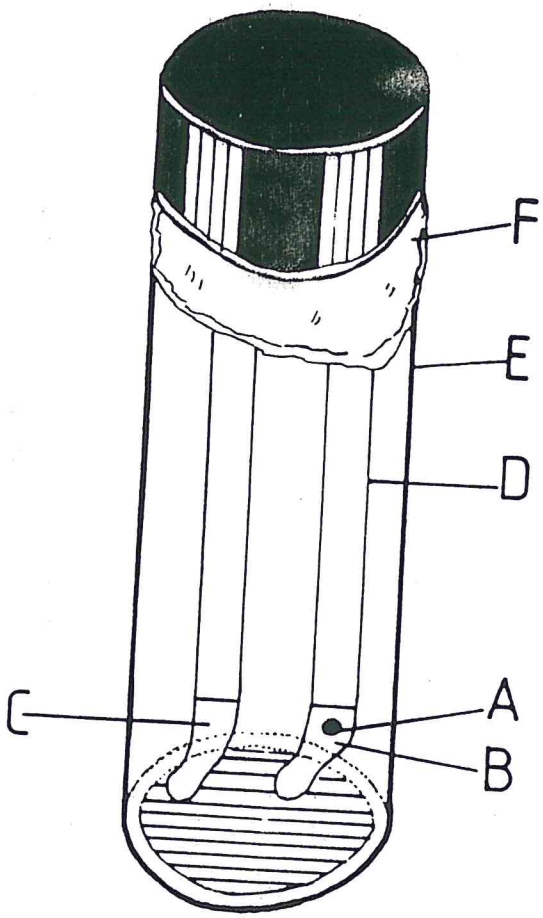
that most of the enzymes studied did not show any activity or produced faint bands suggested that the preparation methods or electrophoretic conditions may have altered the molecular structure of the isoenzymes in such a way that labile proteins lost their enzymatic properties (Hopkinson and Harris, 1976; Allsopp and Gibson, 1983). Some enzymes may also have interfered with the staining reaction to give faint bands (Anon, 1972). Banding patterns of the three isoenzymes did not show a very high degree of polymorphism for the sandflies studied. Sandflies showing no polymorphism were mainly the ones belonging to the genus Phlebotomus. This suggests that there is very little intra-specific variation within this genus except for the known Martini complex and to some extent the Pedifer group. Comparison of P. elgonensis and P. pedifer, two species morphologically very similar, did show clear differences, as Rogo (1985) and Rogo et al. (1988) reported in their study based on conventional IEF. GPI and PGM gave banding patterns which were very useful, so it is important to consider IEF based on Phast System™ for biochemical identification of sandfly or other insect species in future studies.

# CHAPTER FIVE

# PATTERN ANALYSIS OF CUTICULAR COMPONENTS

## 5.1. Introduction

Cuticular components analysis is a technique based on quantitative and qualitative analyses of the components of extracted cuticular waxes. It has been shown that this method can be an important taxonomic tool for distinguishing between insect species and sibling species of complexes (Carlson and Service, 1979; 1980; Phillips, Walsh, Garms, Molyneux, Milligan and Ibrahim, 1985; Phillips, Ward, Ryan, Molyneux, Lainson and Shaw, 1986; Kamhawi, Molyneux, Killick-Kendrick, Milligan, Phillips, Wilkes and Killick-Kendrick, 1987). The major advantages of the technique is that the specimens used for the analysis are not destroyed and old specimens can also be used. The study reported here is an attempt to develop a computerized chromatographic system based on the qualitative and quantitative differences of peak patterns of cuticular components belonging to different sandfly species.


F
E
D
C
A
B

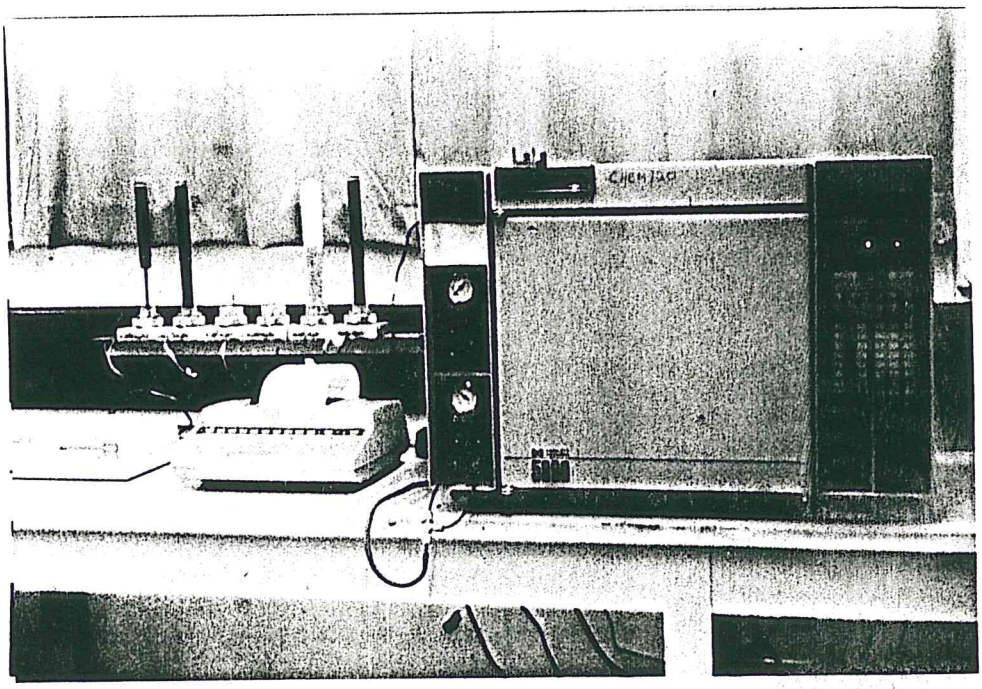
## 5.2. Methodology

Phlebotomine sandflies species used during this study were collected and reared in the same manner as reported in chapter 2.

The following species were analyzed: Phlebotomus elgonensis from Mount Elgon, P. duboscqi from Marigat, P. martini from WRAI, Nairobi, P. pedifer from Kimilili, Sergentomyia africanus from, S. antennatus from Marigat, S. garnhami from Tseikuru, S. ingrami from Marigat and S. schwetzi from MVRP colony. The sandflies were kept at room temperature in glass vials just before the start of the investigations. They were treated individualy. A total of forty individuals were analysed for each species, 20 to 25 from laboratory reared insects and the rest were field collected.

### 5.2.1. Sample preparation

Each sandfly was placed into a 2.5 cm Pyrex tube sealed at one end and 20 µl of doubled distilled hexane (Aldrich Chemical, England) was added as solvent. The tube was left at room temperature in a sample extraction vial (Figure 8) to extract the cuticular components. After 15 minutes of extraction, 5 µl of the extract was injected into the injection port of a Hewlett Packard 5890 A Gas Chromatograph (Plate 11).



5.2.2. Conditions of the HP 5890 A (

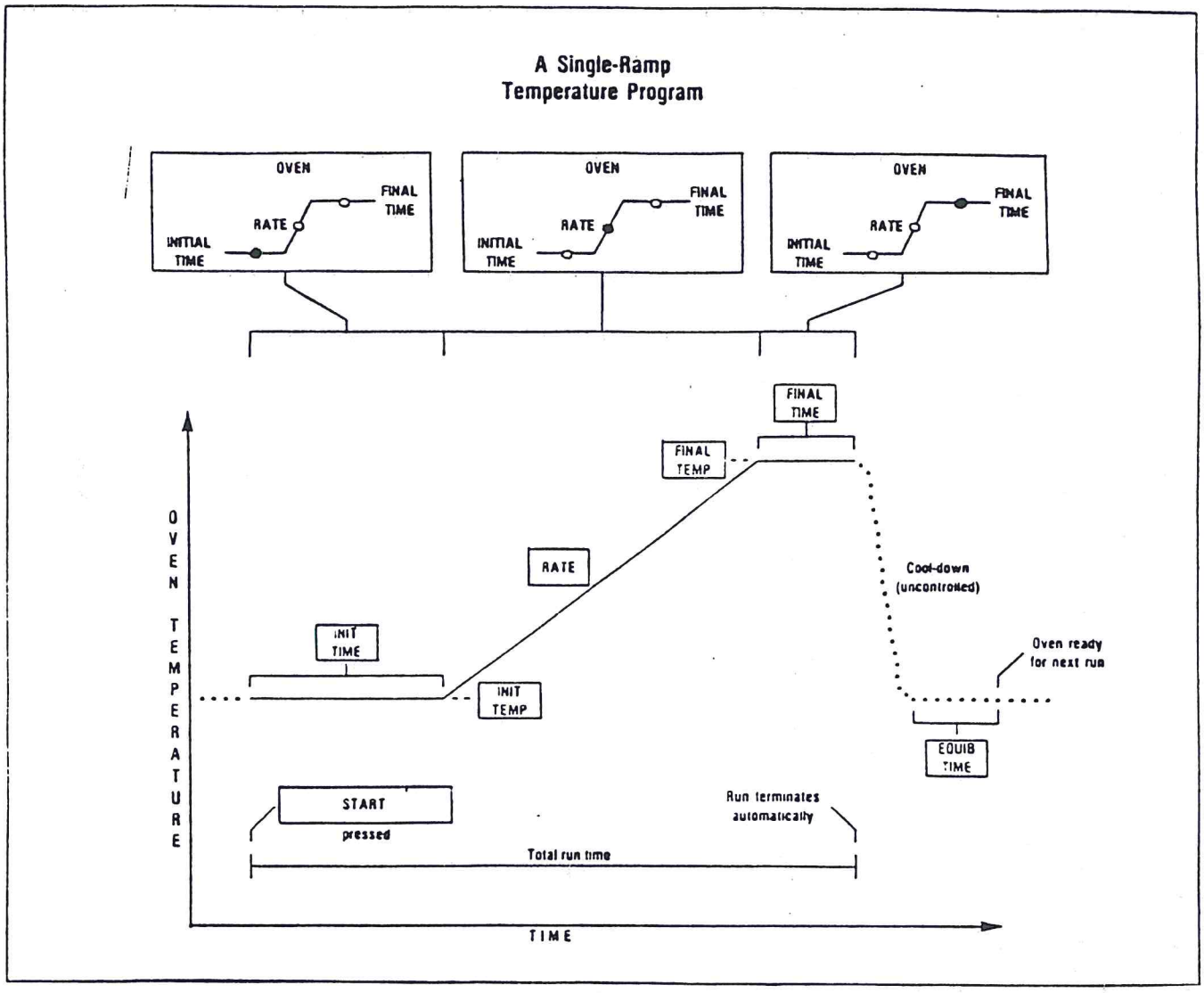
The Hewlett Packard 5890 A Gas chromat(
connected to a Hewlett Packard 3393 A (
integrator (Plate 11). The gas chromat
fitted with a 25 m Hewlett Packard fuse
capillary column crosslinked with methy
column had a 0.31 mm internal diameter
thickness. The chromatograph was fitte
ionization detector (FID). The FID res
compounds which produce ions when burne
($H_2$) - air flame. The sensitivity of t
on the ratio of $H_2$ to the flow of the c
this case nitrogen ($N_2$). After experim
carrier gas $N_2$, air and hydrogen, a sta
giving maximal response was found. Hyd
as fuel for the FID.

5.2.3. Programming conditions of HP

The HP 5890 A GC was programmed as foll

| | |
|---|---|
| . Injector temperature | 240 °C |
| . Detector temperature | 280 °C |
| . Initial temperature | 90 °C |
| . Maximal temperature | 300 °C |
| . Initial time | 4 minutes |

Single ramp running temperature program
chromatograph HP 5890 A was as follows


A Single-Ramp
Temperature Program
OVEN
FINAL TIME
RATE
INITIAL TIME
OVEN
FINAL TIME
RATE
INITIAL TIME
OVEN
FINAL TIME
RATE
INITIAL TIME
FINAL TIME
FINAL TEMP
RATE
Cool-down (uncontrolled)
INIT TIME
Oven ready for next run
INIT TEMP
EQUIB TIME
START
pressed
Run terminates automatically
Total run time
OVEN TEMPERATURE
TIME

| Rate | Final temperature | Final time |
|---|---|---|
| 10 °C | 280 °C | 20 minutes |

| | |
|---|---|
| . Equilibrium time | 3 minutes |
| . Range | 2 |
| . Attenuation | 0 |
| . Signal | A - Column compensation |

Initial temperature is a sentient temperature value at which the oven is maintained at the beginning of a temperature - programmed run. this is also the temperature to which the oven returns at termination of the temperature programmed run.

