MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *CULICOIDES* SPECIES, THEIR HOST PREFERENCE AND INVOLVEMENT IN THE TRANSMISSION OF FILARIAL PARASITES IN BENUE STATE, NIGERIA

BY

PHILIP OLADELE OKE

Matric. No.: 181299

DVM (Makurdi); M.Sc. (Ibadan)

A Thesis in the Department of

Veterinary Parasitology and Entomology,

Submitted to the Faculty of Veterinary Medicine

in partial fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

March, 2023

CERTIFICATION

I certify that this work was carried out by Dr P. O. OKE in the Department of Veterinary Parasitology and Entomology, University of Ibadan

……

Supervisor

J. O. Adejinmi

DVM (Ibadan), M.Sc. (Edinburgh), MSc., Ph.D. (Ibadan), FCVSN

Professor, Department of Veterinary Parasitology and Entomology,

University of Ibadan, Nigeria

DEDICATION

The thesis is dedicated to my darling wife – Dr. (Mrs.) OKE Brenda Engo, and my lovely children (OKE Emmanuel Olaoluwa Iranyohe and OKE David Ireoluwa Onahinyohe) with love for being one of the best things that God gave me.

ACKNOWLEDGEMENTS

My appreciation is foremost to God Almighty, for keeping me alive and provided for me throughout this study.

I am most thankful to my supervisor Professor J. O. Adejinmi who aroused my interest in *Culicoides* biting midges. I appreciate your mentorship and all the freedoms I enjoyed as your student.

My appreciation goes to all the departmental staff Veterinary Parasitology and Entomology, University of Ibadan, starting from the HOD (Prof. I. O. Ademola), Prof. (Mrs.) Adediran, Prof. A. B. Ayinmode, Dr. N. A. Sadiq, Dr. Sola Obebe and all other staff of the Department.

I am obligated to my wife, Dr. (Mrs.) OKE Brenda Engo for the full support and encouragements that enable me complete my study. To my children (Emmanuel and David) who were deprived of daddy's presence during this study. Daddy loves you all.

My sincere appreciation to Dr. Samuel Olawale Michael for the roles he played in the course of my study. Your advice and assistance have enabled me to achieve these feats. I also acknowledge Prof. Adamu Mathew; you have been highly generous in sharing from your wealth of experiences that have encouraged me in this study. The same apply to Dr. Emmanuel Ngbede who was always available to listen and assist with solutions whenever challenged. You have been a strong source of inspiration. My appreciation also goes to Dr. Christopher I. Ogbaje for his support and encouragement.

I gratefully acknowledge the encouragement and excellent teaching I received from my dear lecturers and colleagues. I am most grateful to my former Dean present Dean Prof J. N. Abenga, Prof. J. O. Uko, Prof. A. H. Abu, Prof. S. A. Saganunwa, Prof. M. I. Adah, Dr. F. A. Gberindyer and other colleagues, of Joseph Sarwuan Tarka University Makurdi, for their support all through.

Beginning from sampling to completion, many people rendered indispensable supports without which much of this work would not have been possible. Amongst them are Mr. Yusuf Madaki, Mr. Deakpe Ephraim, Mrs. Yemi Fajire, Mrs. Temitope Shounde (IITA).

To my friends and colleagues who played one role or the other, you are highly acknowledged for your supports. Amongst these are Dr. S. A. Ode, Dr. T. T. Apaa, Dr. Felix Kundushima, Dr. Odeniran Paul, Dr. T. T. Ordourough and many others.

Worthy of appreciation are the farmers and households who permitted me to use their locations for sample collection.

I cannot forget Prof. A. J. Dadah and his entire family. Your supports, encouragement and prayers are exceedingly cherished.

I thank my immediate family members (Alapo Sunday Abioye, Oke Okunlola Williams, Oke Richard Akinbola, Oke Bolaji Johnson, Oke Taiwo Folaranmi, Oke Kehinde Folake, Oke Paul) and my in-laws (Uma Linda, Wodah Ann, Agana Eriba, Agana Egbodo, Agana Isaac, Agana George, Oke Felicia Ronke and Mr. John Ugolo and Engr Francis Wodah. I appreciate your supports.

To all those who in one way or the other contributed to this work but were inadvertently omitted from this acknowledgement, I wish them God's blessings. Thank you.

This acknowledgement would not be complete without appreciating the financial support received from TetFund, Federal Government of Nigeria, through Federal University of Agriculture, Makurdi.

Warm appreciation goes to my mother, mother-in-Law, father-in-Law and Dr. & Mrs. Jenrola.

Finally, I want to give God all the glory for His sustenance, protection and His hand of mercy all through.

ABSTRACT

Culicoides (biting midges) are small blood-sucking dipteran flies inhabiting various regions of the world. They are biological vectors of numerous economically important pathogens, especially filarial nematodes and a serious source of annoyance to humans and animals. Despite their importance and status as neglected vectors, there is limited information on morphology and molecular identification of *Culicoides* species in Nigeria, especially Benue State. Therefore, this study was aimed at identifying *Culicoides* species, their host preference and their possible involvement in filarial worm transmission in Benue State, Nigeria.

A purposive sampling technique was used for the collection of adult *Culicoides* species in thirty locations across the three geopolitical zones (Benue South, Benue Northeast, and Benue Northwest) in Benue State, Nigeria. In weekly overnight collections using two CDC black-light suction traps, *Culicoides* species were trapped between January and December, 2018 and corresponding environmental data recorded. The trapped *Culicoides* were morphologically identified using a stereomicroscope to determine their sex and parity status. Polymerase chain reactions were carried out and characterisation of dominant species was achieved using sequence analysis targeting the *ITS1* gene. Their sources of blood meals were investigated using mitochondrial *MT-cyt b* gene and their role in the transmission of filarial parasites was probed using *Cox-1* gene. Data were analysed using descriptive statistics and correlation coefficient at $\alpha_{0.05}$.

A total of 30,163 *Culicoides* species were trapped, with the highest collection of 13,700 (45.4%) recorded for Benue South geopolitical zone. There was positive association between rainfall and the number of *Culicoides* species trapped (r = 0.96), while the number of catches correlated negatively with wind speed and temperature (r = -0.1586 and r = -0.4789) respectively. Twenty-one species were morphologically identified. Females represents 87.9% (n = 26,502) of the total collection of which 31.4% (n = 8,314) were parous. The two dominant species were *Culicoides imicola* (37.6%) and *C. oxystoma* (13.8%). *Culicoides indistinctus* (0.2%) identified in this study area is a new species reported in Nigeria. The Nigerian *C. imicola* and *C. indistinctus* strains were 95.8% and 97.3% related to French strains respectively, while *C. oxystoma* was (95.2%) related to Israeli strains. Cattle (60%), humans (37%) and dogs (3%) were their preferred hosts. The filaria, *Onchocerca gutturosa* was found in *C. imicola* and *C. oxystoma* that fed on cattle, while *Mansonella perstans* was obtained only from *C. oxystoma* that fed on humans.

Culicoides imicola and *Culicoides oxystoma* were dominant species while *Culicoides indistinctus* was identified for the first time in Nigeria. The potential roles of *Culicoides* in the transmission of filarial parasites of humans and animals were confirmed. Hence, control of *Culicoides* is recommended.

Keywords: *Culicoides*, feeding preference, Filarial transmission, Benue State

Word Count: 437

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LIST OF ABBREVIATIONS

DNA	-	Deoxyribonucleic acid
rDNA	-	ribosomal Deoxyribonucleic acid
RNA	-	ribosomal ribonucleic acid
COI	-	cytochrome c oxidase I
COII	-	cytochrome c oxidase II
Cytb	-	cytochrome b
ITS-1	-	Internal Transcribed Spacer 1

ITS-2	-	Internal Transcribed Spacer 2
CAD	-	Caspase-activated DNAse
CO_2	-	carbon dioxide
SCo	-	sensilla coeloconica
ELISA	-	enzyme-linked immunosorbent assay
PCR	-	Polymerase chain reaction
MALDI-TOF MS mass spectrometry	-	matrix assisted laser desorption/ionization time of flight
µm	-	Micro metre
^{o}C	-	degree celcius
km	-	kilometre
km/h	-	kilometre per hour
&	-	and
%	-	percentage
RNA	-	ribonucleic acid
BT	-	bluetongue
AHS	-	African horse sickness
EE	-	equine encephalosis
EHD	-	epizootic haemorrhagic disease
AKA	-	Akabane
SBV	-	Schmallenberg virus

GPS	-	Global positioning system
UV	-	Ultraviolet
ml	-	millimetre
cm	-	centimetre
AR	-	antennal ratio
PR	-	palpal ratio
am	-	ante meridiem
pm	-	post meridiem
rpm	-	revolution per minute
ng/µl	-	nanogram per microlitre
NaEDTA	-	sodium boric ethylene diamine tetra-acetic acid
w/v	-	weight per volume
NCBI	-	The National Center for Biotechnology Information
BLAST	-	Basic Local Alignment Search Tool
MEGA	-	Molecular Evolutionary Genetic Analysis
C.I	-	confidence interval
Min	-	minimum
Max	-	maximum
OR	-	odds ratio
RR	-	relative risk
Ns	-	not significant

Nj	-	neighbour joining
bp	-	base pairs

CHAPTER ONE
INTRODUCTION

1.1 Background

Biting midges are minute haematophagous dipteran flies belonging to the family Ceratopogonidae. According to Borkent (2004), the family Ceratopogonidae can be identified by the following combination of features: There is no medial longitudinal groove in the postnotum. The wing has 1-3 radial and two median vein branches that reach the wing margin. Each leg's first tarsomere is either longer than its second tarsomere or equal to it, and ocelli are not present. The black flies (Simuliidae) and the non-biting midges (Chironomidae), which the Ceratopogonidae are related to, make up the superfamily Chironomoidea.

While the most renowned members of the family are notorious for attacking people and animals, other members of the family are crucial pollinators of cocoa and rubber. Ceratopogonids thrive in a range of settings. However, most species consume other insects, and it's probable that some of these may spread illnesses to their hosts and have some influence over the species' populations.

There are currently five recognized subfamilies, with the Ceratopogoninae having 98 of the 103 reported genera, making it by far the most diverse (Borkent, 2004). Borkent (2000) came to the conclusion that the most fossil species fed on vertebrates and that the Ceratopogonidae were pleisiomorphic vertebrate feeders using a variety of mouthpart characters that are unique to females of extant species that feed on vertebrate hosts. However, this conclusion was not tested. However, feeding on vertebrate blood is now restricted to just four genera within extant animals (Borkent, 2004). *Culicoides* Latreille is the most economically significant and extensively researched member of the family among the four genera that contain species that feed on vertebrates: Austroconops Wirth, Leptoconops Skuse, Forcipomyia Meigen, and *Culicoides*. This is

because of their importance in medicine and veterinary science (Mellor *et al*., 2000). With the exception of Antarctica, every continent has members of the genus *Culicoides*.

Owing to their diminutive size and propensity to go unnoticed despite their uncomfortable bites, they are also known as no-see-ums. The general health of household animals and wildlife can be impacted by the dermatitis and skin sores that female *Culicoides* species' bites inflict on cattle. The resulting scorching feeling is compared to that of fire's ash when it touches the skin. The genus *Culicoides* has more species, and wider variety of nesting, and more biting tendencies, making it more prevalent. They live in areas that are either aquatic or semi-aquatic, such as the mud or wet soil that surrounds swamps, ponds, and marshes (Mullen and Murphree, 2019). With a few notable exceptions, *Culicoides* midges are present almost everywhere. About 1-3 mm long, adult insects are tiny and black. The antennae of males are plumose and females pilose. They have a slightly humped and a pair of wide, distinctively speckled wings. There are only two separate longitudinal veins.

Among *Culicoides* species, there are some which do not feed on vertebrates while some species, including the few that are a severe annoyance to humans, bite cattle and other animals in particular. There are over one thousand four-hundred (1,400) biting midge species worldwide (Borkent, 2015). Majority of the species are serious nuisance of humans or livestock or as arthropod vectors of disease agents to various hosts. The bites of *Culicoides* are felt as a painful prick, and they are frequently followed by itchy lumps that may remain for days or go away in a few hours. Their habitats include freshwater margins and cattle excrement. Most species feeding on humans choose tidal and estuarine habitats as breeding sites. As haematophagous insects and carriers of infections in humans, animals, poultry, and wildlife, several species of biting midges have ecological, economic, and hygienic implications (Mellor *et al*., 2000).

One of the most prevalent *Culicoides* species connected with cattle is *Culicoides imicola*. In addition to portions of the Mediterranean basin, it has been observed throughout the Afrotropical, Saharo-Arabian, and Oriental regions (Meiswinkel *et al*., 2004). Additionally, it was proposed that its geographic range may perhaps extend more westward in Europe than what is now known (Guichard *et al*., 2014). Weather variables including temperature, humidity, and rainfall, in addition to the availability of suitable hosts, control

the geographic range and abundance of *Culicoides* species (Purse *et al*., 2015). The success of blood-feeding and the risk of disease transmission posed by *Culicoides* species depend on their capacity to remain active when suitable hosts are available. Peak flight activity coincides with twilight and/or dawn for the vast majority of *Culicoides* species (Sanders *et al*., 2012). In cooler or nocturnal patterns in hotter conditions, respectively, this peak may change (Barnard *et al*., 1980). However, diurnal activity is not particularly unusual, as seen in various species (Bellis *et al*., 2004; Gerry *et al*., 2009). Flight activity is influenced by environmental variables like temperature, humidity, wind, and rainfall, just like regional dispersion (Carpenter *et al*. 2009).

Morphological identification of *Culicoides* species is routinely based on microscopic examination of various features prepared on permanent slide using high magnification. This is however a complex task and involves great taxonomic knowledge. Field study of *Culicoides* species is arduous and has lagged behind mainly as a result of their minute size and the availability of numerous *Culicoides* species (Mellor *et al*., 2000).

The key features used in morphological identification include wing pigmentation, number, size and shape of spermathecae in females, male genitalia, the placement of sensory pits on the antennae, the size and shape of the antennal segments, and shape of third palpal segment. Female wings are more intensely marked, shorter and broader than the wings of male of the same species when observed under stereomicroscope and can be readily distinguished.

Accurate speciation is recently achieved through the use of molecular tools as described by various authors (Pagés *et al*., 2005). This is a very important tool in differentiating between species complex or morphologically indistinguishable species. Molecular tools have been routinely and broadly adopted in various fields of entomology (Armstrong *et al*., 1997; Wells *et al*., 2001; Besansky *et al*., 2003). A critical initial step to a successful molecular technique is the extraction of deoxyribonucleic acid (DNA). Obtaining DNA for molecular studies is a decisive initial step. Traditional approaches of extracting DNA using toxic chemicals like chloroform is becoming outdated and newer methods using swift more reliable commercial DNA extraction kits (Ball and Armstrong, 2008).

Mitochondrial DNA is considered the ideal target for speciation using cytochrome c oxidase I (*COI*) commonly called DNA barcoding. This is the 'global standard' for characterization (Hajibabaei *et al*., 2007). The species is confirmed by comparing obtained sequence with those already known species in the GenBank. Such species is identified with the closely matched sequence in the barcode library. Cytochrome b (*Cyt-b*) gene is highly sensitive to differentiate between various host species, and it is being generally used in taxonomic studies to resolve several deviations.

Culicoides species are biological vectors of several disease agents including nematodes, protozoa and viruses. The females obtain blood from variety of hosts such as humans, other mammals, reptiles, birds and even some feed on other engorged insects (Ma *et al*., 2013). They are pests of great veterinary importance.

It is challenging to define the sylvatic transmission pathways of vector-borne illnesses since they involve a variety of factors, including several host species. In response to environmental change and the availability of hosts, many blood-feeding arthropods that act as vectors, particularly those in the family Diptera, display plastic feeding behaviours (Kilpatrick *et al*., 2007; Lefèvre *et al*., 2009). This complexity is further complicated by our ignorance of the range of potential hosts (Harrup *et al*., 2015; Parham *et al*., 2015; Purse *et al*., 2015). For the purpose of predicting future outbreaks, it is necessary to fully characterize the range of host species that vector-borne pathogens feed on (LoGiudice *et al*., 2003).

The vector capability of *Culicoides* species (Diptera: Ceratopogonidae) is primarily influenced by abundance, lifespan, frequency of feeding, and host preference (Mullen *et al*., 2004). The effort put forth by the vector to find blood meals and the relative availability of acceptable hosts both influence host preference (Killeen *et al*., 2001). Understanding *Culicoides* species' host preferences can help with disease prevention and vector management. Environmental elements like host availability, host variety, and dispersal in the insect environment have an impact on host selection.

1.2 Statement of Problem

The importance of *Culicoides* species notwithstanding, however, the weeness of the adults has played prominent role in the low attention given to them by taxonomists. The most recent studies reported by Oke *et al*., (2017) and Dipeolu's (1976) final study on the taxonomy of *Culicoides* species in Nigeria are signs that research on the genus *Culicoides* needs to be revived immediately. Identification using wing patterns alone becomes unreliable as the patterns of wing pigmentation become more broadly similar within subgenera or species complexes. Because of this, along with extremely time-consuming microscopic analysis of specimens on permanent slides, even seasoned taxonomists find morphological identification to be a challenging task (Meiswinkel and Goffredo, 2008).

There are also cryptic species that are hard to distinguish morphologically but are genetically distinct, according to reports. These are not unique to *Culicoides* species from Nigeria, but the prolonged period of inactivity in the study of these vector species has made morphological identification practically more difficult.

1.3 Justification

The lack of information on Nigerian *Culicoides* species taxonomy serves as an example of the unusually large information gap that exist in fundamental vector biology. The phylogeny of Nigerian *Culicoides* species is entirely unidentified, the extent of host preferences for these species are still indefinable, and their vector abilities have not been investigated.

Advanced methods in genetic analysis are currently important tools in vector and vector-borne disease research. The techniques are being used for the determination of varieties in species and prominent among them are polymerase chain reactions (PCR) and mass spectrometry (matrix assisted laser desorption/ionization time of flight mass spectrometry, MALDI-TOF MS) have been considered as molecular tools for vectors such as *Culicoides*.

Culicoides species attract attention as parasites of both medical and veterinary importance due to their haematophagous habits and their capacity to vector diseases of global recognition. As such, various authorities frequently conduct surveillance that help to forestall outbreaks. Such investigation always requires appropriate collection of samples

for analyses and screening to identify possible invasion of new species. This enables adequate prevention and control measures to be put in place.

The four-decade period of inactivity in the study of *Culicoides* species in Nigeria has created a knowledge gap. Additionally, there is no information on *Culicoides* species in Benue State, Nigeria, and the breadth of hosts choice of Nigerian *Culicoides* species is still unknown. Consequently, this study was designed to provide fundamental data that subsequent researches could build upon.

1.4 Aims

The definite aim of this project is to provide an update on *Culicoides* species in Benue State, Nigeria and to create baseline information suitable for future research.

1.4.1 Objectives

The followings are the objectives of this study:

i. To determine the occurrence, abundance and distribution of *Culicoides* species in Benue State, Nigeria.
ii. To determine seasonal variation of *Culicoides* species and the effects of environmental factors.
iii. To determine trap efficiencies in the collection of *Culicoides* species.
iv. To identify any morphological abnormalities associated with *Culicoides* species.
v. To establish the possibility of the presence of any external parasites on *Culicoides* species.
vi. To determine the risk associated with the presence of *Culicoides* species in the study area.
vii. To morphologically identify various *Culicoides* species in Benue State, Nigeria using peculiar characteristic features
viii. To molecularly characterise the members of the Imicola and Schultzei groups
ix. To determine the phylogenic relationship of the molecularly identified *Culicoides* species.
x. To investigate the sources of blood meals of *Culicoides* species in Benue State, Nigeria.

xi. To investigate their potential role in the transmission of filarial nematodes in Benue State, Nigeria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of *Culicoides* Species

Taxonomic hierarchy of *Culicoides* species

Kingdom:	Animalia
Subkingdom:	Eumetazoa
Phylum:	Arthropoda
Subphylum:	Hexapoda
Class:	Insecta
Order:	Diptera
Suborder:	Nematocera
Infraorder:	Culicomorpha
Superfamily:	Chironomoidea
Family:	Ceratopogonidae
Subfamily:	Ceratopogoninae
Tribe:	Culicoidini
Genus:	*Culicoides* (Borkent, 2012a)

2.2 General Characteristics of *Culicoides*

Culicoides are tiny blood-sucking nematoceran flies belonging to Ceratopogonidae family. They are considered to be of extreme importance in veterinary medicine as a result of severe irritations produced by their bites. They also possess the ability to biologically transmit various live-threatening pathogens that cause diseases in livestock. They measure between 1 to 3 mm in length, having penetrating mouthparts and long thirteen-segmented antennal flagellum. *Culicoides pulicaris* was the first species to be described in Europe by Linnaeus in 1758.

Ceratopogonidae are differentiated from other members of the Nematocera sub-order by combination of the following features:

- Characteristic wing pigmentation of black and white patches.
- Wings has r-m cross vein and additionally, the median vein is forked and typically has two radial cells (except in the Leptoconopinae).
- Wings are closed across the abdomen when not active.
- Antennae usually with 14 observable segments.
- Pilose antennae in females and male having plumose antennae.
- Short female mouthparts designed for sucking and piercing.
- Legs are typically short and stout.
- Most species are brown or black although other colours such as orange or yellow have been observed in some.

Among the members of the Ceratopogonidae family, *Culicoides* species can be separated based on their wing pattern and the presence of two radial cells on the wings. However, some species don't have the black and white wing patterns or are poorly defined. Chaker *et al*., (1980) established that *C. circumscriptus* has a slightly different pattern which may vary significantly from distinct spots to a nearly clear wing as the spots become wider and merges into one.

Culicoides species are usually crepuscular preferring to be in full activity just before sunset to just after sunrise. Adults are mainly inactive during the day. They are always found resting on grasses, in cracks on tree trunks, poles in animals' shed and some species in the

sand's top layer (Kettle, 1995). Only the females are haematophagous requiring blood for development of their ovaries for eggs production and completion of their gonotrophic cycle (Chitra, 2002). Other than feeding on blood, the females also feed on plant nectar and sap which serve as the main food for males. The blood-hungry females can attack any available animal - mammals, birds and reptiles. The female *Culicoides* have preference for outdoor hosts (exophagic) and they rarely feed indoors (exophilic). The males are recognized by their slender abdomen which terminates as genitalia and also by their plumose antennae. The females on the other hand have pilose antennae with robust abdomen.

Morphological identification of *Culicoides* species with very distinct wing pigmentation is easily and simply carried out (Meiswinkel, 1996). However, among species complex, identification becomes very difficult and permanent mounting of slides is required to enhance microscopic visibility of other characteristic features including the female spermathecae, antennae's coeloconica, shape of third segment of the palps etc. and the male genitalia (Meiswinkel, 1995).

2.3 Structure and Function of Adult *Culicoides* species

Head: Compound eyes, which make up the majority of the head, are huge. They are close together or just barely apart, and the distance between them can serve as a species-level distinguishing characteristic. The arching supraorbital suture, which is lacking in some *Culicoides* species, runs between the eyes and separates the frons from the vertex (Battle and Turner, 1971). Males and females have different amounts of the frons, which divides the antennae. All families in the Culicomorpha suborder lack ocelli (Wood and Borkent, 1989). The clypeus, that moves in concert with the mouthpieces that make up the proboscis, is underneath the frons and antennae.

Culicoides' antennae are a treasure trove of taxonomic, evolutionary, and ecological data. The basal, ring-shaped scape, a sizable pedicel, and the flagellum, which is secondary divided into 13 flagellomeres, make up the three parts that make up the antennae. The secondary segments of the flagellum are treated as real segments in earlier literature, which frequently refers to the antennae as having 15 segments. There is sexual dimorphism in the antennae. The enormous setal plumes on the flagellomeres 1–10 of the male antennae are

whorls of elongate verticils. In other species, the antennae resemble those of the females and the antennal plume is lacking (Wirth, 1977).

Culicoides antennae have five different sensilla types, which are present in both sexes and include *sensilla chaetica*, *sensilla trichodea*, *sensilla basiconica*, *sensilla ampulacea*, and *sensilla coeloconica* (Felippe-Bauer *et al*., 1989; Blackwell *et al*., 1992). The verticils on the antennae are made of the *sensilla chaetica*, which have mechanoreceptor or mechano- and chemoreceptor functions (Wirth and Navai, 1978). Although they have not been extensively used in the taxonomy of *Culicoides*, the *sensilla trichodea* and basiconica are possible chemoreceptors, and in certain species, their occurrence and patterning can be instructive (Wirth and Navai, 1978).

Light microscopy has some difficulty detecting the *sensilla ampulacea*, and it is unknown what they do. The classification of *Culicoides* has made considerable use of the flagellomeres bearing *sensilla coeloconica*, which has taxonomic relevance. Additionally, these sensilla determines host relationship. The majority of species are either ornithophilic (having *sensilla coeloconica* on flagellomeres 8–13) or mammalophilic (have sensilla on 4-6 flagellomeres) (Blackwell *et al*., 1992). *Sensilla coeloconica* are thermoreceptors that respond to temperature changes in mosquitoes (Davis and Sokolove, 1975). These are thought to serve the same purpose in *Culicoides* (Wirth and Navai, 1978), but it has been demonstrated that these *sensilla coeloconica* respond to carbon dioxide and relative humidity (Blackwell *et al*., 1992). *Sensilla coeloconica* presence should vary between species which feed on hosts with homeothermic and poikilothermic metabolism if these do serve as thermoreceptors, but no such pattern has been seen (Borkent, 1995).

A pair of maxillary palps are situated each lateral to the proboscis. Like the antennae, these attachments offer important taxonomic and ecological data. The palps of *Culicoides* have five segments. The third segment has several *sensilla basiconica* which are either arranged in a hole, an uneven area, or extend out over the segment's whole surface. Females have palps that are larger and more developed in the third segment due to sexual dimorphism. The third segment's length to width ratio, as well as the size and the sensory pit’s depth or region, provide taxonomically useful information.