Initial time is the time at which oven temperature is held at initial temperature. It allows the solvent to go through.

Rate is the degree of speed at which the oven is to be heated or cooled.

Final temperature is the temperature which the oven attains at the end of heating or cooling ramp. In case of multiple ramps for example, a two ramp temperature programme, the final temperature for one ramp is also the initial temperature for next ramp.

Final time is the time period over which the oven temperature is held at the final temperature. In multiple ramp temperature programme, final time for one ramp is also the initial time for the next ramp.

The HP 5890 A GC is connected to the Hewlett Packard 3393 A computing integrator through the Instrument Network (INET)

### 5.2.4. Condition of the HP 3393 A computing integrator

The HP 5890 A GC was connected to the HP 3393 A computing integrator, a multipurpose instrument that can be used for quantitative and qualitative analyses. The HP 3393 A is a digital electronic integrator for plotting, integrating and quantitating chromatographic signals and data from compatible analytical instruments. It plots a chromatogram of the signal and prints a report of quantitization. It features versatile calculation, plot presentation and reporting on its built-in printer / plotter and also provides methods, sequences, calibration files and reintegration. The HP 3393 A computing integrator permits analytical instrument control through the Instrument Network (INET) and bidirectional RS-232-C data communications. It was programmed using the HP 3393 A Basic programming language. It has internal memory for storing run data, files, and operating parameters. The Hewlett Packard Interface Loop (HP-IL)

provides external data storage on disc drives as well as providing additional printing and plotting capabilities.

The run parameters used to programme the HP 3393 A computing integrator are zero, attenuation, chart speed, area reject, threshold and peak width.

. Zero is the parameter, which determines where the chromatographic base line will be plotted on the printer / plotter chart.

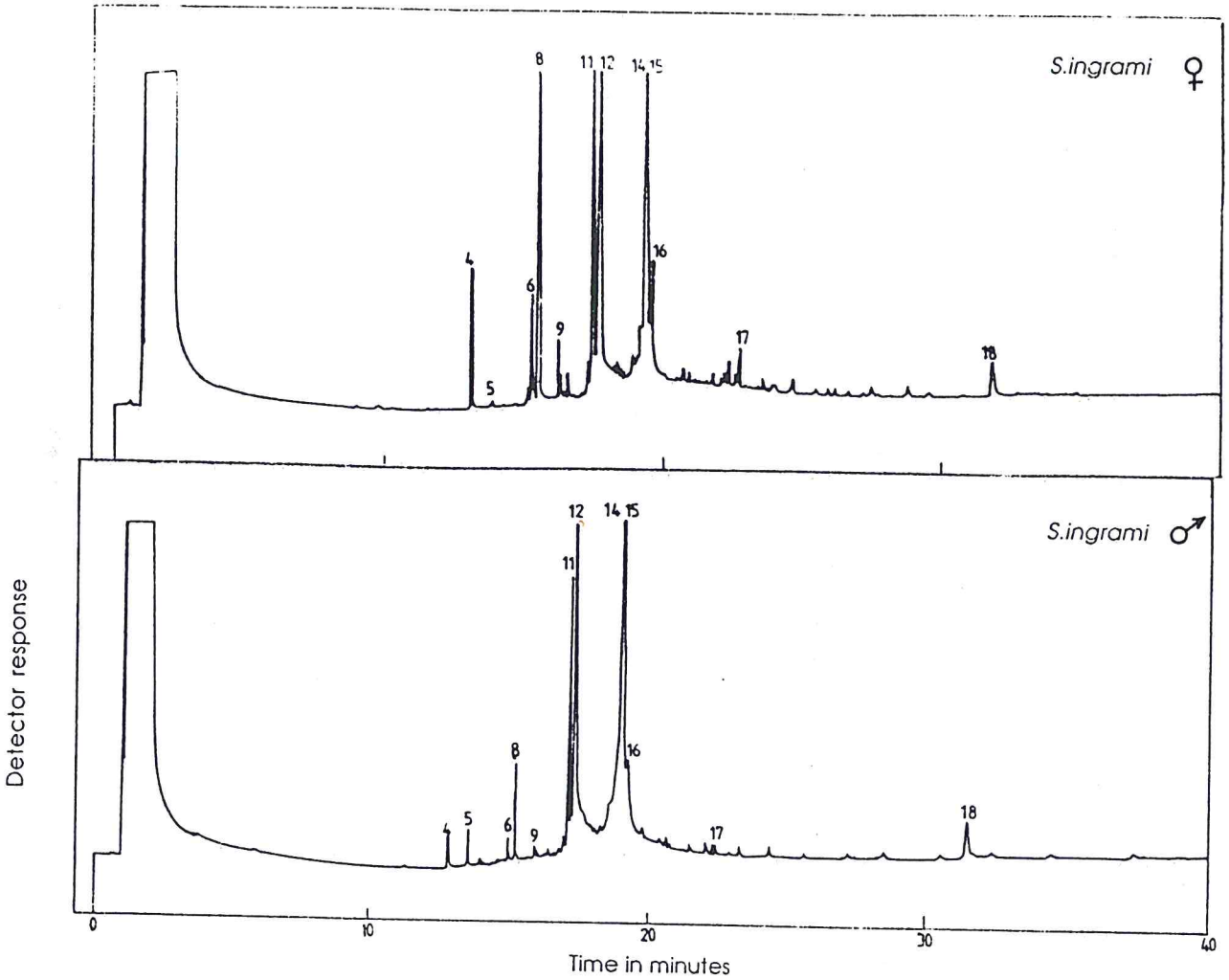
. Attenuation is the parameter, which specifies the maximum amplitude of the plotted signal required to produce full scale printhead.

Chartspeed is the set rate at which the chart paper advances during a plot.

Area reject is the parameter for peaks with less than the area reject limit. They are not reported and stored.

Threshold is the parameter, which determines peak height threshold for peak detection.

Peak width is the parameter, which optimizes peak quantitization, when it is set to match the expected width (in minutes) of a chromatographic peak at one


S.ingrami ♀
4
5
6
8
9
11 12
14 15
16
17
18
S.ingrami ♂
4
5
6
8
9
11
12
14 15
16
17
18
Detector response
0
10
20
30
40
Time in minutes

half the peak's height above the base line.

The HP 3393 A was programmed as follow:

| | |
|---|---|
| Zero | 10 |
| Attenuation | -2 |
| Chart speed | 0.5 cm/minute. |
| Area reject | 100 |
| Threshold | -1 |
| Peak width | 0.05 |

Before the sample was injected, a chromatographic "blank run" was performed. It was assumed that the baseline profile is consistent from run to run and that this may be subtracted from sample run data to remove baseline drift (usually caused by column bleed).

### 5.2.5. Data analysis

Data (based on the following variables of the peaks; for both male and female S. ingrami and for P. elgonensis and P. pedifer (Appendix 2) were examined using the multivariate analysis (SAS programme). Furthermore data of all the nine species (Appendix 2) were analysed to quantify the discriminatory power of the cuticular component chromatograms for the species identification by performing a canonical analysis of discriminance (SAS programme DISCRM) on the species data using specific measurements derived from the numbered peaks in Figure 11, Figure 12 and Figure 13.

Four separate analyses were done using the variable - area, area percent, width alone or area percent and width combined. This procedure finds an optimal multivariate combination of the variables that discriminate between the different species groups taking into account the relationship between the different variables. The results of the analysis were used to produce plots of the Data from individual flies for the first three canonical axes for simple visual interpretation of relationships. The canonical data were also used to predict the species group for each individual fly using the classification procedures provided by the programme.

Table 7: Analysis of Variance (ANOVA) of the following dependent variables (area, peaknumber, area percent, retention time, width) for S. ingrami males and females

---

Dependent variable: Area

| Source | DF | SS | Mean square | F-Value | Pr>F |
|---|---|---|---|---|---|
| Sex | 1 | 18281838 | 18281838 | .05 | .8296 |
| Type | 7 | 16978647190 | 2425521027 | 6.18 | |

| Duncan's grouping | Mean | Sex |
|---|---|---|
| A | 19404 | m |
| A | 18595 | f |

---

Dependent variable: Peak number

| Source | DF | SS | Mean square | F-Value | Pr>F |
|---|---|---|---|---|---|
| Sex | 1 | .8413255 | .8413255 | .05 | .8235 |
| Type | 7 | 844.7896743 | 120.6842392 | 7.17 | |

| Duncan's grouping | Mean | Sex |
|---|---|---|
| A | 10.761 | f |
| A | 10.587 | m |

Table 7: continued

Dependent variable: Area percent

| Source | DF | SS | Mean square | F-Value | Pr>F |
|---|---|---|---|---|---|
| Sex | 1 | 1.226233 | 1.226233 | .02 | .8813 |
| Type | 8 | 2411.610238 | 344.515748 | 6.29 | |

| Duncan's grouping | Mean | Sex |
|---|---|---|
| A | 7.794 | m |
| A | 7.585 | f |

---

Dependent variable: Retention time

| Source | DF | SS | Mean square | F-Value | Pr>F |
|---|---|---|---|---|---|
| Sex | 1 | .0261424 | .0261424 | .00 | .9737 |
| Type | 8 | 548.9220038 | 78.4174291 | 3.28 | |

| Duncan's grouping | Mean | Sex |
|---|---|---|
| A | 19538 | f |
| A | 19509 | m |

Table 7: continued

Dependent variable: Width

| Source | DF | SS | Mean square | F-Value | Pr>F |
|---|---|---|---|---|---|
| Sex | 1 | .00000314 | .00000314 | .00 | .9563 |
| Type | 8 | .02054700 | .00293529 | 2.82 | |

| Duncan's grouping | Mean | Sex |
|---|---|---|
| A | .06091 | m |
| A | .06058 | f |

Remark: same letter means there is no significant difference

## 5.3. Results

The cuticular components of the species Phlebotomus elgonensis, P. duboscqi, P. martini, P. pedifer, Sergentomyia africanus, S. antennatus, S. garnhami, S. ingrami and S. schwetzi were analyzed. Different features of cuticular components peaks were examined and analysed by multivariate techniques to determine if:

(a) males and females of a given sandfly species can be differentiated;

(b) the nine different species studied can be effectively distinguished, and that cuticular component patterns provide a basis for grouping the sandfly species.

(a) Sex differences

The males and females of all the species analysed showed some quantitative differences. For example, Figure 11 shows an apparent quantitative difference between males and females of S. ingrami, with the height of some peaks being higher for females than for males. However, analysis of variance of male and female S. ingrami showed no significant differences between the sexes in peak variables (Table 7). Five variables (peaknumber, retention time, area, width and area percent) of the chromatograms obtained from sandfly species were considered but showed no significant differences ($P > 0.05$).

(b) Species differentiation and grouping inspection of

Table 8: Analysis of variance (ANOVA) of the following sandfly species, P. elgonensis, P. pedifer and S. ingrami, based on the variables; area, area percent and width

---

Dependent variable: Area

| Source | DF | SS | Mean square | F-value | Pr>F |
|---|---|---|---|---|---|
| Model | 17 | 74155477600 | 4362086918 | 19.15 | .001 |
| Error | 202 | 46016752430 | 227805705 | | |

| Duncan's grouping | Mean | Species |
|---|---|---|
| A | 30617 | P. elgonensis |
| B | 18913 | P. pedifer |
| C | 12580 | S. ingrami |

---

Dependent variable: Area percent

| Source | DF | SS | Mean square | F-value | Pr>F |
|---|---|---|---|---|---|
| Model | 17 | 10157.40379 | 597.49434 | 28.88 | .001 |
| Error | 202 | 4178.68094 | 20.68654 | | |

| Duncan's grouping | Mean | Species |
|---|---|---|
| A | 10.132 | P.pedifer |
| AB | 9.255 | P. elgonensis |
| B | 7.667 | S. ingrami |

Table 8: continued

Dependent variable: Width

| Source | DF | SS | Mean square | F-value | Pr>F |
|---|---|---|---|---|---|
| Model | 17 | .032107196 | .01888659 | 49.61 | .001 |
| Error | 202 | .7690795 | .00038073 | | |

| Duncan's grouping | Mean | Species |
|---|---|---|
| A | .07551 | P. elgonensis |
| A | .07275 | P. pedifer |
| B | .06071 | S. ingrami |

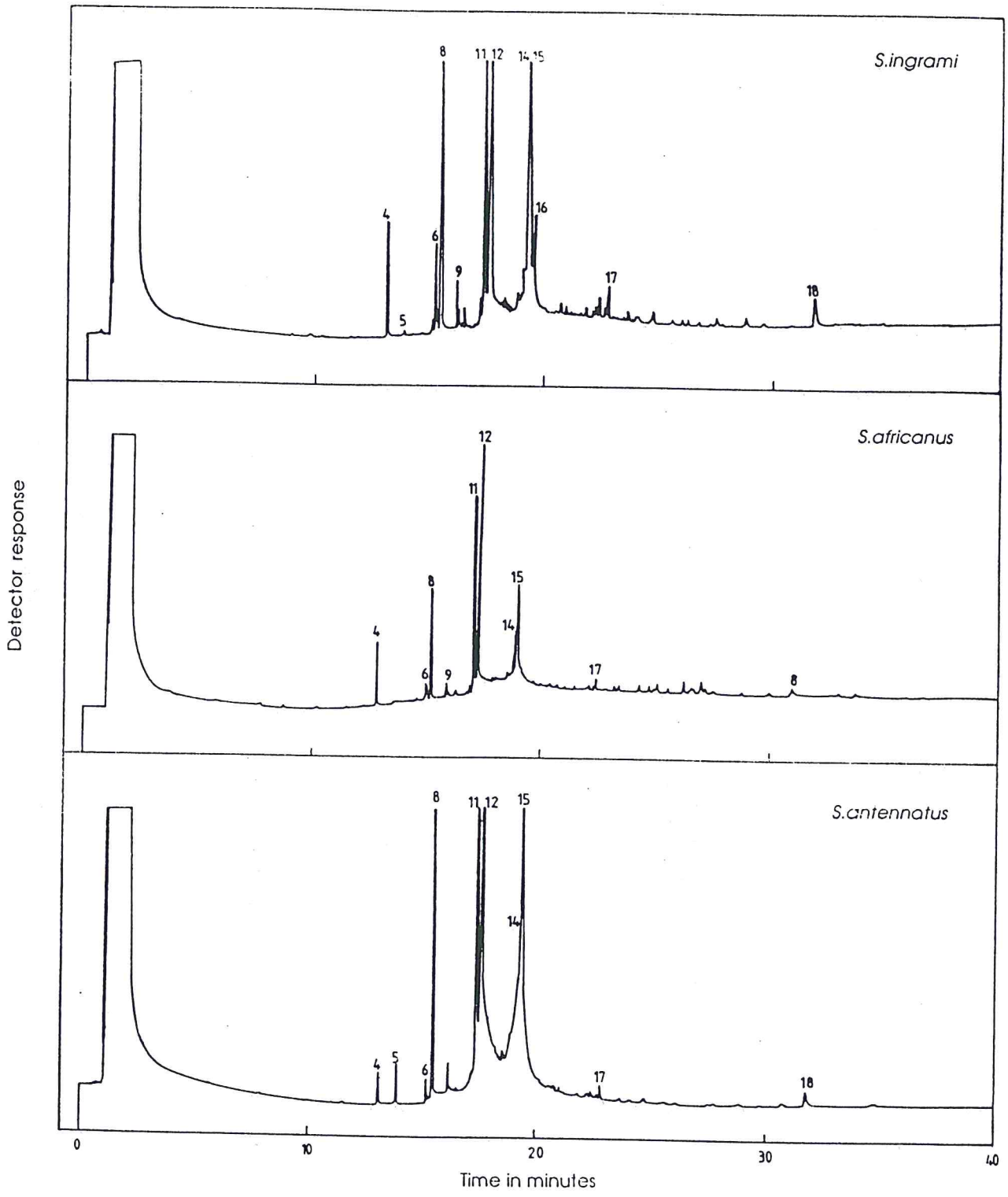
Remark: Same letter means there is no significant difference

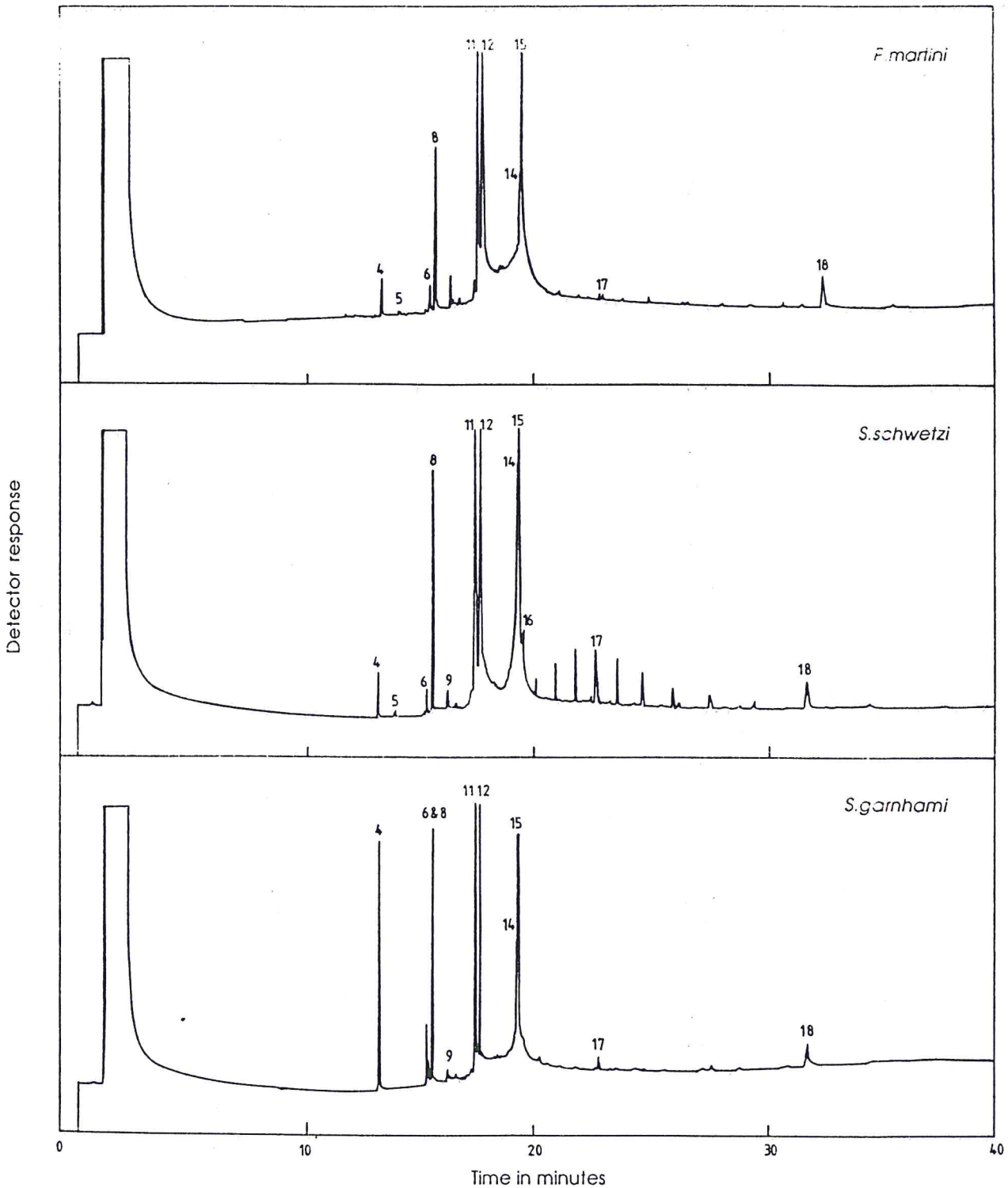
patterns of females of Phlebotomus elgonensis, P. duboscqi, P. martini, P. pedifer, Sergentomyia africanus, S. antennatus, S. garnhami, S. ingrami and S. schwetzi showed clear visual differences between these species (Figure 11, Figure 12 and Figure 13). Even in morphologically similar species, such as P. elgonensis and P. pedifer there were distinct qualitative and quantitative differences which were clearly discernible.

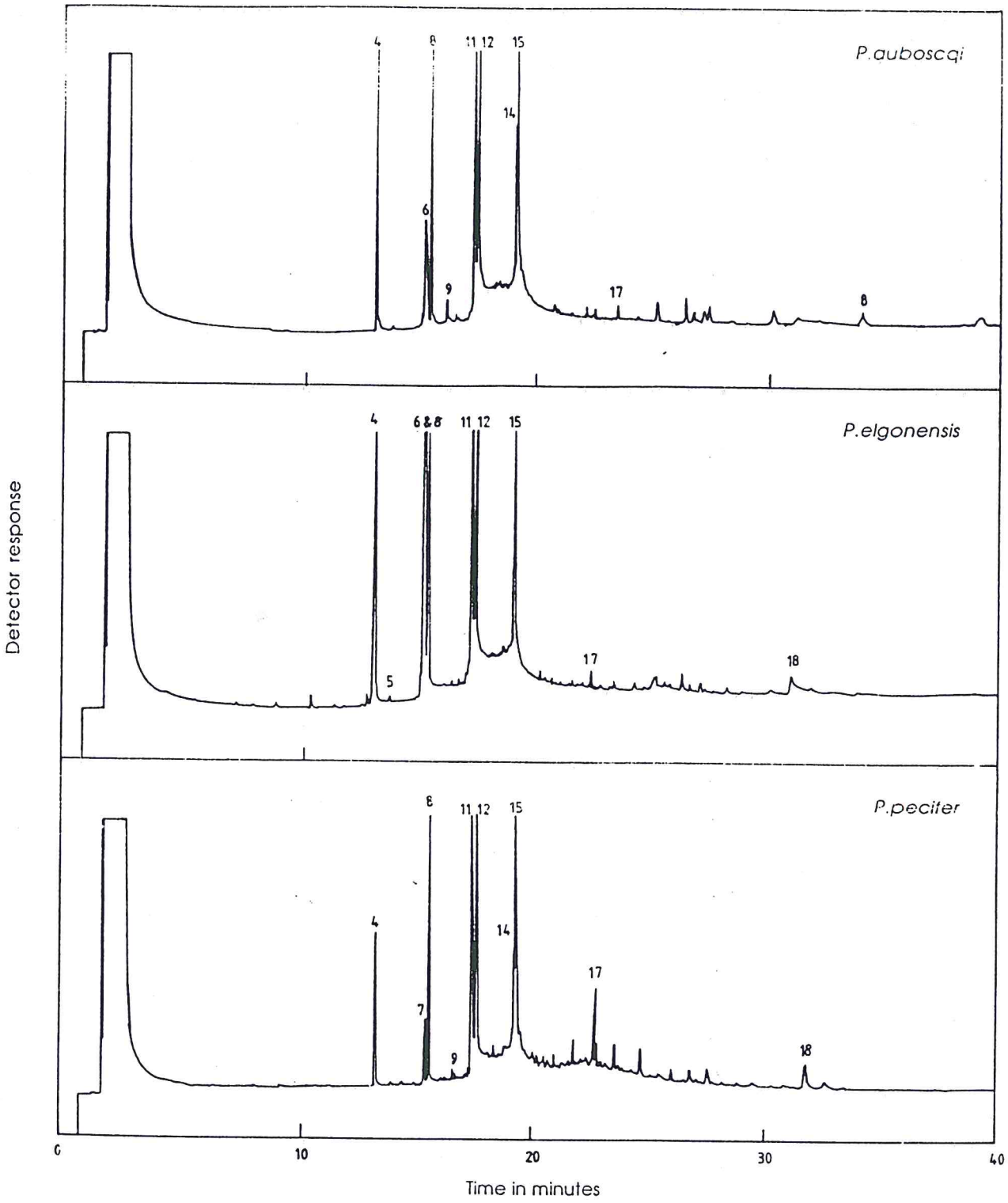
Analysis of all peak data from P. elgonensis, P. pedifer and S. ingrami showed significant differences at the 0.001 level, when area, width and area percent were taken into consideration (Table 8).