Grant and Kline (2003), stated that *sensilla basiconica* are sensitive to variations in carbon dioxide concentration, a crucial indicator of host location. Sensilla count is correlated with host size and can indicate host relationships (Rowley and Cornford, 1972). Sensilla are more common in species that feed on smaller hosts (>29) than in species that feed on larger hosts. (Braverman and Hulley, 1979). The ability to detect lower carbon dioxide outputs from small hosts is presumably the adaptive benefit of having more sensilla. The larger emission of carbon dioxide is not as difficult to detect for organisms that feed on large hosts, though. The third palpal segment's morphology can also serve as a sign of host affiliation (Borkent, 1995). Ornithophilic species are comparably shorter and wider than mammal-feeding species, while non-biting species tend to be short and squat in the third segment (Borkent, 1995).

Adult mouthparts are extended into a proboscis that is made up of the labrum, mandibles, hypopharynx, maxillary laciniae, and labium from anterior to posterior. The labrum has two lateral flaps and a central cuticular strip, and it arches anteriorly. On the distal end of the labrum, there are several lateral teeth and two apical tricuspid teeth. Mandibles with distal serrations are located posterior to the labrum. The mandibles overlap and lock together when a posterior cuticular projection on the superior jaw fits into a depression on the inferior mandible.

The mandibles act as the instrument for slicing through the skin as well as the labrum-formed floor of the food channel and the hypopharynx-formed ceiling of the salivary channel. The distal tip of the hypopharynx has a row of teeth., similar to the labrum. The laciniae of the maxillae are located anterior to the hypopharynx. The laciniae wrap medially around the mandibular and hypopharyngeal margins and are equipped distally with retrorse teeth. The labium, which encircles the other mouthpieces, especially at the distal tip, forms the posterior of the proboscis.

Information about the ecology can be gleaned from the mouthparts' anatomy. The hypopharyngeal, labral, mandibular, and lacinial teeth are typically absent in non-biting species (Borkent, 1995). In addition, the labrum's distal tip is mushy and poorly sclerotized. The kind of hosts that a specific species of *Culicoides* feeds on can be determined by the mandibular teeth. Several species that feed on invertebrate hosts species possess more tiny,

fine teeth (Wirth and Hubert, 1989) while those which feed on vertebrates have a number of massive, coarse teeth (Borkent, 1995). The mandibles of flies that feed exclusively on amphibians are sharply serrated, but the laciniae are toothless (Borkent, 1995).

The thorax is made up of the pro-, meso-, and metathorax appendages, which include the legs, wings, and halters. In *Culicoides*, thoracic sclerite characteristics have not been frequently considered in taxonomy. Pronounced prescutal pits have been employed as a diagnostic characteristic for *Culicoides* (Downes and Wirth, 1981), albeit it can be challenging to tell them apart as they are present in other taxa as well. In addition to the scutellum's pollinosity, the scutellum's color patterns, scutellum, and post-scutellum have been utilized to identify species (Blanton and Wirth, 1979). On the thorax, other characteristics of diagnostic and evolutionary significance are probably present and require description.

The anterior border of the wing is formed by the costa. The sub-costa is either reduced or absent. At roughly the midway of the wing, vein R1 and the radial sector merge with the costa. There are typically two radial cells that are fully grown in *Culicoides* species. Vein R1 forms the proximal boundary of the first radial cell, which is the first vein to enter the costa. The second radial cell's distal boundary is formed by vein R3, which connects to vein R1 transversely and divides the first radial cell into two. Although *Culicoides* no longer have their R4 and R5 veins, these veins are still found in other Ceratopogonidae, both living and inexistant, and are sometimes referred to as the intercalary vein or fake vein (Szadziewski, 1996). Distal to the r-m cross-vein, the medial vein divides into two branches and is highly developed. There are two branches and a well-developed anterior cubitus vein. There is no medio-cubitus cross-vein. The anal and posterior cubitus veins are not developed enough to extend to the wing border.

The wing patterns of *Culicoides* have substantial taxonomic value since they are both light and dark. The size and colour of the macrotrichia on the surface of the wing produce these patterns (Blanton and Wirth, 1979). The present subgeneric classification system for *Culicoides* draws some of its foundation from these patterns. Six segments make up a leg: the coxa, trochanter, femur, tibia, basitarsus, and tarsus. The tarsus is further divided into four tarsomeres, the fourth of which has two claws. Many of the changes seen in other

genera of Ceratopogonidae's legs are absent from *Culicoides'* legs (*e.g*., enlarged empodium, femoral armature, tarsal batonnets). Contrary to some species of black flies (such as those with teeth or lobes), the female's claws are simple, equal, and unaltered Adler *et al*., (2004). Males have equal claws with bifurcated apical points.

Segments II through VII of the abdomen's ten segments each contain spiracles (Downes and Wirth, 1981). The tergites of the abdomen are fully formed. Compared to tergites, the sternites have less sclerotization. As a result of the membrane pleural region, the abdomen can expand during blood feeding and oogenesis. The ventral abdomen is covered with mechano- and chemoreceptors that help with host location and oviposition (Sollai *et al*., 2010). *Trichoidea* and *sensilla chaetica* are a couple of these. Despite the fact that Sollai *et al*., (2010) used the terms "*chaetica*" and "*trichoidea*" interchangeably, their descriptions of the sensilla correspond to the two different sensilla groups classification of the antennae identified (Wirth and Navai, 1978).

The female terminalia's outward characteristics include two hypogynial valves that come from sternite VIII and two fully developed cerci that articulate with segment IX. The female terminalia's exterior characteristics have not been widely used to diagnose species. These characteristics are commonly used for taxonomy in the black flies for species identification (Adler *et al*., 2004).

Differently preparing the terminalia of ceratopogonids may disclose novel diagnostic features. The interior features of the terminalia are taxonomically instructive, in contrast to the outward features. Depending on the species, there are one to three spermathecae present, and species with two spermathecae can have an incomplete third (Blanton and Wirth, 1979). The shape of the spermatheca(e), the neck's length and thickness, the neck's sclerotization, and its presence are all diagnostic. Each spermatheca's individual spermathecal ducts join together to produce a single duct. In certain species, the duct area at this intersection is sclerotized, resulting in the formation of a ring.

Numerous phylogenetic and taxonomic traits can be found in the male terminalia. Tergite and sternite IX have been fused, and a pair of gonopods that articulate with these are utilized to hold the female when mating. They are segmented into two and comprise a distal

gonostylus and proximal gonocoxite. A dorsal root and a ventral root are the two structures the gonocoxite bears close to its surface. The ventral root's purpose is unknown; the dorsal root and parameres articulate (Wirth and Blanton, 1979).

The aedeagus is made up of a membranous region dorsally and a sclerotized plate ventrally. The sclerite, also known as the aedeagus in the literature, has a U-, V-, or Y form due to the distal convergence of two anteriorly directed arms into a plate distally. Depending on the species, the parameres are either made of two rods or one fused plate.

2.4 Life Cycle/Biology/Ecology of *Culicoides* species

All biting midges undergo complete life-cycle involving four biological stages: eggs, larvae, pupae and adults. The duration depends on both environmental temperature and the *Culicoides* species involved although it occurs more rapidly in the tropics (Mellor *et al*., 2000). The eggs appear slender and small in size with a measurement of 350 - 500 µm in length and width of 65-80 µm (Kettle, 1995). They are white when oviposited but within few minutes turn dark brown to black. Eggs are laid in batches of 30 – 40 eggs each although some variations where certain species in some regions lay more eggs than same species in another region (Kettle, 1995). Other than number of eggs per batch, variation in the time of egg maturation could also vary. For instance, with appropriate temperatures, most eggs hatch within a few days. Some enter diapause and takes 7-8 months for hatching to occur (Kettle, 1995).

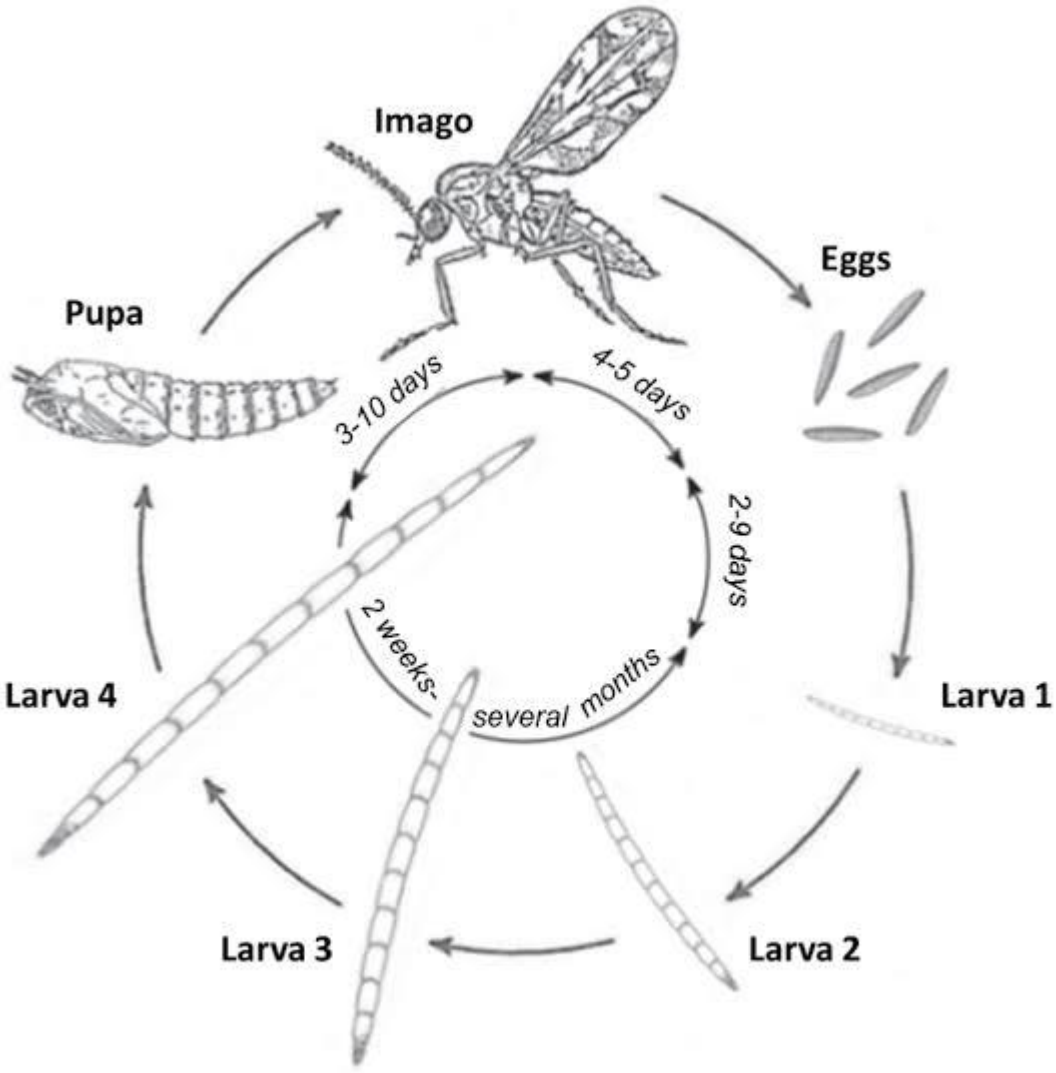

Figure 2.3: Life cycle of *Culicoides* biting midges (Purse *et al*., 1985).

Culicoides species are nematocera hence, their larvae have conspicuous head capsule that can be easily differentiated from the posterior portion. They have three thoracic (prothorax, mesothorax and metathorax) and nine abdominal segments (Kettle, 1995). The larvae are aquatic or semi-aquatic and have four larval instar stages. Temperature is the principal climatic factor that affect larval development. Emergence of most *Culicoides* species occurs between 25-36^{o} C. This was further reaffirmed that larval development is temperature dependent and it occurs rapidly at higher temperature. The larvae of certain species feed on protozoa and nematodes, hence they are considered to be are carnivorous. Also, some fourth larval instars feed on their second larval instars.

Pupae are light brown to black and comma-shaped and equipped with a duo of breathing siphons on the dorsal surface of the first thoracic segment. There are abundant spines and processes that are of diagnostic importance. Most pupae live in water from where they emerge to adults after a short-live period (Kettle, 1995). Pupation occurs rapidly once larval habitats begin to dry. The adult midges have life span of approximately twenty days although this depends on ambient conditions and may seldom live up to, 2 to 3 months (Mellor *et al*., 2000).

Females are anautogenous requiring at least single blood consumption for development of separate batch of eggs. Consequently, the regularity of blood meals is related to the proportion of egg maturity, this in turn is connected to the species and surrounding temperature. They are weak fliers, usually staying within the vicinity of their larval habitation (Kettle, 1995), but due to their minute size can be dispersed by wind to a very far distance of possibly up to 700 km.