Discriminant analysis of all the nine species based on the different characteristics for each peak (area, area percent and width) also showed significant differences.

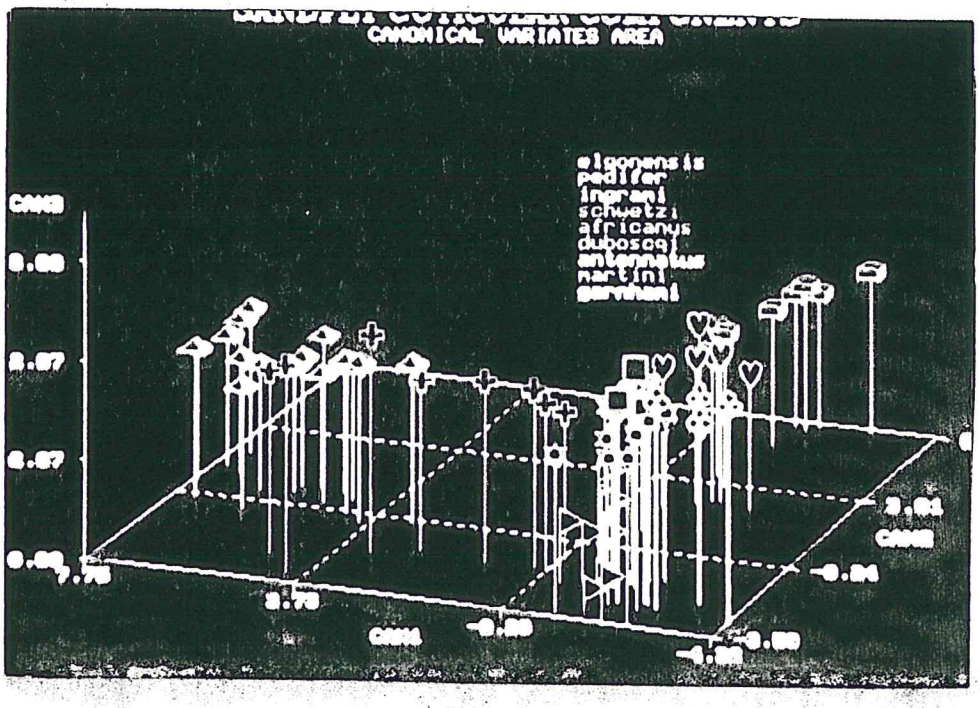
Based on peak area, the sandflies were scattered in practically the whole surface of the plot (Plate 12) and it was difficult to group the sandflies into genera. The variable area could not discriminate between the majority of the sandfly species (Table 9) and the correlation with morphological identification was very poor (Table 9). This is to be expected since large differences between the absolute quantities of components are normally found between individuals of a given species.


S.ingrami
S.africanus
S.antennatus
Detector response
Time in minutes
0
10
20
30
40


F.martini
S.schwetzi
S.garnhami
Detector response
Time in minutes
0
10
20
30
40


P.auboscqi
4
8
11 12
15
14
6
9
17
8
P.elgonensis
4
6&8
11 12
15
5
17
18
P.peciter
8
11 12
15
4
14
17
7
9
18
Detector response
0
10
20
30
40
Time in minutes

Discrimination based on the variable area percent (Plate 13) was more revealing as was the correlation with morphological identification was also better (Table 10). In addition, the sandfly species were grouped into three main clusters: P. elgonensis and P. pedifer; S. ingrami and S. schwetzi; and P. duboscqi, P. martini, S. africanus, S. antennatus and S. garnhami. Plots of scores of peak width showed the groupings: P. elgonensis and P. pedifer; S. garnhami, S. schwetzi, P. duboscqi and P. martini; S. ingrami; S. africanus and S. antennatus (Plate 14). Plots of the canonical variates showed scores that were practically the same as those based on area percent (Table 10 & Table 11). Matching between the results obtained from peak widths and morphological data gave 85 % correlation (Table 11). The combination of area percent and width (Table 12) gave 100 % correlation between the two methods for all species except P. duboscqi, which gave 89 % matching. Thus of the various canonical variates based on (a) area, (b) area percent, (c) width and (d) area percent combined with width, used to differentiate the flies collected from the field, (b) and (c), and particulary the combination of the two, provided the best discrimination between the species and the best correlation between the two methods. The relatively low correlation between the two methods for P. duboscqi may be due to the fact that




S. garnhami

S. africanus

S. antennatus

P. pedifer

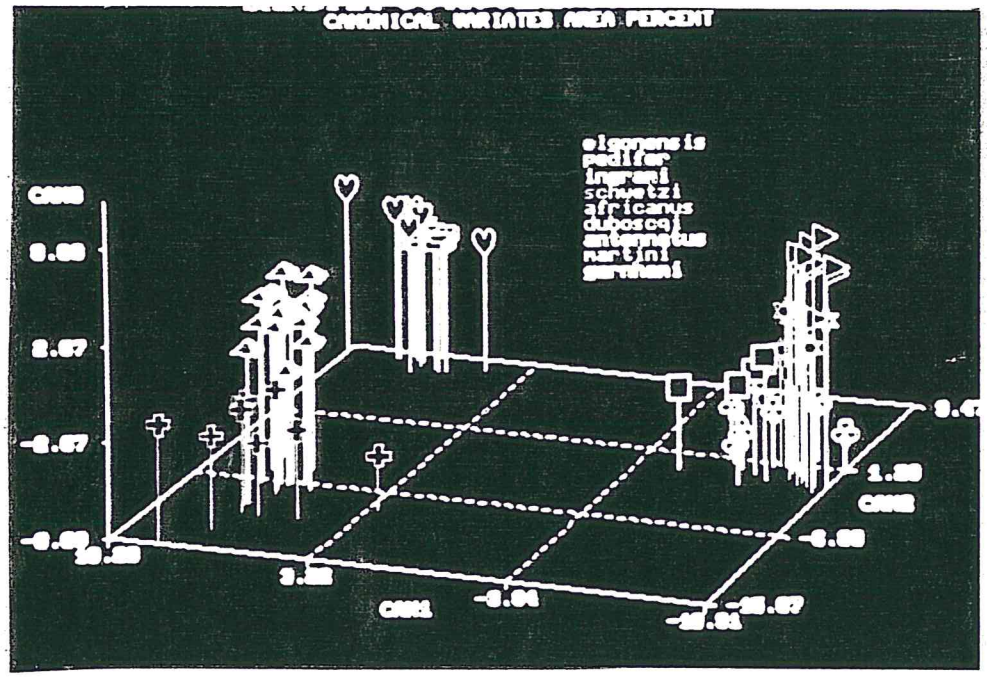
P. elgonensis

S. ingrami

S. schwetzi

P. martini

P. duboscqi




S. garnhami

S. africanus

S. antennatus

P. pedifer

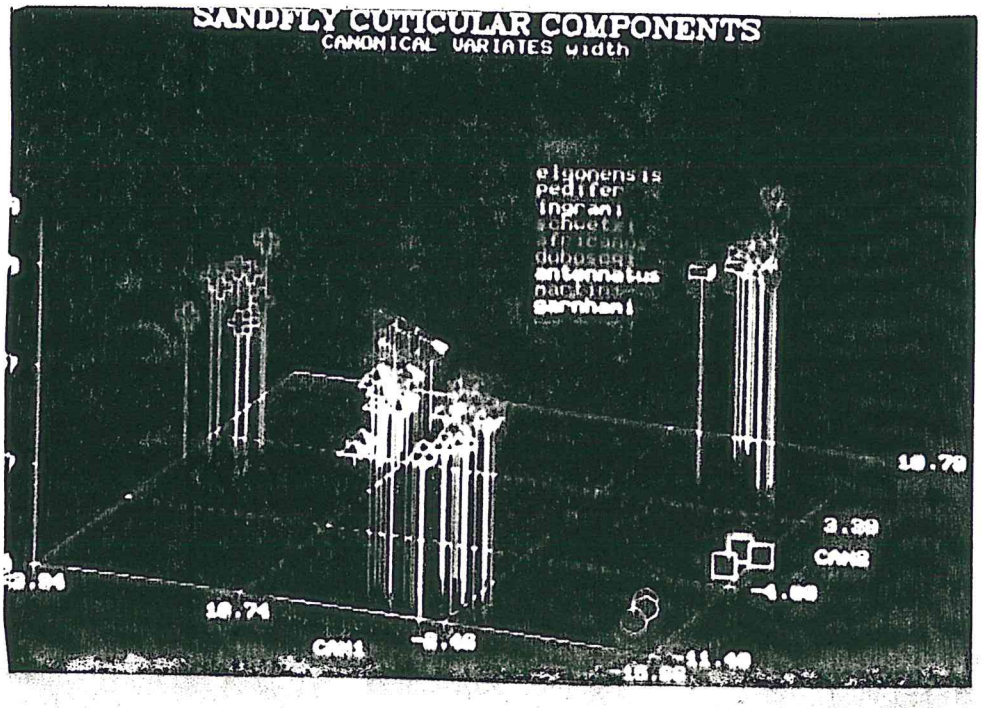
P. elgonensis

S. ingrami

S. schwetzi

P. martini

P. duboscqi




*S. garnhami*

*S. africanus*

*S. antennatus*

*P. pedifer*

*P. elgonensis*

*S. ingrami*

*S. schwetzi*

*P. martini*

*P. duboscqi*

some individuals might have been misidentified morphologically. Closer examination of the individuals in this group is clearly warranted.

It is interesting to note that the canonical variates plots for P. elgonensis and P. pedifer are particularly closely related. S. garnhami showed close association to the genus Phlebotomus in Plate 12 and 14. Other species such as S. africanus and S. antennatus known from the literature to be subspecies, were also very close to each other.