2.5 Habitats of immature stages of *Culicoides* species

The immature stages of biting midges are generally aquatic or semi-aquatic, undergoing their life cycle around pools or streams. Although some are found in varieties of habitats such as animal dungs and foliage. Moisture and certain organic media remain the basic prerequisites for larval habitats but some species prefer highly specialized environment (Nevill *et al*., 2009).

Culicoides larvae breed in four principal habitats:

i. Shallow water and a soil interface: Majority of biting midges in Africa utilize this combination as their breeding sites. Any type of soil (loamy, sandy or clay) in mixture of decomposed plant materials together with any water source (stream, contaminated stagnant pools with varying degree of pH are suitable for breeding sites. *Culicoides imicola* breed in humid pastures of *Pennisetum clandestinum* (Kikuyu).

ii. Excrement from large mammals: Many *Culicoides* species have been associated with fresh dungs of large animals including African buffalo, Elephant, Zebra, Cattle, Horses, blue wildebeest and black and white rhinoceros (Nevill *et al*., 2009).

iii. Tree pits, plants and rock hollows: These are hollows where small pockets of water are collected. Some cavities appear deep and dark, containing collection of water (up to 2 liters) and are always contaminated with rotting leaves and other materials while other cavities are exposed but soggy.

iv. Rotting fruits and plants: These have also served as breeding sites for larvae of different species. These include rotten stems of banana plant and other falling fruits.

2.6 Feeding Behaviour of adult *Culicoides* species

Although there have been reports of species that are diurnally active, *Culicoides* females typically spend the night or crepuscular hours (Bellis *et al*., 2004). There is evidence that some *Culicoides* species are exophilic and hesitant to penetrate structures for food. Extrapolating to non-vector species is challenging because research on this behaviour has been focused on preventing viraemic or valuable stock from coming into contact with vectors. This is further complicated by the fact that this phenomenon differs among the species that have been researched (Doherty *et al*., 2004; Melville *et al*., 2005a, 2005b; Meiswinkel *et al*., 2000). The use of light traps in several of these investigations, which, as Carpenter *et al*., (2008a) point out, may draw otherwise exophilic species into buildings, further complicates these findings. Furthermore, there is

proof that some species behave differently when dealing with hosts beneath roofs compared to light traps under roofs (Melville *et al*., 2005b).

2.7 Geographical Distribution of *Culicoides* species

2.7.1 Worldwide distribution

Culicoides biting midges occur almost in every country of the world besides Antartica (Meiswinkel *et al*. 2004). However, some species are predominantly or restricted to certain zoogeographical areas of the world. For example, the main vector of orbiviruses (*Culicoides imicola*) is copious in Africa and Europe whereas *C. sonorensis, C. insignis* are found in South and Central America, *C. brevitarsis, C. actoni* in Australia, *C. fulvus, C. schultzei* in Asia, *C. pulicaris* and *C. obsoletus* in Europe. *Culicoides imicola* is the most widely distributed species around the world occupying Africa, many European countries, Mediterranean Sea, Sri Lanka, Thailand, Laos and Vietnam (Meiswinkel *et al*., 2004).

Land use has a negative effect on the density of Ceratopogonidae larvae. Abundance of larvae have been reported to occur in forested areas in comparison with less forested regions (Ngai *et al*., 2008). Less forested or desolated habitats are generally exposed hence, absent or reduced egg laying of many species (Yanoviak, 1999a, 2001). However, some *Culicoides* species were documented to exist at different development levels in varying number in some human dwellings (Rubio *et al*., 2013; Oke *et al*., 2017).

Culicoides imicola is established as the number one most widely spread livestock-linked species world over occurring across the African continent, Saharo-Arabian, Mediterranean basin and Oriental regions (Meiswinkel *et al*., 2004) and with the possibility of an extension in west of Europe than presently reported along with regions of the Mediterranean basin.

Availability of preferred hosts and adequate climate conditions of optimum temperature, rainfall, wind speed and humidity are among the leading factors that influence the geographical spreading and abundance of *Culicoides* species. Their ability to transmit pathogens is dependent on their successful blood-feeding which is also a factor of their activeness when suitable hosts are available. This in turn to some extent regulated by weather conditions.

2.7.2 Seasonal Distribution of *Culicoides* species

Climatic factors are the foremost influencing the spreading of *Culicoides* species (Mellor *et al*., 2000). For instance, low temperature limits distribution of biting midges. Precipitation may influence distribution when there are favourable temperatures by contributing to the lack of suitable breeding grounds. In Africa, *Culicoides imicola* is known to breed where annual rainfall is between 300 – 700 mm, moist and organically enhanced soil, although other factors such as water spillage from water troughs and irrigation may also play role in creating dampness that could aid breeding.

Seasonal distribution, survival and activeness of *Culicoides* species are affected by both temperature and rainfall (Mellor *et al*., 2000). Temperature is a requirement for the maintenance of *Culicoides* populations while rainfall play roles in preserving suitable breeding sites for larvae (Meiswinkel *et al*., 2004). Even in dry season, the number of *Culicoides* species will gradually increase if other factors such as irrigation create semi-aquatic habitats for development of larvae and thus sustain high midges' populations (Meiswinkel *et al*., 2004). Apart from those factors mentioned, drought (Yee and Juliano 2012, Ptatscheck and Traunspurger, 2015), predators, oxygen concentration, water pH and temperature may have serious effect on biting midges' fauna (Yanoviak, 1999a).

The height of the light trap may have an effect on the catch of *Culicoides* because Braverman and Linley (1993) demonstrated that *C. imicola* and some species were caught in greater numbers in the higher than in lower light traps, while more females of the *C. schultzei* group were caught in lower than higher traps. As a matter of fact, species that are regularly caught in higher traps may be more likely to be carried over large distances by air currents and hence are more likely to be crucial dispersal vectors.

Changes in the distribution and abundance of *Culicoides* are likely to be amongst the most important and immediate effects of climate change (Mellor, 1996). This is particularly concerning when it comes to insects that spread pathogens or parasites to people or animals because it could have an impact on the prevalence

of insect-borne diseases. The effect of climate change on distribution and abundance of *Culicoides* has been discussed by Bethan *et al*., (2005).

Given that vector-borne viruses are especially climate-sensitive, there has been considerable and ongoing conjecture that human climate change would enhance the frequency and intensity of their transmission.

Many different types of farmyards provide a variety of wet microhabitats (including irrigation channels, drainage pipes, and dung heaps) where vector species of *Culicoides* can breed. The distributions of these species should have consequently remained largely unaltered by modifications to agricultural methods or land usage.

A capable adult *Culicoides* vector must feed on a viraemic host and consume enough virus to surpass its infection threshold in order for pathogen transmission to take place. The vector must then endure the virus's extrinsic incubation period as well as the time between that blood meal and the next, before feeding on a vulnerable host. Temperature and moisture availability influence these crucial pathogen transmission cycle events, and there is a rather low likelihood that they will occur in time to actually affect transmission.

This is frequently made up for by the massive abundances of *Culicoides* vectors under favorable conditions, which impose extremely high vector-host contact and bite rates. If we examine these climatic effects in greater detail, we find that temperature modulates the majority of the *Culicoides* life cycle stages.

The competence of the *Culicoides* vector and the rate of virogenesis within humans are both directly impacted by temperature. This is partially due to the fact that in vitro virus production, which depends on the activity of the RNA-dependent RNA polymerase, is suppressed below 10°C but performs best above 28 – 29°C. The effectiveness of heritable barrier mechanisms that prevent the spread of a virus by a certain vector at different stages after oral infection may also be impacted by temperature. Even if the virus enters the haemocoel, these barriers may stop it from infecting the ovaries or the midgut, or they may limit it to the fat cells or the midgut cells. Even at low temperatures, the disease can survive inside adult vectors for up to 35 days at low titres before being able to multiply and spread when the temperature rises.

The second most significant extrinsic factor affecting pathogen transmission is moisture availability. Rainfall has an impact on semi-aquatic breeding sites' size and persistence as well as the availability and duration of humid microhabitats, which allow adults to carry out crucial tasks and find refuge from desiccation. Initially, adult *Culicoides* that are passively transported on the wind travel into suitable habitat patches. Wind patterns determine this dispersion's direction and size (for example, speed, direction and frequency). Increased vector dispersal could result from more frequent extreme weather events (especially winds), which could promote colonization and cause disease outbreaks in new locations (Randolph and Rogers 2010).

2.8 Methods of Collection of biting midges

Several methods of collection exist for trapping insects. Insects are generally attracted to carbon-dioxide, heat, light, moisture and certain odours. Commercial trapping devices producers take advantage of these and incorporate them into traps for easy collection of insects and the traps are useful for nuisance reduction, monitoring and surveillance study of dipteran flies.

Ultra violet light is another very strong insects' attractant that has been widely used in light traps for monitoring. Light traps remain the most commonly used collection devices and efficiencies of different colours have been tested in the field and as such, there are light traps with white, black, yellow or blue bulbs. Black-light has proven to be very efficient in attracting flies and has been adopted as standard by researchers. Other component of light traps that enhance collection is the fan system which blow the attracted flies into collecting beakers containing killing agents and this is usually a down-draught mechanism.

Black-light traps have several advantages which include collection of huge number of flies, user friendliness by not requiring regular monitoring, possibility of collecting either live or dead. Wide range of usefulness of trapped specimens which include viral isolation, identification of hosts, age grading and taxonomic studies. Consequently, light traps have been employed as tools for collection of dipteran flies for multiple studies. On the other hand, black-light traps have several disadvantages, among which are limitation of their usage in rural areas due to lack of electricity, weak light source with resultant low number of catches for those using rechargeable batteries. Additionally, there is possibility of

suction fan mutilating trapped midges. Furthermore, the collections may be extremely 'dirty' due to presence of larger insects which could be easily excluded using fine mesh around the traps. Also, black-light traps have been considered to be biased samplers (Meiswinkel *et al*., 2004). They are also negatively affected by wind by become less efficient with increasing wind speed.

Mechanical aspiration from bait animals is another method of collection. This is the best method for detection of biting rates on the hosts and it is useful for pathogen transmission by arthropods. In this method, females are collected during or attempted blood feeding on hosts by lightly touching them using gauze or cotton wools soaked in light alcohol or by use of specialized aspirators or fine-mesh net. A single animal host can be used and several investigations can be conducted from such host. Insects are usually collected intact this way. However, this method is adversely affected by weather conditions and it is highly labour intensive.

Apart from ultraviolet light traps and mechanical aspiration, other trapping strategies include bait traps which employ the use of baits such as pheromones or host odours like carbon-dioxide and 1-octen-3-ol and CO_2. However, these have not given satisfactory results for collection of *Culicoides* species due to their reluctance to enter an enclosed space.

Vehicle-mounted trap is another tool for capturing *Culicoides* species. This utilizes a large net through which insects are trapped as the vehicle moves. Specimens are usually collected during the day and it's useful for swarming males or specimens around wildlife. Such collections could be used for taxonomy. Vehicle mounted traps are the least biased trapping device.

Emergence traps placed around the breeding sites of *Culicoides* species assist in trapping newly emerged midges. Also, this can be used to retrieve both larval and pupal stages when investigating larval habitats (Meiswinkel *et al*., 2004).

Since majority of *Culicoides* species are crepuscular, a successful trapping would take into consideration the time of their activeness and should be performed just before the dusk to just after the dawn. This also would suffice for those species which are nocturnal.

2.9 Tools for identification of *Culicoides* species

Traditionally, the identification of *Culicoides* species has been based on the morphology of adult specimens especially the females. Morphology of the immature stages also has been employed but in the recent times, genetic characterisation is gradually becoming the main tool for taxonomic studies due to its higher specificity and less time consuming. Furthermore, molecular characterisation is revealing the deficiencies of morphological identification (Harrup *et al*., 2015).

2.9.1 Morphological Identification of *Culicoides* species

During morphological identification of *Culicoides* species, little consideration is given to the use of immature stages. Higher number of biting midges have been identified to species level using pupae (17%) than with larvae (13%) possibly due to the ease of nurturing pupae. When conducting larval taxonomy, the fourth larval instars are commonly used and attention is given to their "colour, dimension of cranium capsule, dimensions and sum of epiphygeal teeth, thoracic colouration and relative measurements of anal setae. However, for identification using pupae, the following characters are commonly used: the pattern of abdomen and rear thoracic protuberances, the number and distribution of spikes, the degree of scaling and apertures on the prothoracic horn, and the shape of the posterior region (Bellis *et al*., 2013). On the other hand, field-collected immature stages could be nurtured in the laboratory till adults emerged and then they are identified.

The larvae of *Culicoides* appear whitish, slender and lacking appendages. Occasionally, thoracic pigments pattern may be observed. In aquatic environment they swim to produce a characteristic eel-like motion (Kettle, 1995). They have a sclerotized head capsule that houses the pharyngeal skeleton, which is a key characteristic for species differentiation.

Immature stages exclusively have not been used to describe *Culicoides* species however, Nevill *et al*., (2009) recently described two African species using their pupae. Majority of *Culicoides* species are identified by their adults since they are the frequently encountered around hosts or during surveillance using suction traps.