Table 9: Percentage of matching (discriminant analysis) of nine sandfly species after canonical variates analysis of area of cuticular component peaks with morphological identification

| SPECIES | Correlation | No correlation |
|---|---|---|
| S. africanus | 100 % | - |
| S. antennatus | 100 % | - |
| P. duboscqi | 44.44 % | 55.55 % |
| P. elgonensiss | 85.71 % | 14.29 % |
| S. garnhami | 100 % | - |
| S. ingrami | 95 % | 5 % |
| P. martini | 92.86 % | 7.14 % |
| P. pedifer | 100 % | - |
| S. schwetzi | 100 % | - |

Table 10: Percentage of matching (discriminant analysis) of nine sandfly species after canonical variates analysis of area percent of cuticular component peaks with morphological identification

| SPECIES | Correlation | No correlation |
|---|---|---|
| S. africanus | 100 % | - |
| S. antennatus | 100 % | - |
| P. duboscqi | 66.67 % | 33.33 % |
| P. elgonensis | 85.71 % | 14.29 % |
| S. garnhami | 100 % | - |
| S. ingrami | 95 % | 5 % |
| P. martini | 92.86 % | 7.14 % |
| P. pedifer | 100 % | - |
| S. schwetzi | 100 % | - |

Table 11: Percentage of matching (discriminant analysis) of nine sandfly species after canonical variates analysis of width of cuticular component peaks with morphological identification

| SPECIES | Correlation | No correlation |
|---|---|---|
| S. africanus | 100 % | - |
| S. antennatus | 100 % | - |
| P. duboscqi | 88.69 % | 11.11 % |
| P. elgonensis | 100 % | - |
| S. garnhami | 100 % | - |
| S. ingrami | 100 % | - |
| P. martini | 92.86 % | 7.14 % |
| P. pedifer | 100 % | - |
| S. schwetzi | 100 % | - |

Table 12: Percentage of matching (discriminant analysis) of nine sandfly species after canonical variates analysis area percent and width of cuticular component peaks with morphological identification

| SPECIES | Correlation | No correlation |
|---|---|---|
| S. africanus | 100 % | - |
| S. antennatus | 100 % | - |
| P. duboscqi | 88.89 % | 11.11 % |
| P. elgonensis | 100 % | - |
| S. garnhami | 100 % | - |
| S. ingrami | 100 % | - |
| P. martini | 100 % | - |
| P. pedifer | 100 % | - |
| S. schwetzi | 100 % | - |

## 5.4. Discussion

Cuticular components analysis has previously been shown to be of important taxonomic value for distinguishing between different insect species (Carlson and Service, 1979; 1980; Phillips et al. 1985; 1986). In the present study , this technique was used to complement the analysis of isoenzymes of sandfly species based on thin layer starch gel electrophoresis and isoelectric focusing. Analyses were performed after chromatograms of cuticular components were obtained for the different species studied. It is interesting to note that chromatograms of males and females of the same species were qualitatively the same. This finding is different from that reported by Nelson et al. (1981), who found that individual males of the house fly have a much simpler hydrocarbon composition as well as smaller amounts of these compounds compared to females. In a number of dipteran species cuticular hydrocarbons components act as pheromones (Carlson et al., 1978 and Jallon, 1985) and the sexes are readily discernible.

Comparison of the patterns of Phlebotomus elgonensis, P. duboscqi, P. pedifer, Sergentomiya africanus, S. antennatus, S. garnhami, S. ingrami and S. schwetzi demonstrated qualitatively differences which were visible. Multivariate statistical analysis and discriminant functions were shown to be of great importance in helping to analyse data recorded from the

samples after a chromatographic run. Thus Ready and da Silva (1984) showed the possibility of quantitatively separating the sympatric Psychodopygus wellcomei from other Psychodopygus females. Likewise quantitative analysis of cuticular components of members of the Simulium damnosum Enderlein, S. sirbanum, S. yahense and S. sanctipauli also showed differences between these species (Lane and Ready, 1985).

In the present study, multivariate analysis of the species P. elgonensis, P. pedifer, which are morphologically very difficult to differentiate (Mutinga and Ngoka, 1978; Rogo, 1985; Rogo et al. 1988) have shown significant differences. Thus cuticular component pattern analysis may be used to distinguish not only between distinctly related species but also closely related sibling species. Further work in this area will throw more light in the degree of the effectiveness of the method.

This suggests that cuticular components analysis is a very useful taxonomic tool with distinct advantages. In one day, up to a dozen runs (chromatograms) can be obtained for individual flies. Dried individual insects can be used for analysis without being destroyed. The same individual can also be mounted for morphological examination or kept as voucher specimen.

# CHAPTER SIX

## GENERAL DISCUSSION

Until recently, the taxonomy of sandflies has relied mainly upon morphological criteria based on the features defined by Kirk and Lewis (1952). Perlifi'ev (1968) showed that various measurements based on these features were of value for the characterisation. Indeed, these criteria were used to describe a series of new species of sandflies in Kenya following the discovery of visceral leishmaniasis in Machakos District and other areas after World War Two (Anderson, 1943; Fendahl, 1952; Heisch, 1954; Mckinnon and Fendahl, 1956; Heisch, Guggisberg and Teedale, 1956; and Mutinga and Ngoka, 1978). To date, 40 sandfly species from Kenya belonging to the genera Phlebotomus and Sergentomyia have been described. However studies by different authors have demonstrated that many of the sandflies examined were subject to wide variations within a single species and many difficulties have been encountered in differentiating between some groups.

For example, two sandfly collections originally from Lake Naivasha (Lewis et al., 1974) and Mount Elgon (Ngoka et al., 1975) areas, respectively,

were identified as different species on the basis of the number of hairs on the coxite. The distance between the two locations is about 200 km. The Lake Naivasha collection, named Phlebotomus aculeatus, had 30-45 hairs whereas the Mount Elgon collection, named Phlebotomus elgonensis had 65-75 hairs. However, it was later shown by isoenzyme analysis that the two belong to the same species (Lewis, 1982).

Interestingly, P. elgonensis is morphologically very similar to P. pedifer found near Kimilili, about 240 km from Mount Elgon (Lewis et al., 1974; Ngoka, Madel and Mutinga, 1975). P. pedifer transmits the causative agent of cutaneous leishmaniasis whereas P. elgonensis is not a vector. Morphological differentiation between the two species has always presented a problem.

Many cases of misidentification have also been reported for sibling species belonging to the Synphlebotomus complex i.e. Phlebotomus celiae, P. martini, P. vansomeranae (Minter, 1963; 1964), which are also very difficult to find. Likewise, Sergentomyia africanus, S. antennatus, S. bedfordi and S. squamipleuris, considered to be subspecies, are very difficult to differentiate because they are separated only by very minor morphological characters (Abonnenc, 1972).

A critical study of the literature (Abonnenc, 1972; Perfili'ev, 1968; Ngoka et al., 1975; Rogo, 1985 and Rogo et al., 1988) shows that the source of the difficulties is invariably associated with the nature of morphological features. For example, the number of cebarial teeth is normally expressed as a range which may be wider than the true range, because an exact count is usually not practical (some lateral teeth are usually hidden). These ranges for different species often overlap considerably. In addition, it is difficult to be precise when measuring morphometric characters on mounted sandfly specimens and environmental factors can also influence organisms in ways that may alter morphological characters resulting in misidentification..

In this study, the use of biochemical techniques was explored to identify, map and classify sandfly species of known origin as a basis for developing, in the future, a data bank which will help in the exact identification of the sandflies. The computer connected with the GC will have all the chromatograms of the species studied, saved in the hard disk. After injecting a new sample in the GC and the total run the computer will compute the data and show if the sample injected is from a known species. This will

lead to better understandin of the epidemiology of the disease leishmaniasis.

Two methods, viz isoenzyme analysis (based on thin layer starch gel electrophoresis and isoelectric focusing) and pattern analysis of cuticular components were used in comparison with morphological criteria.

Three isoenzymes, namely GPI, MDH and PGM, were selected for the development of dendrograms based on the Jaccard's and simple matching© coefficients. Two patterns were obtained: the dendogram obtained from the Jaccard's matching showed two groupings corresponding to the genera Phlebotomus and Sergentomyia whereas the Simple matching dendogram showed more or less three groupings corresponding to Phlebotomus, Sergentomyia and P. duboscqi. Interestingly, P. duboscqi is generally accepted as a member of the Phlebotomus genus. In both dendrograms, S. garnhami, a species generally classified under the genus Sergentomyia was found to be associated with the genus Phlebotomus. It is interesting to note that S. garnhami was reported to be a possible vector of visceral leishmaniasis (Mutinga and Odhiambo, 1982; Mutinga and Kyai, 1985). This and the results of our analysis raise some interesting questions regarding the biology and epidemiology of this species. Studies are currently under way

to establish the vectorial capacity and the exact status of this species based on these findings.

The alternative electrophoretic technique based on isoelectric focusing (IEF) on the Phast System™ (Pharmacia) evaluated in the present study demonstrated that this technique can be useful with certain advantages in the identification of sandfly species and other insects. Of the nine isoenzymes screened (ICD, 6-PGD, G-6-PD, GAPDH, ASAT, HK, MDH, GPI and PGM), GPI, ME and PGM showed greater promise. All the nine sandfly species examined could be separated neatly by these enzymes although there was a tendency for the bands to fade quite rapidly. The Author's experience with the Phast System™ is in line with the observation of Allsopp and Gibson (1983) who found that conventional isoelectric focusing improved discrimination. Contrary to Rogo (1985) who found that the conventional IEF is less discriminatory than the thin layer starch gel electrophoresis, the Phast System™ IEF in this study was very useful for taxonomic purposes. It is clear, however, that this system based on precast gels constitutes an improvment of the IEF technique in two ways. First, the amount of samples required is relatively low, suggesting that a broader profile of the enzymes of an insect may be detected. Second, the time taken to run

IEF on the Phast System™ is considerably shorter. Moreover, it is relatively easy to operate. Thus it is a very useful additional tool for the study of the taxonomy and population genetics of the sandflies and other insects.

Gas chromatographic patterns from sandfly cuticular components were obtained with the aim of finding features useful for species differentiation. Both quantitative and qualitative differences of cuticular component patterns were found to be of potential value for taxonomic purposes. Indeed the cuticular component patterns were characteristic for each species examined. Previously, Phillips et al. (1985, 1986) and Kamhawi et al. (1987) analysed the hydrocarbon fractions of the cuticular waxes of the adults of Simulium damnosum complex and populations of Phlebotomus ariasi respectively and found the technique effective in differentiating between these species or biotypes. In our study we examined chromatographic patterns obtained from the whole extracts which include all classes of compounds present. Species differentiation was possible by simple visual examination suggesting that profiles obtained from the whole wax extracts provide richer and characteristic sets of information. Multivariate and discriminant function analyses of selected peaks were

undertaken to quantify the differences between the sandflies species studied. The usefulness of these statistical methods were also demonstrated by Phillips et al. (1985, 1986) and Kamhawi et al. (1987). Comparison between morphological taxonomy and cuticular component patterns analysis gave 100% correlation for all the species studied except P. duboscqi. This may be probably the result of the presence of a different sandfly species in the P. duboscqi population studied. This peculiarity of the population is being investigated to establish the exact identity of the few insects whose cuticular component patterns did not match with their classification based on morphological examination. It was interesting to note that the pattern of cuticular components of male and females of the same sandfly were qualitatively the same. The quantitative differences that were found between males and females of the same species were not significant. This finding differs from those obtained for Musca domestica (Nelson et al. 1981), where females have a more complex cuticular components than the males. The cuticular waxes of the females of these insectes are also a source of sex stimulating pheromones.

Comparison of isoenzymes, cuticular components pattern analyses and morphometrics

suggests that the two biochemical techniques (thin layer starch gel electrophoresis and cuticular component analyses) are more diagnostic and reliable taxonomic tools especially in distinguishing between closely related sandfly species. These two techniques made it possible to differentiate between morphologically closely related species which are most important in the transmission of the disease i.e. P. elgonensis and P. pedifer. It will be interesting to try these techniques on P. longipes which is not found in Kenya and which is very similar to the other two. P. longipes is known to be a vector of Leishmania tropica which causes dermal leishmaniasis in the Ethiopian highlands (Lewis et al. 1982). The effectiveness of the two biochemical methods (isoenzymes and cuticular components pattern analyses) is further illustrated by their ability to differentiate unambiguously between S. africanus, S. antennatus and S. bedfordi which are closely related subspecies.

Comparison of the results presented in this thesis suggets that if the 3 methods i.e. morphological taxonomy, isoenzyme and cuticular component analyses were used, the cuticular component analysis is the most useful technique for the identification of sandfly species because

of the ease with which the species studied can be differentiated in a relatively clear manner and its very high reproducibility of 90%. The inclusion of all classes of compounds in the analysis provides a broader profile of the components for comparison and is recommended rather than analysis of only the hydrocarbon fraction undertaken by most workers. It is significant that the chromatogrammes of two morphologically very similar species P. elgonensis/ P. pedifer and S. africanus/S. antennatus showed clear differences that were dicernible visually. It was, however, unfortunate that during this study only P. martini of the Synphlebotomus complex was available. The other two species P. celiae and P. vansomeranae were unavailable for comparison. The location of these rare species and their study could help solve the difficulties associated with their identification. An additional advantage of the method is that, unlike the other two methods, the test specimen need not to be sacrificed and the individual flies can be preserved for morphological examination. Moreover, unlike isoenzyme analysis, no special arrangements such as for blood ( as diet for haematophagous insects) or liquid nitrogen (for preservation of the insects) need to be made during transportation of

the flies from the field to the laboratory. Clearly it will be useful to extend this study to other sandfly species to determine the extent of the method's relative advantage and perhaps to discover new species which may have been misidentified by morphological examinations.

The cuticular component analysis is a relatively new method. Recently it has been used in the study of the taxonomy of related plant and insect species and has shown great promise in solving sibling species identification problems. The technique could be refined by linking a gas chromatograph to a computer system that would allow storage of all reference chromatograms belonging to identified species to be made thus facilitating quick comparison and identification of new sandfly collections. The sophistication in instrumentation involved, however, means that only a few specialized institutions such as the International Centre of Insect Physiology and Ecology (ICIPE) would have the resources to instal the system. This would make the ICIPE one of the reference centres in the taxonomy of sandflies and other insects.

In summation, it is hoped that this study has made some useful contributions toward the development of new alternative techniques for the

identification, classification and population genetic studies of sandfly species.

Suggestions for future work

Biochemical techniques can be used for the identification of sandfly species. However, in this study, sandfly isoenzyme analyses based on thin layer starch gel electrophoresis and Phast System™ IEF gave various degrees of polymorphism for the enzymes GPI, MDH, ME and PGM, suggesting that the sandfly population in Kenya is heterogenous. It is advocated that more sandfly species and enzymes be screened with the purpose of developing a more comprehensive system for distinguishing between a larger profile of sandflies. However, in view of its usefulness in genetic grouping in data obtained from such extended work, isoenzyme analysis should be used to study genetic variations in sandfly populations. In view of the particularity shown by S. garnhami in both dendograms studies, the morphology and the feeding habit of this species need to be further investigated to determine if there is a subspecies of S. garnhami.

Pattern analysis of cuticular components has shown a lot of promise as a taxonomic tool. This work needs to be extended to other sandfly species with the objective of developing a computerized gas Chromatograph system for sandfly taxonomy. The lower level of correlation between

morphological data and pattern analysis of cuticular components for P. duboscqi needs to be investigated in detail to find out the underlying cause.

# CHAPTER SEVEN

## SUMMARY

1. Phlebotomus elgonensis, P. duboscqi, P. martini, P. pedifer, Sergentomyia africanus, S. antennatus, S. bedfordi, S. garnhami, S. graingeri, S. ingrami and S. schwetzi collected from the field and from the laboratory were used to identify useful isoenzyme banding patterns for taxonomic purposes.

2. Eighteen isoenzymes were assayed using thin layer starch gel electrophoresis and only nine, namely ICD, GAPDH, ASAT, GPI, G-6PD, HK, MDH, 6-PDG and PGM, showed visible banding patterns.

3. These enzymes showed many bands at different position from the origine, however only three enzymes GPI, MDH and PGM were selected for the identification of sandfly species.

4. GPI, MDH and PGM showed differences in the number of bands of males and females of the same species, which can be an important factor for genetic variations.

5. Isoenzyme banding patterns based on GPI, MDH and PGM showed capabilities to distinguish between sandfly species including P. elgonensis and P. pedifer, two morphologically similar species.

6. Phenetic relationships between the sandfly species based on the three enzymes GPI, MDH and PGM using Jaccard's coefficient and the simple matching coefficient grouped the flies into the genera Phlebotomus and Sergentomyia.

7. It was noted that S. garnhami, a species known morphologically to belong to the genus Sergentomyia was found to be associated with the genus Phlebotomus, a finding to be investigated to etablish the importance of this species.

8. Phast System™ isoelectric focusing of the following nine enzymes, namely ICD, GAPDH, ASAT, GPI, G-6PD, HK, MDH, 6-PDG and PGM was carried out to determine which enzymes could be used for the identification of sandfly species.

9. Three enzymes GPI, ME and PGM showed visible banding patterns after isoelectric focusing. Banding patterns of these enzymes could distinguish between the flies effectively.

This technique could also be used for genetic studies of members of the same species.

10. The cuticular component pattern of Phlebotomus elgonensis, P. duboscqi, P. martini, P. pedifer, Sergentomyia africanus, S. antennatus, S. garnhami, S. ingrami and S. schwetzi showed qualitative and quantitative differences between males and females of the same species and different species.

11. Cuticular component peaks of S. ingrami illustrated quantitative differences between males and females , that were not significant at 0.05 level for the variables area, area percent, peak number, retention time and width. This is to suggest that future work could be concentrated on female sandflies only.

12. P. elgonensis and P. pedifer, two morphologically similar species were distinguished through the qualitative differences of their cuticular component pattern.

13. Analysis of variance of the variables area, area percent and width for P. elgonensis and P. pedifer showed significant diffences at the 0.001

level, thus confirming, the clear qualitative differences in point 12.

14. Discriminant functions analysis compared to morphological taxonomy showed some differences in classification results based on the variables area, area percent, width and area percent combined with width.

15. The best variable for species discrimination was area percent combined with width, followed by width, area percent and area.

16. Cuticular components pattern analyses can be used to identify morphologically identical sandfly species belonging to a complex (e. g. the Pedifer group) and subspecies.

LITERATURE CITED

Abonnenc, E. (1972): Les Phlebotomes de la region ethiopienne (Diptera: Psychodidae).
Mem. Off. Rech. Sci. Tech. Outre mer 55; 1-289

Agatsuma, T., Uemoto, K. and Onofre Ochoa A. J. (1986): Biochemical genetics of blackfly isozymes I. Isozyme variation among three species, Simulium ochraceum, S. metallicum and S. horacioi from Guatemala.
Jpn. J. Sanit. Zool. 37 (1); 1 - 9

Allsopp, B.A. and Gibson, W.C. (1983): Isoelectric focusing in agarose: a highly discriminatory method for the detection of enzyme heterogeneity.
Ann. Trop. Med. Parasit. 77; 169 - 173

Anderson, T.D. (1943): Kala-azar in the East African Forces.
Afr. Med. J. 20; 172 - 175

Anon. (1972): Enzyme Nomenclature. Recommendations of the international Union of Pure and Applied Chemistry and the International Union of Biochemistry. Amsterdam; Elsevier

Ayala, F.J. and Jeffrey, R.P. (1972): Allozymes as diagnostic characters of sibling species of Drosophila.

Proc. Nat. Acad. Sci. USA 69 (5); 1094 - 1096

Ayele and Mutinga, M.J. (1989): Studies of the epidemiology of Kala-azar in Ethiopia. - a new record of Phlebotomus celiae (Diptera: Psychodidae).

Insect Sci. Applic. in press

Bagster, I.A. and Parr, C.W. (1977): Trypanosome identification by electrophoresis of soluble enzymes.

Nature 244; 364 - 366

Basimike, M. (1988): Studies on factors affecting the distribution and abundance of leishmaniasis endemic focus Marigat area, Baringo District, Kenya.

Ph.D. Thesis, RSUST, Nkpolu, Port harcourt

Beach, R., Kiilu, G., Hendricks, L. and Leeuwenberg, J. (1984): Cutaneous Leishmania major to man by the bite of infected Phlebotomus duboscqi.

Trans. R. Soc. Trop. Med. Hyg. 78; 747 - 751

Beach , R., Young, D.G. and Mutinga, M.J. (1982): Phlebotomus (Phlebotomus) duboscqi from Kenya a new record.
Trans. R. Soc. Trop. Med. Hyg. 76; 707 - 708

Beach , R., Young, D.G. and Mutinga, M.J. (1983): A new phlebotomine sandfly colonies: Rearing Phlebotomus martini, Sergentomyia schwetzi and Sergentomiya africanus (Diptera: Psychodidae).
J. Med. Ent. 20; 579 - 584

Blomquist, G.J. and Jackson, L.L. (1979): Chemistry and biochemistry of insect waxes.
Prog. Lipid Res. 17; 319 - 345

Bullini, L. and Colluzi, M. (1973): Electrophoretic studies on gene enzyme systems in mosquitoes (Diptera: Culicidae).
Parassitologia 15; 221 - 248

Byrne, G.I. (1976): Requirements for ingestion of Chlamidia psittaci by mouse fibroblasts (L cells).
Infect. Immun. 14: 645 - 651

Carlson, D.A., Langley, P.A. and Huyton, P.M. (1978): Sex pheromone of the tsetse-fly: Isolation, identification and synthesis of contact aphrodisiacs.
Science 201; 750 - 753

Carlson, D.A and Service, M.W. (1979): Differentiation between species of the Anophele gambiae Giles complex (Diptera: Culicidae) by analysis of cuticular hydrocarbons.
Ann. Trop. Med. Parasit. 73 (6); 589 - 592

Carlson, D.A and Service, M.W. (1980): Identification of mosquitoes of the Anopheles gambiae species complex A and B by analysis of cuticular components.
Science 207; 1089 - 1091

Carlson, D.A. and Walsh, J.F. (1981): Identification of two West African blackflies (Diptera: Simuliidae) of the Simulium damnosum species complex by analysis of cuticular paraffins.
Acta tropica 38; 235 - 239

Chance, M.L., Schnur, L.F., Thomas, S.C. and Peters, W. (1978): The biological and serological taxonomy of Leishmania from the Ethiopian Region of Africa.
Ann. Trop. Med. Parasit. 72; 533 - 542

Coluzzi, M. (1968): Cromosomi politenici delle cellule nutrici ovaricje nel complesso gambiae del genere Anopheles.
Parassitologia 10; 179 - 184

Coluzzi, M. ad Sabatini, A. (1967): Cytogenic observations on species A and B of Anopheles gambiae complex.
Parassitologia 9; 73 - 88

Dedet, J.P., Desjeux, P. and Derouin, F. (1980): Ecologie d'un foyer de leishmaniose cutanee dans la region de Thies, (Senegal, Afrique de l'Ouest). 4.- Infestation spontanee et biologie de Phlebotomus duboscqi Neveu-Lemaire 1906.
Bull. Soc. Path. Exotique 73; 266 - 276

Fendall, N.R.E. (1952b): Kala-azar in East Africa with particular reference to Kenya and Kamba country.
J. Trop. Med. Hyg. 55; 220 - 223

Fendall, N.R.E. (1961): The spread of Kala-azar in Kenya.
E. Afri. Med. J. 38; 417

Freeman A. B. (1979): Burrows Textbook of Microbiology. 21ST Edition, W. B. Saunders Company, Philadelphia. London. Toronto.

Gardener, P.J. (1977): Taxonomy of the genus Leishmania: A review of nomenclature and classification.
Trop. Dis. Bull. 74; 1069 - 1088

Garnham, P.C.C. (1977): Global ecology of leishmaniases: Introctory remarks.
Colloques, Int. Cent. Nat. Rech. Scient. 239; 17

Gatty, M., Santini, G., Pimpinelli, S. and Colluzzi, M. (1977): Fluorescence banding techniques in the identification of sibling species of the Anopheles gambiae complex.
Heredity London 38; 105 - 108

Gibson, W.C., Mehlitz, D., Lanham, S.M. and Godfrey, D.G. (1978): The identification of Trypanosoma brucei gambiense in Liberian pigs and dogs by isoenzymes and resistance to human plasma. Trop. Med. Parasit. 26; 253 - 282

Githure, J.I., Beach, R. and Lightner, P.K. (1984): The isolation of Leishmania major from rodents in Baringo District of Kenya. Trans. R. Soc. Trop. Med. Hyg. 78; 283

Godfrey, D.G. and Kilgour, V. (1976): Enzyme electrophoresis in characterizing the causative organism of Gambian trypanosomiasis. Trans. Roy. Soc. Trop. Med. Hyg. 70; 219 - 224

Harris, H. and Hopkinson, D.A. (1976): Handbook of enzymes electrophoresis in human genetics. Amsterdam; North Holland.