The most common characteristic features for taxonomic classification of adult biting midges of both sexes are the wing venations and pigmentations. Other features are either

specific to males or females. In females, other useful features aside wing veinations are the spreading of microtrichia across wing margin, mesonotal patterning, distance between eyes, the third segment of the palps' shape and sensilae distribution on it, the number as well as the form of mandibular teeth (Bellis *et al*., 2013). Other features include the dimensions, sum and outline of the spermathecae and proportions of various segments of the antenna, fraction of measurements of the foremost and subsequent segments of the hind tarsus, relationship between the size of the costal vein and the wing, as well as the proportion between the head and proboscis lengths. Important features of the spermathecae include the sum, outline, level of sclerotisation, dimension of the collar attached to the tubes and the occurrence of a sclerotised ring close to the gonopore (Bellis *et al*., 2013). Possibly the most suitable taxonomic support for morphological identification of females *Culicoides* species is the accurate figure and distribution of the seven forms antennal sensillae. *Sensillae coeloconica* and *chaetica* are the most significant for species identification.

The genitalia and their accessories, such as the aedeagus and parameres, the presence of spiculae on the ninth sternite, the degree of development of the posterolateral processes on the ninth tergite, and the shape and degree of advancement of the dorsal and ventral roots of the gonocoxite, are examples of anatomical features that are used to identify males (Bellis *et al*., 2013) and these are clearly visible in permanent slide specimens (Kremer *et al*., 1988). The outlines of various fragments of the genital organ are extremely species-specific and are constantly useful for morphological identification.

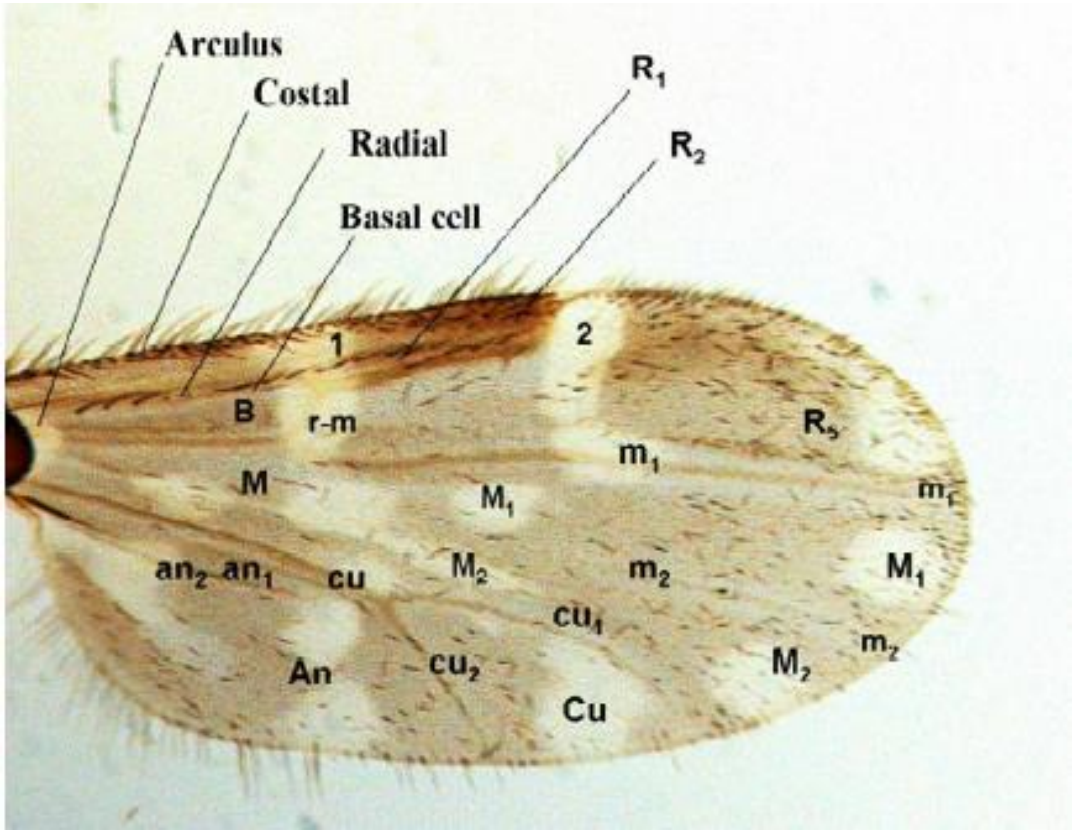

Figure 2.2: Characteristic wing of *Culicoides* species showing veins and cell

Keys - Cells: R_1: 1° radial; R_2: 2° radial; R_5: 5° radial; M: Medial; M_1: Medial cell 1°, M_2: Medial cell 2°; Cu: Cubital cell; An: Anal cell; 1: First costal spot; 2: Second costal spot; B: basal cell

Venation: m_1, m_2, cu, cu_1, cu_2, an, an_1, an_2, r-m, costal vein, radial vein, arculus

(Gonzalez, 2012)

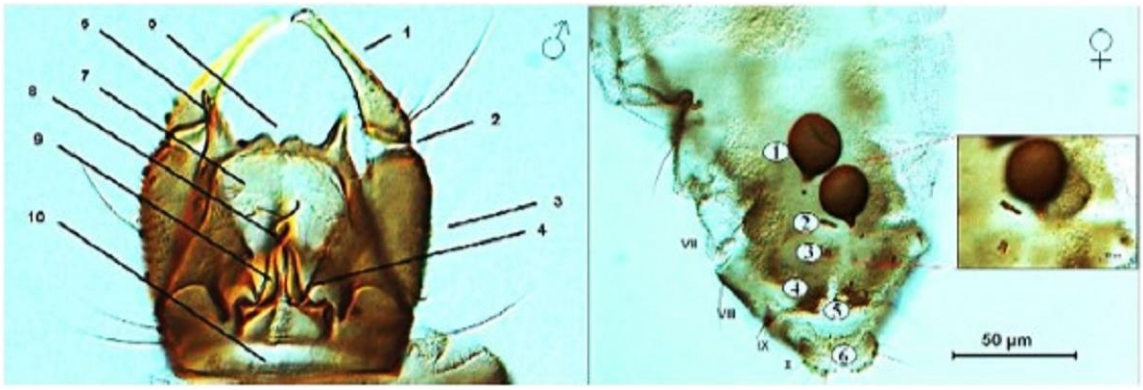

Figure 2.3: Genitalia of male and female *Culicoides* species (Gonzalez, 2012)

Keys - Male: (1) Dististyle (2) Joint (3) Basistyle (5) Lobes and caudomedian excavation (6) Apicolatera process (7) Cercus (8) Aedeagus (9) Parameres (10) Ninth sternite

Female: (1) Spermathecae (2) Rudimentary spermatheca (3) Anal sclerite (4) Chitinous plate (5) Genital space between plates (6) Cercus. Abdominal segments represented with Roman numerals

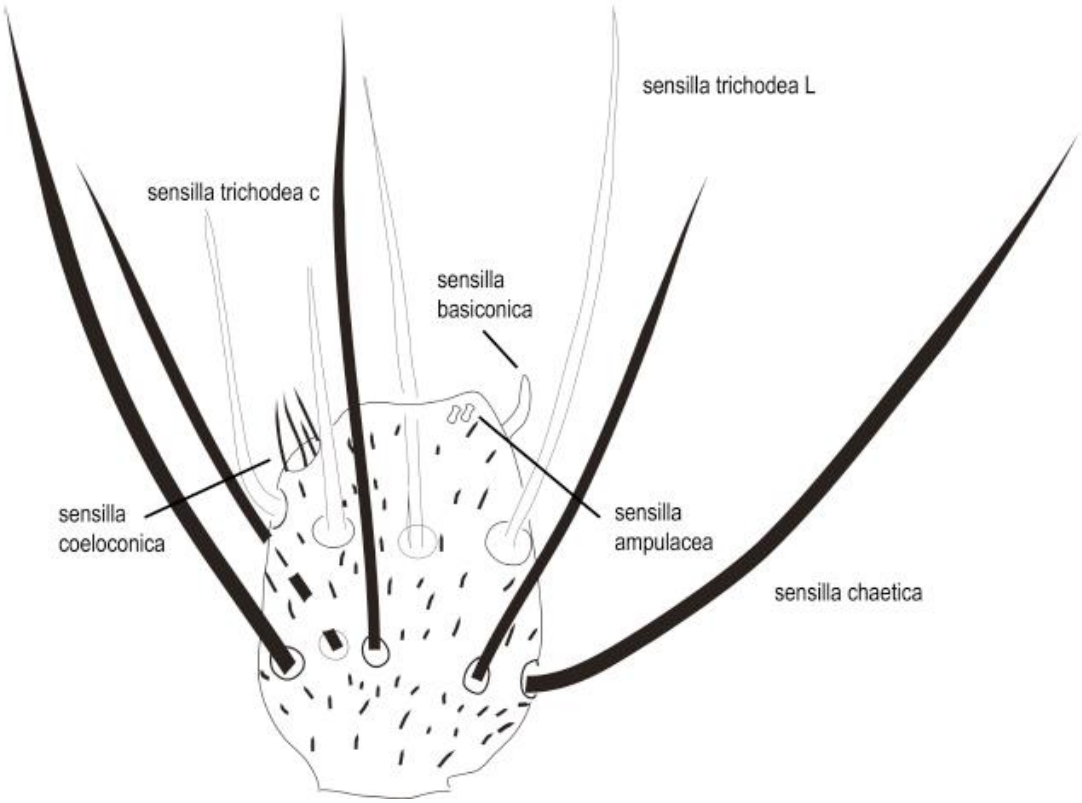

Figure 2.4: Flagellomere of *Culicoides* illustrating the five basic forms of sensilla (Bellis *et al.*, 2004)

Negligence in the study of *Culicoides* species is caused by two things - their minuteness and species diversity (Mellor *et al*., 2000). Among species complex, identification becomes very tasking and requires great taxonomic expertise. Identification is made more difficult by the lack of information on the taxonomy of *Culicoides* in Western Africa.

The production of the first readily accessible interactive key to a regional *Culicoides* fauna signals an entrance to a new age for *Culicoides* identification tools. These keys supply a noteworthy perfection on conventional dichotomous explanations as they classically provide a complement of photographs of the vital features for individual species that enable user in selecting features to progress through the key. Despite the noticeable improvement on the traditional identification method, the need to use computer alongside the microscope constitute its main set back.

2.9.2 Molecular Characterization of *Culicoides*

Recently, molecular techniques have provided a better alternative to the morphological means of identifying *Culicoides* biting midges hence, more accurate species identification is now realizable. Several genes are targeted for genetic investigation of arthropods however, mitochondria genes are regularly used because they evolve faster than nuclear genes (Watanabe *et al*., 1999). Also, as a result of their abundance in the cells, they are considered ideal for measuring genetic discrepancies and are readily amplified using polymerase chain reactions. Furthermore, they are hereditary from the mother and contain specific regions that are highly variable. The 16S ribosomal RNA and cytochrome oxidase I (COI) genes are the mitochondrial sequences that are most frequently used in gene studies of arthropod vectors (Schouche and Patole, 2000). Among others, the advantages of mitochondrial gene sequences include high resolution in separating between species complex and within genera as well as revealing subpopulation in specific species (Schouche and Patole, 2000).

The COI gene from mitochondria is quickly becoming into a useful and efficient tool for *Culicoides* species molecular identification and for other animals generally especially within species complexes where morphological identification is not too reliable as obtained among the *Culicoides obsoletus* species complex. Here, COI barcodes has differentiated 95% of species studied and this has also been used in the phylogeny study of Imicola group

and to identify members of the Pulicaris group (Pagès *et al*., 2009). Erroneous identification of arthropod vectors has significant epidemiological consequences. Consequently, understanding vectorial capacity of certain species and their accurate identification is necessary to enable risk assessment for disease transmission into disease-free areas.

Numerous researchers have also highlighted the speed and dependability of the ribosomal DNA (rDNA) Internal Transcribed Spacer 1 (ITS-1) area for PCR (Cetre-Sossah *et al*., 2004, real-time PCR, or multiplex PCR, and to a lesser degree on the Internal Transcribed Spacer 2 (ITS-2) rDNA and 18S rDNA.

Probe hybridization in DNA microarray format is another molecular technique (Schena *et al*., 1995) with numerous benefits over the different PCR techniques. Those advantages include parallel analysis of several species, high degree of sensitivity and specificity, and very minimal background signal noise (Zhou and Thompson, 2004).

Finally, the application of matrix assisted laser desorption/ionization time of flight mass spectrometry; MALDI-TOF MS has recently become popular for species identification and characterisation of biting midges due to its speed, simplicity, and cost-effectiveness.

Table 2.1: Molecular markers in use for identification of *Culicoides* species

Genomic region	Molecular marker	References
Mitochondrial	*COI* *COII* *28S* *18S rRNA* *16S rRNA* *Cyt-b*	Pages *et al*., 2009; Lasen *et al*., 2012; Slama *et al*., 2014; Hey *et al*., 2003; Matsumoto *et al*., 2009; Henni *et al*., 2014; Jan *et al*., 2010.
Ribosomal	*ITS-1* *ITS-2*	Morag *et al*., 2012; Perrin *et al*., 2006
Nuclear	*CAD*	Bellis, 2013

2.10 Host preferences of *Culicoides* species

Both sexes feed on sugar from plant juice, however, only adult females are haematophagous, requiring blood meals for maturation of the ovaries and production of eggs. They feed on varieties of hosts especially domesticated animals, and also haemolymph of other engorged insects. They are intermittent feeders, taking blood at intervals of 3 to 5 days and may ingest blood about three times throughout its entire lifespan. They are most active from dusk to dawn (*i.e*., crepuscular or nocturnal) and it is at this peak time that maximum blood feeding occurs. The preferred sites of bites are the underbelly and legs; particularly area surrounding the coronary bands of the claws.

Host-location and selection during biting involves certain behavioural processes such as visual attractiveness as well as chemosensory mechanisms as influenced by antennal and maxillary receptors thus enabling vectors seek their hosts with non-random biting. *Culicoides* species have various receptors that are highly sensitive to range of host derived products which are very attractive (Marquardt *et al*., 2000; Mordue, 2003). Among these are CO_2, lactic acid and 1-octen-3-ol. Volatile pheromones released by parous females may be an attractant to other females. In addition, parous female pheromones may interact with host-derived volatile components., to produce a repelling or attracting effect. This depends on the proportional doses of each mixture. Also, the infection status of the host is another key feature determining location of host by vectors. Infection could interfere with metabolic processes of the host and subsequently the host-derived products where individual humans with high symptoms of malaria were more attracted to vectors (Torres-Estrada and Rodrı´guez, 2003).

Depending on the species, most females *Culicoides* are active from shortly before dark to just after dawn (crepuscular) or at night (nocturnal), however, those active during the day (diurnal) have been described (Bellis *et al*., 2013). Most *Culicoides* species are exophilic in nature, preferring to feed outside. Knowledge of the hosts preference of *Culicoides* species were obtained from transmission studies of domestic animals' illness or the bothersome potentials of human-biting bugs (Bellis *et al*., 2013).

Potential blood hosts for *Culicoides* species include a wide variety of animals, such as humans, mammals, birds, and amphibians (Lassen *et al*., 2010; Augot *et al*., 2017a). While

certain *Culicoides* species have a clear affinity for particular hosts, others prey on a wider variety of hosts (Braverman, 1994). In terms of several things, including its preferences for blood hosts, *C. obsoletus* is regarded as a generalist among the *Culicoides* midges. It draws blood from a wide variety of vertebrates, including cattle, sheep, horses, donkeys, birds, mice, rabbits, and even people (Augot *et al*., 2017a). *Culicoides albicans*, in contrast, appears to be a specialist and has only been shown to suck blood on cattle (Elbers and Meiswinkel, 2014).

The mechanisms underlying the blood feeding act are the same in all species of *Culicoides*, despite the wide variation in the range of potential blood hosts. Biting midges, also known as "pool feeders," use their powerful piercing-sucking mouthparts to cut into their hosts' skin and subsequently harm small capillaries present within the skin. The insect is licking up the blood that is oozing out of the damaged blood vessels. The Ceratopogonidae produce salivary fluid during the blood meal, which typically lasts 2 to 5 minutes (Borkent, 2005), to avoid clotting and maintain the blood stream afloat.

Only female midges can feed on blood because they need particular blood proteins for the maturation of fertilized eggs (oogenesis). Depending on species, different blood meals are needed, thus resulting in a variable number of meals and a different volume per meal. The volume of the blood meal varies depending on the species. According to Braverman and Swanepoel (1981), *Culicoides zuluensis* absorbs 0.410 mg blood, *C. imicola* up to 0.139 mg, and *C. variipennis* up to 0.56 mg (Tempelis and Nelson 1972). Similar to the size of the blood meals, the number of egg batches varies depending on the species and can be as many as seven for a single female midge. For instance, *C. variipennis* can produce up to 1000 eggs, each divided into two.

Accordingly, the preferred hosts of those species which do not obtain blood from domestic animals or humans remain unknown in many parts of the world (Hadj-Henni *et al*., 2015). Host preference studies is considered indispensable for the identification of vectors and reservoirs of pathogens to implement appropriate preventive measures. Based on preferential feeding habits, *Culicoides* species are generally grouped into two – those with affinity for mammals (mammophilic) or those with affinity for birds (ornithophilic),

although, some have been reported to exhibit opportunistic behaviour possibly due to availability of hosts and quest for blood meals.

Information on their host preference is extremely important in evaluating their vectorial potential. Olfactometric techniques is a consistent method to determining the host range and preference of *Culicoides* species. This requires, collection of insects directly from baited traps, or directly from animals (Braverman and Frish, 1984).

The antennae of female biting midges bear five different sensory pits. Among these, *sensilla coeloconica* (SCo) has been associated with host preference. Species whose antennae feed on huge mammals have sensilla on flagellar number 4 - 6 while those which preferentially feed on birds have SCo on number 8 -13 flagellomeres. The likelihood of some species obtaining blood from birds and mammals is an indication that the sum of antennal sensilla coeloconica is not entirely reliable for determination of host preference and that the link between host preference antennal morphology is not absolute (Bellis *et al*., 2013).

Various immunological methods have also been used for detection of arthropods' sources of blood meals. The serological techniques commonly used include precipitin test, latex agglutination, and the Enzyme-Linked Immunosorbent Assay (ELISA) as described by Boorman *et al*., (1997) and Gomes *et al*., (2001).

However, polymerase chain reaction provides a more reliable and accurate identification of arthropods' source of blood meals. It is an appropriate substitute for laboratories using DNA-based techniques in a study involving species characterization, detection of pathogens, and vector population genetic studies. It is advantageous in that blood hosts can be detected base on the size specific fragments as observed on agarose gel electrophoresis.

Molecular techniques as methods of detecting arthropods' sources of blood meals is fully gaining ground and have been adapted by many researchers. Not only does it overcome the limitations related to serologic assays, it is also incorporated in the identification of potential reservoir species (Pichon *et al*., 2003). Numerous molecular methods have been employed to detect sources of blood meals of arthropods vectors. Prominent among these are conventional PCR with host-specific primers (Ngo and Kramer 2003), PCR-

heteroduplex analysis, terminal restriction fragment length polymorphism analysis, dot blot hybridization (Sato *et al*., 1992), and reverse line-blot hybridization (Pichon *et al*., 2005 and 2003; Kirstein and Gray, 1999).

2.11 Mating behaviour in *Culicoides* swarms

Adult males of most biting midges are found in swarms during which mating are presumed to take place. Swarms are observed as patches of the earth that stand out as being dark and light in contrast (Bellis *et al*., 2013). Most species formed their swarms at sunset and males are found to be always more than females. Swarming has also been established to stimulate production of spermatophore by the males for speedy movement of spermatids and afterward reduces copulation time.

Finding females within swarms requires the use of auditory signals by males through the *sensilla chaeticae* (mechanoreceptive hairs) which are sensitive to sound waves. These signals when received are recorded through the Johnston's organ on the antenna's pedicel (Bellis *et al*., 2013). The mechanoreceptive hairs are fewer in females and are non-sensitive to auditory signals. Also, some *Culicoides* species have been reported to practice non-swarming mating while some have been observed to exhibit facultative swarming behaviour for example *C. nubeculosus*.

The literature on Ceratopogonidae only briefly mentions the behaviour of the male terminalia before sex, which is well known in mosquitoes. Several *Culicoides* species have been observed with a slight tilting of the hypopygium. After two days, male *C. nubeculosus* housed in confinement from emergence displayed different degrees of torsion up to 90º. The majority of wild-caught individuals had torsion angles exceeding 90°, ranging from 0° to 180°; however, this effect may have been the result of previous sexual encounters. Regarding *C. furens*, it is still possible that male swarm rotation takes place before an approaching female is noticed (Linley and Adams, 1972).

Epigamic recognition - Females entering or approaching swarms of male *Culicoides* and swarming of male *Culicoides* is extensively documented. According to research on mosquitoes, male recognition of the female in such situations is likely dependent on aural detection made possible by the plumose male antenna. It has been determined that there

are two main categories of modified behavioural patterns that depart from this fundamental and widespread swarming process: (i) reduced or "truncated" patterns, in which flight is eliminated and mating occurs on the ground or another substrate; and (ii) male assembly at a waiting station that serves as a platform for quick flights to capture the female. Males occasionally took long naps close to females -about 1 mm away - without appearing to be aware of them.

These three significant events - Male orientation, terminalial rotation, and female receptive behaviour are grouped together because they are so closely related chronologically that no particular order can be determined without additional research. After the male makes contact with his legs, a series of highly complicated actions and manoeuvres that eventually achieve genital contact happen very quickly (Linley and Adams, 1972).

A responsive female replies when a male grab her by the legs in one of two ways: (i) by stopping moving right away, or (ii) by instantaneously assuming a receptive position.

The female's body is positioned so that the abdomen is angled upward posteriorly and the genitalia are elevated much above the level they typically occupy in a resting insect. The head is low, and the proboscis nearly touches the supporting surface. To avoid getting in the way of the male's attempts to achieve union, the rear legs are kept out of the way. The male may otherwise be dragged along the substrate as his position becomes localized at the female's back, which makes copulation easier when the female stops moving. Elevating the female's abdomen allows the male to more easily grab it from underneath.

Once the male has established contact with the female, he must solve three orientation problems: (i) identifying the female's posterior end; (ii) identifying the female's ventral abdominal surface; and (iii) arranging himself such that he can achieve union with a rotating hypopygium. According to visual data, the legs are crucial to orientation.

The male usually always releases the female with his legs once coitus has been established and then instantly assumes a stance facing the opposite direction. The female may move briefly during this adjustment, but the pair eventually settles down and almost invariably does so for the duration of the entire copulation session until separation starts.

Description of stance: Although the male may adopt a square and level stance with all four legs in contact with the ground, torsion from the rotated terminalia causes the body to slant in one of two directions much more commonly. The legs on the raised side very commonly are not in contact with the substrate if the degree of twist is severe enough.

The clasping phase - A few seconds after the male and female unite, rhythmic telomere extensions and retractions start as soon as the male takes a stance. The basimeres are unaffected by these motions, which exclusively affect the telomeres; one telomere extends while the other retracts (Linley and Adams, 1972).

The movement stops abruptly during the post-clasping phase, when both telomeres are deeply pressed against the female's dorsal abdomen surface. Until the start of separation, this position is maintained for the duration of the copula.

Separation's beginning has one thing in common with union, namely that it is finished very quickly. The amount of time needed varies, but is rarely less than 4 seconds and occasionally as much as 25 seconds; the majority of numbers fall between 5 and 10 seconds. Males frequently exhibit the earliest symptom of approaching separation through solitary restless motions of just one leg. The first separation-related observable motions appear very soon after that.

Regardless of the preliminary actions, both insects' hind legs may immediately start to move, however the female uses hers far more frequently and effectively. In essence, these leg motions are grooming reactions that, in the case of the female in particular, have evolved to serve a specific purpose during separation. Since the female is getting rid of the adhering male, her actions could be seen as a unique case of body cleansing.

2.12 Flight Activity Rates of *Culicoides* species

Adverse meteorological conditions affect biting midges’ activities separately even in their most active time of the day. Various trapping techniques have proposed different activity patterns and some meteorological conditions have been established to affect not only the activities of midges but also the efficiency of trapping. For instance, light traps have reduced proficiently when other sources of light are available and number of trapped midges be lessened by moonlight.

Higher wind speed adversely affects light suction traps by lessening their capacity to suck air is reduced. Human and animal baits or vehicle-mounted traps for investigations on bite rates are considered better alternatives sampling methods.

Culicoides species exhibit either crepuscular or nocturnal activity and their onset of activeness is prompted typically by falling light intensity. Hence, total darkness may hinder their activity.

At extremely high temperatures air can tolerate significant quantities of humidity and biting midges are predisposed to dryness. Some *Culicoides* species have reduced activities at low moisture levels, such is the case for *C. imicola* and *C. impunctatus*. The nocturnal activeness of several *Culicoides* species could be an adaptive strategy to overcome risk of desiccation hence, they exploit the combine effect of low temperature and high relative humidity present at night. In arid environment, peak activities are recorded at dawn saturation deficit is diminished. Rainfall hinders the activities of all biting midges.

Peak period of flight activity coincides with dusk and/or dawn, although, in the cooler period the peak may swing to a diurnal pattern while to a nocturnal pattern in hotter weather. However, diurnal activity is not strange. Flight activity is dependent on environmental factors for instance temperature, rainfall, wind speed and relative humidity (Carpentar *et al*., 2007). Since arthropods respond directly to wind, rain, relative humidity, temperature and light intensity, weather is considered very significant. Daily weather varies from location to location even within relatively short geographical distances.

2.13 Dispersal of *Culicoides* species

Implementing effective control techniques to stop the spread of a vector-borne disease during an outbreak requires a precise understanding of the vector species' ability to disperse. It is well established that the structure and composition of the terrain affect dipteran dispersal. When a landscape feature (also known as "environmental factor") favours an organism's ability to disperse, the latter is referred to as a conductance factor Manel and Holderegger, (2013).