Heisch, R.B. and Guggisberg, C.A.W. (1952): Notes on the identification of Kenya sandfles. E. Afr. Med. J. 29; 423 - 432

Heisch, R.B. (1954): Studies in leishmaniasis in East Africa. 1. The epidemiology of the out-break of Kala-azar in Kenya. Trans. Roy. Soc. Trop. Med. Hyg. 48 (5); 449 - 464

Heisch, R.B. (1955): The vector of an out break of kala-azar in Kenya.
Nature 175: 433

Heisch, R.B. (1963): Is there an animal reservoir of kala-azar in Kenya.
E. Afr. Med. J. 40; 360 - 362

Heisch, R.B., Guggisberg, C.A. and Teedale, C. (1956): Studies in Leishmaniasis in East Africa. II. The sandflies of the Kitui kala-azar area in Kenya, with description of six new species.
Trans. Roy. Soc. Trop. Med. Hyg. 50; 209

Jackson, L.L. (1981): Cuticular lipids of insects -IX. Surface lipids of the grasshoppers Melanoplus bivittatus, Melanoplus femurrubrum, and Melonoplus dawsoni.
Comp.Biochem. Psysiol. 70B; 441 - 445

Jallon, J.M. (1985): A few chemical words exchanged by Drosophila during courtship and mating.
Behav. Genet. 14; 441 - 478

Kaddu, J.B. and Mutinga, M.J. (1981): Leishmania in kenyan phlebotomine sandflies. - I. Leishmania aethiopica in the midgut of naturally infected Phlebotomus pedifer.
Insect Sci. Appli. 2; 245 - 250

Kaddu, J.B. and Mutinga, M.J. (1984): Leishmania in Kenyan phlebotomine sandflies. -II. Natural infection in the malpighian tubules of Sergentomyia garnhami and Sergentomyia antennatus.
Insect Sci. Appli. 5 (4); 239 - 243

Kambona, K.O. (1989): A biochemical investigation of the taxonomy of the cassava green mite Mononychellus spp. (Acari: Tetranychidae) in Kenya.
M.Phil Thesis, RSUST, Nkpolu, Port Harcourt

Kamhawi, S., Molyneux, D.J., Killick-Kendrick, R., Milligan, P.J.M., Phillips, A., Wilkes, T.J. and Killick-Kendrick, M. (1987): Two populations of Phlebotomus ariasi in the cevennes focus of Leishmaniasis in the South of France revealed by analysis of cuticular hydrocarbons.
Med. Vet. Entomol. 1; 97 - 102

Killick-Kendrick, R. and Ward, R.D. (1981): Ecology of Leishmania.
Parasitology 82; 143 -152

Kirk, R. and Lewis, D.J. (1951): The phlebotomine of the Ethiopian region.
Trans. R. Ent. Soc. London 102: 383 - 510

Kirk, R. and Lewis, D.J. (1952): Some ethiopian Plebotominae.
Ann. Trop. Med. Parasit. 46: 337 - 350

Kirk, R. and Lewis, D.J. (1955): Studies in leishmaniasis in the Anglo-Egypt Sudan. Phlebotomus in relation to leishmaniasis in the Sudan.
Trans. R. Soc. Trop. Med Hyg. 49 (3): 229 -240

Kreutzer, R.D. and Galindo, P (1980): Isozyme studies of two Melanoconion mosquitoes Culex ocassa and Cx. panocassa.
Mosquito News 40; 605 - 613

Kreutzer, R.D., Posey, F.T. and Brown, P.A. (1977): A fast and sensitive procedure for identifying genetic variants of phosphoglucomutase in certain genera of mosquitoes.
Ibid 37; 407 - 409

Kungu, A., Mutinga, M,J. and Ngoka, J.M. (1972): Cutaneous Leishmaniasis in Mount Elgon, Kenya. E. Afr. Med. J. 49; 458 - 465

Lane R. and Ready, P.D. (1985): Multivariate discrimination between Lutzomyia wellcomei the vector of mucocutaneous leishmaniasis and Lutzomyia complexus. Ann. Trop. Med. Parasit. 79; 469 - 472

Lainson, R. and Shaw, J.J. (1978): Epidemiology and ecology of Leishmania in Latin America Nature 273; 595 - 600

Lee, J.J., Hunter, S.H. and Bovee, E.C. (1985): An illustrated guide to the protozoa. Society of Protozoologists

Lewis, D.J. (1976): Host-parasite interactions of Leishmania in vitro. Ph.D. thesis, University London

Lewis, D.J. (1982): A taxonomic review of the genus PhlebotomusA(Diptera, Psychodidae). Bulletin of British Museum (Natural History) Entomology Series, 45 (2): 122 - 209

Lewis, D.J., Minter, D.M. and Ashford, R.W. (1974): The subgenus Larroussuis of Phlebotomus (Diptera: Pschychodidae) in the Ethiopian Region. Bull. Ent. Res. 64; 435 - 442

Lewis, D.J.; Mutinga, M.J. and Ashford, R.W. (1972): Phlebotomus longipes Parrot and Martin (Diptera: Phlebotomitidae). J. Ento. 41; 119

Lewis, D.J. and Ward, R.D. (1987): Transmission and vectors. In Peters and Killick-Kendrick (eds). The Leishmaniasis in Biology and MedIcine, Vol. I. 235 - 262. (Academic press)

Lockey, K.H. (1978): The adult cuticular hydrocarbons of Tenebrio molitor L.and Tenebrio obscurus F. (Coleoptera: Tenebrionidae). Insect. Biochem. 8; 237 - 25

Lockey, K.H. (1982): Hydrocarbons of adult Physamesdia globosa (Haag) and Stenogara gracilipes (Haag) (Coleoptera: Tenebriomidae). Insect. Biochem. 12 (3); 331 - 342

Lok, J.B., Cupp, E.W. and Blomquist, G.J. (1975): Cuticular lipids of the imported fire ants Solenopsis invicta and S. richteri. Insect. Biochem. 75; 821 - 829

Mahon, R.J., Green, G.A. and Hunt, R.H. (1976): Diagnostic allozymes for routine identification of adults of the Anopheles gambiae complex (Diptera; Culicidae).
Bull. Entomol. Res. 66; 25 - 31

May, B. Bauer, L.S., Vadas, R.L. and Granett, J. (1977): Biochemical genetic variation in the Family Simuliidae: electrophoretic identfication of the human biter in the isomorphic Simulium jenningsi group.
Ann. Ent. Soc. Amer. 70 (5); 637 - 640

McKinnon, J.A. and Fendall, N.R.E. (1956): Kala-azar in the Baringo District of Kenya. Progress Report.
J. Trop. Med. Hyg. 59; 208 -212

Meredith, S.E.O. (1982): Enzyme identification of SImulium damnosum S. I. caught biting man.
Ann. Trop. Med. Parasit. 76 (3); 375 - 376

Miles, M.A., Toye, P.J., Oswald, S.C. and Godfrey, D.G. (1977): The identification by isoenzymes pattern of two distinct strain groups of Trypanosoma cruzi circulating independently in a rural area of Brazil.
Trans. R. Soc. Trop. Med. Hyg. 71; 217 - 225

Miles, M.A. and Ward, R.D. (1978): Preliminary isoenzyme studies on phlebotomine sandflies (Diptera: Psychodidae).
Ann. Trop. Med & Parasit. 72; 398 - 400

Minter, D.M. (1962): Phlebotomus (Phlebotomus) celiae sp. nov (Diptera; Psychodidae) a new sandfly from Kenya.
Ann. Trop. Med. & Parasit.A56; 19 - 23

Minter, D.M. (1963): Studies on the vector of Kala-azar in Kenya. III. Distributional evidence.
Ann. Trop. Med. & Parasit. 57; 7 - 18

Minter, D.M. (1964): The biology of sandflies (Phlebotomus) in Kenya.
Ph.D. thesis, University of London

Minter, D.M. (1964a): The distribution of sandflies (Diptera; Psychodidae) in Kenya.
Bull. Ent. Res. 55; 205 - 217

Minter, D.M. and Wijers, J.B. (1962): Studies on the vector of Kal-azar in Kenya. IV. Experimental evidence.
Ann. Trop. Med. Parasit. 57; 24 - 31

Minter, D.M. and Wijers, J.B., Heisch, R.B. and Manson-Bahr, P.E.C. (1962): Phlebotomus martini probable vector of Kala-azar in Kenya. British Med. J. 2; 835

Mutinga, M.J. (1971): Phlebotomus longipes. - A vector of cutaneous Leishmaniasis in Kenya. Trans. Roy. Soc. Trop. Med. & Hyg. 65; 106

Mutinga, M.J. (1972): Cutaneous lieshmaniasis and its transmission factors in Kenya. Ph.D. thesis, University of Nairobi

Mutinga, M.J. (1975): The animal reservoir of cutaneous leishmaniasis on Mount Elgon, Kenya. E. Afr. Med. J. 52 (3): 142 -151

Mutinga, M.J. (1975a): Phlebotomus fauna in the cutaneous leishmanianis focus of Mt. Elgon, Kenya E. Afr. Med. J. 52 (6); 342 - 347

Mutinga, M.J. (1980): Leishmaniasis vector behaviour in Kenya. In Isotope and Radiation on Animal Diseases and Their Vectors, IAEA SM 240/19, 195 - 204. International Atomic Energy Agency, Vienna

Mutinga, M.J. (1985): Leishmaniasis in Kenya. Medicus 4 (2): 11 - 22

Mutinga, M.J. (1986): Epidemiology of leishmaniasis in Kenya. Advances in research on vectors and animal reservoirs, and possible control measures.
Insect Sci. Applic. 7 (2): 199 - 206

Mutinga, M.J. (1986): Leihsmaniases.
Insect Sci. Applic. 7 (3): 421 -427

Mutinga, M.J. and Kaddu, J.B. (1982): Investigation on the vectors of Leishmania major in Baringo District, Kenya.
Proc. 4Th. Annual Scientific Conference of the African Association of Insect Scientits. 14Th -A 18Th Dec. 1982, Nairobi, Kenya

Mutinga, M.J. and Kaddu, J.B. (1983): Studies on the vectors of Leishmania major in Kenya.
Proc. of the 58Th Annual Meeting of the American Society of Parasitologists, pp 57, San Antonio, Texas USA December 1983

Mutinga, M.J., Kaddu, J.B., Olobo, J., Kyai, F.M. and Omogo, D. (1983): Studies on the vectors of Kala-azar in Kenya. Phlebotomus heischi (Diptera; Psychodidae) Kirk and Lewis. A new male record in Kenya.
Insect Sci. & Appl. 4: 253 - 254

Mutinga, M.J. and Kamau, C.C. (1986): Investigations of the epidemiology of leishmaniasis in Kenya. II. The breeding sites of phlebotomine sandflies in Marigat, Baringo District, Kenya.
Insect Sci. Applic. 7 (1): 37 - 44

Mutinga, M.J., Kamau, C.C., and Kyai, F.M. (1986): Investigations of the epidemiology of leishmaniasis in Kenya. IV. Breeding habitat of Phlebotomus duboscqi (Diptera, Psychodidae), a vector of L. major in Marigat, Baringo District Kenya.
Insect Sci. Applic. 7 (6): 727 - 729

Mutinga, M.J., Kamau, C.C. and Mwandandu, J. (1987): Laboratory investigations on the survival and fecundity of P. duboscqi (Diptera, Psychodidae), a vector of Leishmania major in Kenya.
Trop. Med. Parasit. 38: 86 - 88

Mutinga, M. J., Kihara, S. M., Ngindu, A, Loghing A, Mutero, T. A. and Karanu, F. (1988): Epidemiology of leishmaniases in Kenya. The domestic goat as a possible reservoir
Abstract, XII. th International Congress for Tropical medicine and Malaria. Amsterdam, Netherlands.