On the other hand, a landscape feature might be classified as a resistance factor if it restricts dispersal Manel and Holderegger, (2013); Manel *et al*., (2007). However, other methods

of dispersal, such as "stratified dispersion," enable organisms to get beyond barriers to resistance. Due to a variety of dispersal processes that operate actively over short distances and passively or semi-actively over longer distances, *Culicoides* dispersal is defined as stratified Murray and Kirkland, (1995).

Previous mark-release-recapture experiments on *Culicoides* species shown that the distance traveled over two nights after release ranges from 1 to 2.5 km and is connected to the gradual search for hosts or oviposition locations (Kluiters *et al*., 2015). For *Culicoides mohave* in a specific desert environment, the longest recapture distance ever was 6 km (Brenner *et al*., 1984). However, in the context of mark-release-recapture operations, the potentially great dispersing capacity of *Culicoides* and the significant mortality of adults when they are handled pose practical restrictions. There is evidence from numerous studies that the wind-borne transmission of *Culicoides* during outbreaks and the spread of disease are related (Gloster *et al*., 2008).

The dispersion of arthropod vectors is a critical factor in the spread of pathogens (Sellers, 1980). Due to their minute size, prevailing wind is a key factor associated with long-dispersal of *Culicoides* species. Generally, appropriate winds between 0.5 to 2 km altitude, with winds and temperature between 10 to 40 km/h and temperatures of 12^{o} to 35^{o} C respectively tend to disperse biting midges to as far as 700 km.

The dispersal of biting midges and several other flies is classified into two: short- and long-distance dispersals. Short distance dispersal is considered as foraging. Behavioural pattern of vector is responsible for long-distance dispersal thus exposing them to wind current which subsequently transport them to far locations (Bellis *et al*., 2013).

2.13.1 Short dispersals of biting midges

Adults *Culicoides* species are normally as weak fliers. Their movement beyond 10 km is through the aid of wind speed. They prefer to seek their hosts short distance within their breeding sites. When looking for shelter, hosts, mates, and oviposition locations, insects can fly unaided in the absence of wind or at wind speeds of less than 2 m/s (Sellers, 1980). Station-keeping movements, in which the insect remains in its existing environment, and movements that transport it away from the home patch permanently or for an extended length of time are two categories of insect movement (Chapman *et al*., 2011). Finding and

feeding on hosts, finding refuge, mating, and locating breeding environments are the main goals of station-keeping activity. These actions take place within the insect's flight boundary layer because they need effective track control (FBL). This layer of the atmosphere, which is typically close to the ground, has winds that are so weak that an insect can fly freely in any direction.

2.13.2 Long dispersals of biting midges

Direct evidence for the long-distance dispersion of Diptera is not an easy task to establish. Several methods employed for comparing flies at different heights include truck traps, light suction traps (Quinn *et al*., 1991) and mast traps (Johansen *et al*., 2003). The occurrence of flies a long distance away from their breeding environment or at altitude, is an indication of possibility of long-distance spreading.

Indirect and incidental evidence to determine the dispersion of insect vectors over long distances is to correlate an epidemic with wind trajectories or by intrusions/detection of an unusual virus genotypes (Johansen *et al*., 2001). However, in the recent times, application of genetics has been used to establish the movement of blood sucking arthropods over extensive distances.

Range-finding and migration are the goals of long-distance movement (Chapman *et al*., 2011). When ranging, the behaviour ends after exploring a new area and discovering a new home range. The purpose of migration, in contrast, is to actively move the insect to a habitat that is or will be more resource-rich than its current habitat. Utilizing the swift winds above the FBL is the most effective way to move insects over long distances. In order for *Culicoides* midges and mosquitoes to survive transportation by winds above the FBL, the winds must be warm: 15 to 20°C at night or 20 to 40°C during the day. This is because insects are cold-blooded and active at temperatures between 10 and 35°C (Braverman and Chechik, 1996).

2.14 Importance of *Culicoides* species

Haematophagous arthropods such as *Culicoides* biting midges, are intrinsically significant to humans and animals' health due to their annoyance, their roles as vectors of various pathogens and the ability to produce hypersensitivity.

Female biting midges have wide range of hosts from which they obtain blood meals. These include humans, mammals, birds, reptiles as well as blood-engorged insects such as mosquitoes. The genus receives prominence through their roles in the transmission of internationally recognized pathogens affecting animals and humans. Opportunistic biting of *Culicoides* species on humans have been reported to negatively affect tourism, agricultural industries and forestry (Mellor *et al*., 2000). Their bites in humans constitute severe nuisance while in horses they produce acute allergic dermatitis commonly known as sweet itch.

2.14.1 Annoyance/Nuisance Effects of *Culicoides* species

Biting midges have acquired several scientific attributes due to their nuisance value and these are evidenced in the common appellations given to them due to their reputation of being notorious, there now exist appellations such as irritans, damnosus, molestus and diabolicus. Biting midges have long been serious pest of humans causing indescribable pains after bites. By their infuriating bites, in great numbers, they can make life nearly intolerable in some parts of the world. The pains from their bites when compared to that produce by *Phlebotomus* species is similar however, the sensations are prolonged and the inflammation observed may remain for several days unabated with subsequent itching in slightest irritation.

They are primarily nuisance at sunset till dawn howbeit, they are able to strike at any time of day. They selectively attack in shady places when the sunrise is high. They feed on any mammals but preferentially, they attack horses, cattle, buffaloes and goats. Birds, earthworms and other engorged insects have been reportedly attacked by biting midges. Habitually, they take more blood than their abdomen can contain. Their bites in humans are characterized by severe instant burning sensation, that can be compared to that caused by a burning ash. Some species find their way to the scalp to bite and cause substantial irritation.

2.14.2 Summer Seasonal Recurrent Dermatitis

The nuisance value of many *Culicoides* species have capable of producing undeniable consequences of both medical and veterinary significance following attacks. Such are seeing as allergic dermatitis in several hosts although more severe in equines. Repeated

attacks can predispose individuals to secondary bacterial infection with resultant serious medical problem.

In horses, their bites produce a chronic, seasonal recurring, irritating, superficial dermatitis on the withers, mane, tail and ears known as summer seasonal recurrent dermatitis. Depending on countries, the names defer but most commonly referred to as sweet itch, Queensland itch, 'dhobic itch', kascn disease, allergic dermatitis and sommerekzem. Horses respond by biting the sites with their teeth and this intend compromise skin integrity and capable of exposing them to secondary infections.

2.14.3 Filarial Parasites transmitted by *Culicoides*

Filariids are the only nematodes that have been linked to *Culicoides* species. They have been reported to transmit eighteen (18) filarial parasites to humans, cattle, horses and water buffalo and (Meiswinkel *et al*., 2004). Prominent among the filarial nematodes transmitted by biting midges are *Onchocerca* species and *Mansonella* species. Nine *Onchocerca* species have been recorded in cattle and water buffalo while four species have been documented in equines. They are reported to be transmitted by various *Culicoides* species. The genus *Mansonella* affects principally humans. The species include *Mansonella ozzardi, M. perstans* and *M. streptocerca*. These nematodes cause damages of various forms to carcass thus resulting in partial condemnation of carcass at meat inspection.

There are several parasitic nematode species in the genus *Onchocerca* that are spread by blackflies from the genus *Simulium* and *Culicoides* commonly called biting midges (CDC, 2020). It has been hypothesized that these flies' biting behaviours affect the presence and distribution of microfilariae in the subcutaneous tissues of their hosts (Ogbogu *et al*., 1990). *Onchocerca* has been divided into multiple species, and although humans, canids, and felids have also been reported to carry the parasite, ungulates appear to be its primary hosts (Lefoulon *et al*., 2017). In recent years, *Onchocerca* species infecting animals were found in human tissues.

When people and animals coexist in an area, such as when both hosts are present, zoonotic illnesses are more likely to spread between them (McArthur, 2020). In order to avoid and manage the introduction of zoonotic illnesses, it is crucial to take into account humans,

animals, and the environment as a whole, as is stressed in the One Health Strategy. Public health and socioeconomic facets of the world's population are impacted by newly emerging zoonotic diseases (McArthur, 2020). Around the world, 40 cases of people contracting an animal-infecting *Onchocerca* species have been reported so far (Wesolowska *et al*., 2020; Uni *et al*., 2015; Grácio *et al*., 2015; Fukuda *et al*., 2015).

The likelihood of an *Onchocerca* species infecting humans increases due to a number of circumstances. One of those aspects is the high prevalence of the causal agents in the host animals (Takaoka *et al*., 2005). A significant element that supports animal-human interaction is the growth of wild animal habitats brought on by climate change, deforestation, and urbanization (Uni *et al*., 2015). The geographical distribution of possible *Onchocerca* vectors, however, is determined by climate, and environmental changes, such as increasing climate warming, may alter the ranges in which these vectors can exist (Hoberg and Brooks, 2015).

Some *Culicoides* species act as carriers of *Mansonella* filarial worms. Mansonellosis is specifically caused by three species, *M. ozzardi*, *M. streptocerca*, and *M. perstans*, which are pathogenic to humans. Asymptomatically or, very rarely, with little clinical signs, infestations progress. Only humans are known vertebrate hosts for *M. ozzardi*, although *M. streptocerca* and *M. perstans* also cycle through monkeys. Additionally, *Simulium* blackflies seem to be effective carriers of *M. ozzardi*. *Mansonella perstans* is primarily found in Central and West Africa, where the principal vector species include *C. austeni*, *C. inornatipennis*, *C. grahami*, and certain species from the *C. milnei* group.

2.14.4 Protozoan Parasites transmitted by *Culicoides*

Most protozoan parasites transmitted by *Culicoides* species worldwide are found in birds. About thirty-three of such protozoan species have been documented. These include several *Haemoproteus* species, *Hepatocystis* species and avian trypanosomes. However, those affecting monkeys have also been reported and among them is *Hepatocystis kochi* transmitted by *Culicoides adersi* as well as other species of *Hepatocystis* found in several species of monkeys in Africa. Protozoa of squirrels in the genera *Callosciurus* and *Sundasciums* have been associated with *Culicoides* species in Malaysia. In all, about ten

(10) protozoa occur in birds and the most economically important is *Leucocytozoon caulleryi*. They also act as vectors of *Leishmania* species (Slama *et al*., 2014).

2.14.5 Viruses transmitted by *Culicoides* species

The vectorial roles of *Culicoides* species that have given them their prominence is not in their ability to transmit nematodes and protozoa but in the transmission of various viruses of greatest economic impact. Different viruses are transmitted by a variety of *Culicoides* species to a total of seventy-five (Mellor *et al*., 2000). Majority are considered of greatest importance to large animal industry (cattle, sheep, goats and horses) where they cause varying degree of economic losses and have constituted a serious setback in the industry. Among these 75 arboviruses linked to *Culicoides* species, only Orupouche is associated with humans. Twenty of these arboviruses belong to the family Bunyaviridae while the families Reoviridae and Rhabdoviridae have nineteen (19) and eleven (11) respectively (Mellor *et al*., 2000; Meiswinkel *et al*., 2004).

The veterinary significance of these viruses varies greatly. Bluetongue, African horse sickness, equine encephalosis, epizootic haemorrhagic disease and Akabane (AKA) are among the diseases of greatest importance. Schmallenberg virus, a novel Orthobunyanvirus has also been associated with *Culicoides* species. Twenty-five of these arboviruses have been detected from biting midges in Africa hence putting Africa in a state continuous surveillance and watchfulness.

Forty-six of the seventy-five viruses were identified from pools of *Culicoides* species containing single morphologically identified specimens rather than in mixed specimens. *Culicoides imicola* has been reported to be the principal vectors of most of these viruses in Africa. The regularity of feeding of a vector on susceptible and non-vulnerable vertebrate hosts will be strictly correlated to pathogen amplification and transmission risk for different vertebrates.

African Horse Sickness (AHS) is an arthropod-borne, infectious, non-contagious viral pathogen of equines transmitted by biting midges. It is prevalent in sub-Saharan African countries where it presents itself with a seasonal manifestation. All members of the family equidae are susceptible but with varying clinical manifestations. Horses are mostly affected

with severe clinical signs and resultant mortality while zebra and donkeys only show subclinical infection. Zebras and donkeys serve as reservoirs through which the virus is disseminated to a larger population. African horse sickness infection dogs and other carnivores occurs through consumption of infected carcass and infection could be severe cause high mortality. Currently, there is no treatment however, the best approach is prevention and control measures.

Bluetongue (BT) is a vector-borne, infectious, non-contagious viral disease of ruminants transmitted among susceptible hosts by *Culicoides* species (Standfast *et al*., 1985). Sheep are the principal hosts and degree of susceptibility varies between breeds. Infection may go unnoticed or be detected during entomological surveillance. Only certain *Culicoides* species are effective vectors for the biological transmission of bluetongue. The primary amplifying hosts are cattle, which are also likely significant maintenance hosts. Cattle are a more plentiful food source for the capable *Culicoides* vector species. The prevalence of insect vectors plays a significant role in determining the frequency and geographic distribution of bluetongue infections. The main role of the insect in BT epidemiology assures that ecological elements and climatic conditions, such as temperature, humidity, and soil qualities, which are favorable to insect life, control the occurrence of the illness. Therefore, the disease tends to appear seasonally in many parts of the world, typically in late summer and early fall.