Mutinga, M.J. and Kyai, F.M. (1985): A new record of Sergentomyia garnhami (Heisch, Guggisberg and Teedale, 1956) in Kenya
Trans. Roy. Soc. Trop. Med. & Hyg. 79: 137-138

Mutinga, M.J., Kyai, F.M., Kamau, C.C. and Omogo, D.M. (1986): Epidemiology of leishmaniases in Kenya. III. Host preference studies using various types of animal baits at animal burrows in Marigat, Baringo.
Insect Sci. & Appl. 7 (2): 191 - 197

Mutinga, M.J., Kyai, F.M. and Omogo, D.M. (1986): Investigations of the epidemiology of leishmaniasis in Kenya. I. Studies on vectors of Leishmania major in Marigat Baringo District, Kenya.
Insect Sci. & Appl. 7 (2): 181 - 189

Mutinga, M.J. and Ngoka, J.M. (1970): Culture, isolation and description of cutaneous Leishmaniasis in Kenya.
E. Afr. Med. Res. Council.A4: 72 - 74

Mutinga, M.J. and Ngoka, J.M. (1978): Incrimination of the vector of visceral Leishmaniasis in Kenya.
E. Afr. Med. J. 55: 337 - 340

Mutinga, M.J. and Ngoka, J.M. (1981): Suspected vectors of lizard leishmaniasis in Kenya and their possible role in partial immunization of the human population against Leishmania donovaniÅin kala-azar endemic foci.
Insect Sci. Applic. 1: 207 -210.

Mutinga, M.J. and Ngoka, J.M. (1983): Investigations of animal reservoirs of visceral leishmaniasis and the isolation of Leishmania major in Marigat, Baringo District, Kenya.
Insect Sci. Applic. 4: 237 - 240

Mutinga, M.J., Ngoka, J.M, Schnur, L.F. and Chance, M.L. (1980): The isolation and identification of leishmanial parasites from domestic dogs in the Machakos District of Kenya, and the possible role of dogs as reservoir of Kala-azar in East Africa.
Ann. Trop. Med. Parasit. 74 (2); 129 - 134

Mutinga, M.J., Ngoka, J.M and Odhiambo, T.R. (1984): Epidemiological investigations of visceral leishmaniasis in West Pokot District, Kenya.
Insect Sci. Applic. 5 (6): 521 - 525

Mutinga, M.J. and Odhiambo, T.R. (1982): Studies on infection rates of human-baited antropophilic sandflies in Machakos District, Kenya.
Insect Sci. Applic. 3: 211 - 214

Mutinga, M.J. and Odhiambo, T.R. (1986a): Cutaneous leishmaniasis in Kenya. II. Studies on vector potential of Phlebotomus pedifer (Diptera, Psychodidae) in Kenya.
Insect Sci. Applic. 7 (2): 171 - 174

Mutinga, M.J. and Odhiambo, T.R. (1986b): Cutaneous leishmaniasis in Kenya. III. The breeding and resting sites of P. pedifer (Diptera, Psychodidae) in Mount Elgon focus, Kenya.
Insect Sci. Applic. 7 (2): 175 - 180

Molyneux, D.H. and Ashford, R.N. (1983): The biology of Trypanosoma and Leishmania, Parasites of Man and Domestic Animals. Ist Edition
Taylor and Francis, London

Nelson, D.R. and Carlson, D.A. (1986): Cuticular hydrocarbons of the tsetse flies Glossina morsitans morsitans, G. austeni and G. pallidipes.
Insect biochem. 16 (2); 403 - 416

Nelson, D.R., Carlson, D.A. and Fatland, C.L. (1988): Cuticular hydrocarbons of tsetse flies. II: Glossina fuscipes fuscipes, G. palpalis palpalis, G. p. gambiensis, G. tachinoides, and G. brevipalpis.

J. Chem. Ecology. 14 (3); 963 - 987

Nelson, D.R., Dillwith, J.W. and Blomquist, G.J. (1981): Cuticular hydrocarbons of the house fly Musca domestica.

Insect Biochem. 11; 187 -197

Nkoga, J.M., Madel, G. and Mutinga, M.J. (1975): Phlebotomus (Larroussius) elgonensis sp. nov. (Diptera: Phlebotominae). A new sandfly from Kenya.

E. Afri. Med. J. 52; 132 - 141

Ngoka , J.M. and Mutinga , M.J. (1978): Visceral Leishmaniasis animal reservoirs in Kenya.

E. Afr. Med. J. 55; 332 - 336

Olsson, I., Axio-Fredriksson, U., Degerman, M. and Olsson, B. (1988): Fast horizontal electrophoresis. I. Isoelectric focusing and polyacrylamide gel electrophoresis using PhastSystem™.

Electrophoresis 9; 16 - 22

Perfil'ev, P.P. (1968): Phlebotominae. Translation of Perfil'ev, 1966 by Israel Program of Scientific Translations. x + 363 pp Jerusalem

Petersen, J.L. (1982): Technique for isozyme analysis of live mosquitoes (Diptera: Culicidae). J. Econ. Entomol. 75 (4); 719 - 720

Petersen, J.L. (1982): Identification of phlebotomine sandflies (Diptera:Psychodidae) by cellulose acetate electrophoresis
In: Symposium on Application of biochemical and molecular biology techniques to problems of parasites and vectors identification. Geneva 8-10 November 1982

Phillips, A. and Milligan, P. (1986): Cuticular hydrocarbons distinguish sibling species of vectors.
Parasitology Today. 2 (6); 180 - 181

Phillips, A., Walsh, J.F., Garms, R., Molyneux, D.H., Milligan, P. and Ibrahim, G. (1985): Identification of adults of the Simulium damnosum complex using hydrocarbons analysis.
Trop. Med. Parasit. 36: 97 - 101

Quate, L.W. (1964): Leishmaniasis in the Sudan Republic; Phlebotomus sandflies of Paloich area in Sudan (Diptera: Psychodidae).
J. Med. Ent. 1; 213 - 268

Ready, P.D. and da Silva, R.M.R. (1984): An alloenzymic comparison of Psychodopygus wellomei - an incriminated vector of Leishmania braziliensis in Para' State, Brazil - and the sympatric morpho-species Ps complexus (Diptere, Psychodidae).
Cah. ORSTOM ser. Ent. Med. parasit.A22; 3 - 4

Rogo, L.M.N. (1985): Taxonomic studies based on phlebotomine (Diptera: Psychodidae) sandflies in Leishmania endemic area of Kenya.
Ph.D Thesis, University of Nairobi

Rogo, L.M.N., Khamala, C.P.M and Mutinga, M.J. (1988): Biochemical identification of Phlebotomus (Laroussius) pedifer and Phlebotomus (Larroussius) elgonensis.
Biochem. Syst. Ecol. 16 (7/8) 655 - 659

Ryan, L., Phillips, A., Milligan, P., Lainson, L., Molyneux, D.H. and Shaw, J. J. (1986): Separation of female Psychodopygus wellcomei and P. complexus (Diptera: Psychodidae) by cuticular hydrocarbons analysis.
Acta tropica 43; 85 - 89

Saul, S.H., Grimstad, P.R. and Graig Jr, G.B. (1977a): Identification of Culex species by electrophoresis.
Am. J. Trop. Med. Hyg. 26; 1009 - 1012

Saul, S.H., Sinsko, M.J., Grimstad, P.R. and Graig Jr, G.B. (1977b): Identification of sibling species, Aedes triseriatus and A. hendosoni, by electrophoresis.
J. Med. Entomol. 13; 705 - 708

Schmidt, G.; Walter, R.D. and Koenigk, E. (1975): A purine nucleoside hydrolase from Trypanosoma gambiense, purification and properties.
Trop. Med. & Parasit. 26: 19-26

Schnur, L.F. and Zuckermann, A. (1977): Leishmanial excreted (EF) serotype in Sudan, Kenya and Ethiopia.
Ann. Trop. Med. Parasit. 71; 273 - 294

Svensson, H. (1961): Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. I. The differential equation of solute concentrations at a steady state and its solution for simple cases.
Acta Chem. Scand. 15 (2); 325 - 341

Townson, H., Meredith, S.E.O. and Thomas, K. (1977): Studies of enzymes in Aedes scutellaris group.
Trans. R. Soc. Trop. Med. Hyg. 71; 110

Ward, R.D.; Pasteur, N. and Rioux, J.A. (1981): Electrophoretic studies on genetic polymorphism and differentiation of phlebotomine sandflies in France and Tunisia
Ann. Trop. Med. & Parasit. 56; 19-23

Wijers, D.J.B. (1963): Studies on the vector of Kala-azar in Kenya. II. Epidemiological evidence
Ann. Trop. Med. & Parasit. 58; 7-18

Wijers, D.J. and Minter, D.M. (1966): Studies on the vector of Kala-azar in Kenya. V. The outbreak in Meru District.
Ann. Trop. Med. Parasit. 60; 11 - 21

Wraxall, B.G.D. and Culliford, B.J. (1968): A Thin-Layer starch gel method for enzyme typing of blood stains.
J. Foren. Sci. Soc. 8; 81

# APPENDIX

Table Ia: Enzyme banding patterns after staining for PGM based on the histogram (Figure 3). (1) band present and (0) band absent.

| Band number | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sandfly species | | | | | | | | | | | | |
| S. schwetzi | male | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| | female | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| S. ingrami | male | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| | female | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| S. garnhami | male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| P. duboscqi | male | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| | female | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| P. elgonensis | male | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | female | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. bedfordi | male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| P. pedifer | male | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | female | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| P. martini | male | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| | female | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |

Table Ib: Enzyme banding patterns after staining for MDH based on the histogram (Figure 3). (1) band present and (0) band absent.

| Band number | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sandfly species | | | | | | | | | | | | |
| S. schwetzi | male | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | female | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| S. ingrami | male | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | female | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| S. garnhami | male | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | female | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| P. duboscqi | male | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| P. elgonensis | male | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| S. bedfordi | male | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | female | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| P. pedifer | male | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| | female | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| P. martini | male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |

Table Ic: Enzyme banding patterns after staining for GPI based on the histogram (Figure 3). (1) band present and (0) band absent.

| Band number | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sandfly species | | | | | | | | | | | | |
| S. schwetzi | male | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | female | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| S. ingrami | male | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | female | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. garnhami | male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| P. duboscqi | male | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | female | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| P. elgonensis | male | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| S. bedfordi | male | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| P. pedifer | male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| P. martini | male | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| | female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |

Table 2: Method used to analyse cuticular component pattern

```
OPTIONS LINESIZE=132 PAGESIZE-=66;
TITLE "SANDFLY CUTICULAR HYDROCARBORNS';
DATA FLY;
INFILE `C:\USERS\MAHAMAT\HASINGR.PRN';
INPUT
SPEC $ SEX $ PEAK4  AREA4  WIDTH4 APCT4
S2 $ SEX2 $  PEAK5  AREA5  WIDTH5 APCT5
S3 $ SEX3 $  PEAK6  AREA6  WIDTH6 APCT6
S4 $ SEX4 $  PEAK7  AREA7  WIDTH7 APCT7
S5 $ SEX5 $  PEAK8  ATC
A8  WIDTH8 APCT8
S6 $ SEX6 $  PEAK9  AREA9  WIDTH9 APCT9
S7 $ SEX7 $  PEAK11 AREA11 WIDTH11 APCT11
S8 $ SEX8 $  PEAK12 AREA12 WIDTH12 APCT12
S9 $ SEX9 $  PEAK14 AREA14 WIDTH14 APCT14
S10 $ SEX10 $ PEAK15 AREA15 WIDTH15 APCT15
S11 $ SEX11 $ PEAK17 AREA17 WIDTH17 APCT17
S12 $ SEX12 $ PEAK18 AREA18 WIDTH18 APCT18;
PROC DISCRIM CANONICAL LISTERR;
VAR AREA4 WIDTH4 APCT4
AREA5 WIDTH5 APCT5
AREA6 WIDTH6 APCT6
AREA7 WIDTH7 APCT7
AREA8 WIDTH8 APCT8
AREA9 WIDTH9 APCT9
AREA11 WIDTH11 APCT11
AREA12 WIDTH12 APCT12
AREA14 WIDTH14 APCT14
AREA15 WIDTH15 APCT15
AREA17 WIDTH17 APCT17
AREA18 WIDTH18 APCT18;
CLASS SPEC;
PRIORS PROPORTIONAL;
RUN;
```