There are currently 24 traditional BTV serotypes that can induce BT. Because sick animals are typically asymptomatic and the virus can be spread through direct contact, novel serotypes in goats have recently been discovered as abnormal (Maan *et al*., 2011; Zientara *et al*., 2014; Schulz *et al*., 2016; Sun *et al*., 2016; Savini *et al*., 2017; Marcacci *et al*., 2018). Depending on the species, BTV infection has drastically variable clinical results. According to MacLachlan *et al*., (2008), symptoms in sheep can be severe and have a high death rate, however in cattle, goats, and wild ruminants, symptoms are typically minor or nonexistent. It was proven through the use of controlled experimental settings that the host species and individual isolates of the BTV serotype play a crucial role in altering the clinical features (Caporale *et al*., 2014). The most frequent symptoms in clinically susceptible ruminants are various degrees of fever, conjunctivitis, hyperaemia of the

periorbital areas, nasal discharge, anorexia, lameness, prostration, and subcutaneous oedema in the inter-mandibular space, as well as hemorrhagic-necrotic erosions on the dorsal mucosal surface of the tongue, which in the most severe cases displayed a bluish staining. The body temperature rises in experimental BTV infection six days after intradermal inoculation, along with other early clinical (Puggioni *et al*., 2018).

Epizootic haemorrhagic disease is a life-threatening viral illness of ruminants. The aetiologic agent was recovered from wild *Culicoides* species. The disease is classically associated with fever, anorexia, disorientation and lethargy. It is common to observe swelling of the head and neck, dyspnea with protruding tongue, an arched back, and sore hooves.

Schmallenberg disease is a novel viral disease of ruminants caused by Schmallenberg virus (SBV) characterized in pregnancy by abortion and feotal malformation (European Food Safety Authority, 2012). Recently, the virus was found in populations of Palearctic *Culicoides* species captured during entomological monitoring operations. The hallmark symptoms of SBD are the deformities of the offspring, which are caused by the dam's infection at a specific period of gestation, the virus' ability to cross the placenta, and the vulnerability of the embryonic tissues to the virus. According to a review of the studies using pregnant ruminants as test subjects, SBV has a limited ability to cause fetal abnormalities (Collins *et al*., 2019). The estimated range for the percentage of deformed stillborn sheep within a flock was between 3% and 19%, depending on the breeding techniques used at the various farms (Dei Giudici *et al*., 2013).

Data from an experimental investigation on the Simbu serogroup virus Akabane (AKA), which is closely linked to SBV, has been used to help understand the pathophysiology of SBV (Konno *et al*., 1982). It is considered that the gestational stage at which dams are infected rigorously determines the effects of AKA virus (AKAv) infection on embryos. AKAv can cause fetal malformation in the central nervous system and skeletal muscles in pregnant cows after the first month of gestation, with the crucial time falling between the third and sixth month (Konno *et al*., 1982; Kirkland *et al*., 1988). AKAv was demonstrated in the placenta of pregnant sheep after 24 hours of intravenous inoculations, however only in lambs between 46 and 53 days of gestation did foetus deformity occur.

The embryo appears to be sensitive to the SBV between 47 and 162 days of age in naturally infected pregnant cows (Wernike *et al*., 2014). In the central nervous system of the SBV malformed stillborn lambs, kids, and calves, gross morphological examination primarily identified hydrocephalus, cerebellar hypoplasia, and spinal cord hypoplasia; in the musculoskeletal system, brachygnathia inferior, curvature of the vertebral spine (kyphosis, scoliosis, and torticollis); and arthrogryposis. Histologically, some deformed brains exhibit mild focal lymphocytic meningo-encephalomyelitis, with lymphocyte T being the most prevalent inflammatory cell type (Herder *et al*., 2012, 2013). It's interesting to note that only one of the twin lamb pregnancies is of relevance due to a noticeably greater incidence of abnormalities.

2.14.6 Medical importance of *Culicoides* species

In some areas, *Culicoides* biting midges can be a nuisance to people and make outdoor work and enjoyment activities uncomfortable. The bites can hurt, resulting in a minimal brief burning sensation and a minimal swelling that can persist up to a few hours Wongsathuaythong *et al.*, (1977). If a bite is vigorously scraped, secondary infection may result. As the cause of 70–95% of assaults on humans in several nations. This biting midge leaves a human with catastrophic injuries.

2.15 *Culicoides* and climate variables

Climatic factors do not only influence the distribution vectorial capacity of *Culicoides* species but also their prevalence and the number of midges trapped. Wind speed has been established to negatively influence midges' collections. Windy dusks decrease host seeking activities of biting midges. Wind was considered the most significant climatic factor affecting trapping of biting midges. Wind also has strong influence on the distribution *Culicoides* species and can disperse them over a long distance of about 700 km. It was established that collection of *C. imicola* and *C. bolitinos* was positively affected by temperature, but negatively influenced by humidity and wind speed in outdoor collections (Meiswinkel et al., 2000). This also was the same for *Culicoides* species in Kenya and a positive correlation was established between temperature and number of catches of *C. impunctatus*. Likewise, there exist a positive relationship between number of catch and relative humidity (Blackwell, 2001).

Culicoides imicola activity is optimal at air temperature of 18 – 38 °C. Biting midges' activities are also decreased due to high solar concentration (>200 Wm−2) and high humidity. High wind speed (>3ms-1) as well as high degree of wind turbulence (>40º variation) are another influential factor that decrease midges' activities. However, there is an increase in the number of adult biting midges after heavy rainfall (Meiswinkel, 1998) and rainfall above average is associated with occurrences of *Culicoides*-borne orbiviral diseases (Meiswinkel *et al*., 2004).

2.16 Control of *Culicoides* bites

The best approach to preventing *Culicoides* from biting is to protect the animals from contact with them. *Culicoides* species are always found in very large numbers (about 1 million) around livestock during their active period of warmer nights and after heavy rainfall seeking for blood meals. It is estimated that the number of catches per night is about 1% of active midges for that period. This is an indication of what these animals suffer unprotected. The rarity of effective control measures and basic information serves as a severe reminder to be sufficiently prepared for outbreaks of *Culicoides*-borne diseases.

2.16.1 Housing Livestock in Screened Insecticide buildings

As earlier postulated that *Culicoides* species exophagic (feed outdoors) and that, animals and humans indoors are safe from their bites. However, it is becoming uncertain if this is still the case. Several reports have now established their endophagic (indoor feeding) possibility. For example, it has been reported in Europe that the number of some *Culicoides* species trapped in horse stables exceeded those trapped outdoors under certain climatic conditions. This is likely the resultant effects of environmental temperature and which may increase at the beginning of winter.

2.16.2 Treating of either resting sites or host animals with insecticides

The topical application of insecticides directly on animals to control adult biting midges may not be realizable for livestock in extensive farming system, but may be useful for some valued animals for example racehorses which are not fully protected against disease pathogens transmitted by these biting midges. Many groups of insecticides are available but the most common are the pyrethroids and they have proven to be effective against most Diptera. The duration of the residual effects depends on the formulation used. Sprays, spot-

on and pour can be used weekly while insecticide-impregnated ear tags provide good effect in cattle for about six weeks, or more. Subcutaneous injection of a single dose of ivermectin has been reported to cause 99% mortality of engorged females 48 hours after ingestion of blood. This is found to effective even after 10 days of injection. Further research on the use of chemicals to control adult *Culicoides* species is necessary in regions with high rates of attacks (Carpenter *et al.,* 2007).

2.16.3 Environmental interventions in breeding sites of biting midges

This approach has been adopted for control of *Culicoides* species in several places all over the world except Africa. However, only little success was recorded. Adult *Culicoides* biting midges may be reduced by treating their breeding habitats using chemicals such as Temphos. Nevertheless, the rapid rate of reproduction of certain species in suitably moist soil following continuous rainfall will not only increase the financial involvement but will also make the approach impracticable (Schmidtmann *et al.,* 2000).

The effect of insecticides in the environment is one major factor that has hindered researches that could produce more potent chemicals. Also, the continuous use of only a few available active components may result in insecticide resistance.

2.16.4 The use of repellents against adult midges

Several repellents have been used to control arthropod nuisance and these usually remain effective for about 8 hours (Carpenter *et al.*, 2005). Most of them don't produce any side effects (irritation, toxicity etc.) on the hosts. Assessing the efficiency of repellent against *Culicoides* biting midges could be hindered due to their minuscule size and their ability to move at night, direct observation is challenging or nearly impossible (Logan and Birkett, 2007). A model insect repellent would be effective for different classes of arthropods and would have a residual effect of approximately 8 hours. The proposed insect repellents have different mode of action which include:

i. suppression of responsiveness to a stimulus that would otherwise be appealing;
ii. a shift in the sensory message from one of attraction to one of repulsiveness;
iii. the stimulation of a competing behaviour's receptor system;
iv. unpleasant odor receptor activation; and

v. simultaneous activation of many receptor types resulting in the loss of the particular signal for host location.

In recent study in South Africa, Venter e*t al*., (2009) affirmed that repellency can be tested by comparing the number of catches over a period of time following the application of repellents. The studies concluded that mixture of certain chemical oils produced a substantial repellent outcome against *Culicoides* species while few showed no significant repellent effect.

2.16.5 Use of Decoy hosts in Control of biting midges

The use of decoy hosts can be employed in certain conditions. Hosts with high preference but with little significance in terms of disease transmission are normally used. This is necessary to lure *Culicoides* species away from those hosts where their effect could be more devastating. For example, keeping cattle around sheep may have reduced influence on blue tongue infection in the sheep, since cattle are preferred hosts to sheep. However, a very important disadvantage is that, keeping decoy hosts will increase animal biomass thus increasing the number of hosts available to biting midges for feeding. This in turn will increase reproduction and consequently their population. The resultant effect is increasing transmission risk.

2.16.6 Protection of animals against bites from *Culicoides* during transportation

Arthropods are without borders hence they can easily overcome many barriers. This is even more true about *Culicoides* biting midges because of their small size. Thus, it is very important to consider methods to protect animals while on transit. There are several methods that could be used and these include (Doherty *et al.,* 2004; Melville *et al.,* 2005a, 2005b):

i. Treatment of animals with Insecticide repellents before and during transportation;
ii. Loading of animals when vectors' activity is minimal
iii. Ensuring non-stoppage of vehicles during dawn, sunset, or overnight transportation except the animals are fully protected.
iv. Creating a dark background in the inner compartment of the vehicle, by providing shades on the roof and/or sides of vehicles with shade cloth

v. Vectors surveillance at stopping locations and off-loading destinations to obtain data on seasonal variations.
vi. Utilizing historical facts or modeling statistics to detect ports or transport routes of less low risk.

2.16.7 Personal protection against *Culicoides*

There are three approaches described by Venter e*t al*., (2009) to achieve success in this: clothing, insect repellent and avoidance. Simple long sleeves and trousers will be a highly effective technique of defense against midge assault because midges cannot bite through clothing. particularly in combination with a personal repellent for insect on exposed skin using DEET (diethyl toluamide) and picaradin. To protect hands, you can also put on thin cotton gloves while the head and face could be protected with fine meshed netting together with a broad brimmed hat. Avoiding places where *Culicoides* biting midges occur and the of the day they are active will go a long way to save a lot of discomfort.

CHAPTER THREE

METHODOLOGY

3.1 **Experiment One: Morphological Identification of field collected *Culicoides* species**

3.1.1 Sample sites/Location

Benue State is located in the north-central Nigeria. It shares boundaries with Nasarawa to the north, Taraba on the eastern part, Ebonyi and Cross River to the south, Enugu to the south-western part and Kogi to the west as well as an international boundary with Cameroon to the south-east. The State has seasonal wet and dry seasons and is a member of the Koppen's Aw climate group. The dry season begins in November and lasts until March, while rain falls for seven months from April to October, totaling between 12,000 and 20,000mm annually (Ologunorisa and Tersoo 2006; Nyagba, 1995). Temperatures are consistently high, averaging between 280 and 320 degrees Celsius and occasionally rising to 370 degrees Celsius, especially in Makurdi, the state capital. The vegetation of Benue State still contains remnants of the guinea savanna, including many different species of dispersed trees and coarse grasses. In the State, there are only a very small number of dense forests, which are either found in forest reserves, village forests, or gallery forests (Nyagba 1995).

The followings were the criteria for site selection:

a. Availability of animal hosts (in this case equines and ruminant hosts). Various places where animals (horses, cattle sheep, goats) are kept in large numbers – (*i.e.,* >10 animals) were selected for sample collection. These include polo clubs, traditional rulers' farms, nomadic settlements, livestock markets, backyard farms, University farms, *etc*.
b. Locations with livestock around the neighborhood of the suction traps all night.

c. Locations with poles where the traps could easily be hanged and in which security of both the personnel and the traps can be guaranteed.

d. Places having dampness caused by rainfall or irrigation which are suitable habitats for *Culicoides* species.

e. Willingness of the livestock owners to permit setting of traps.

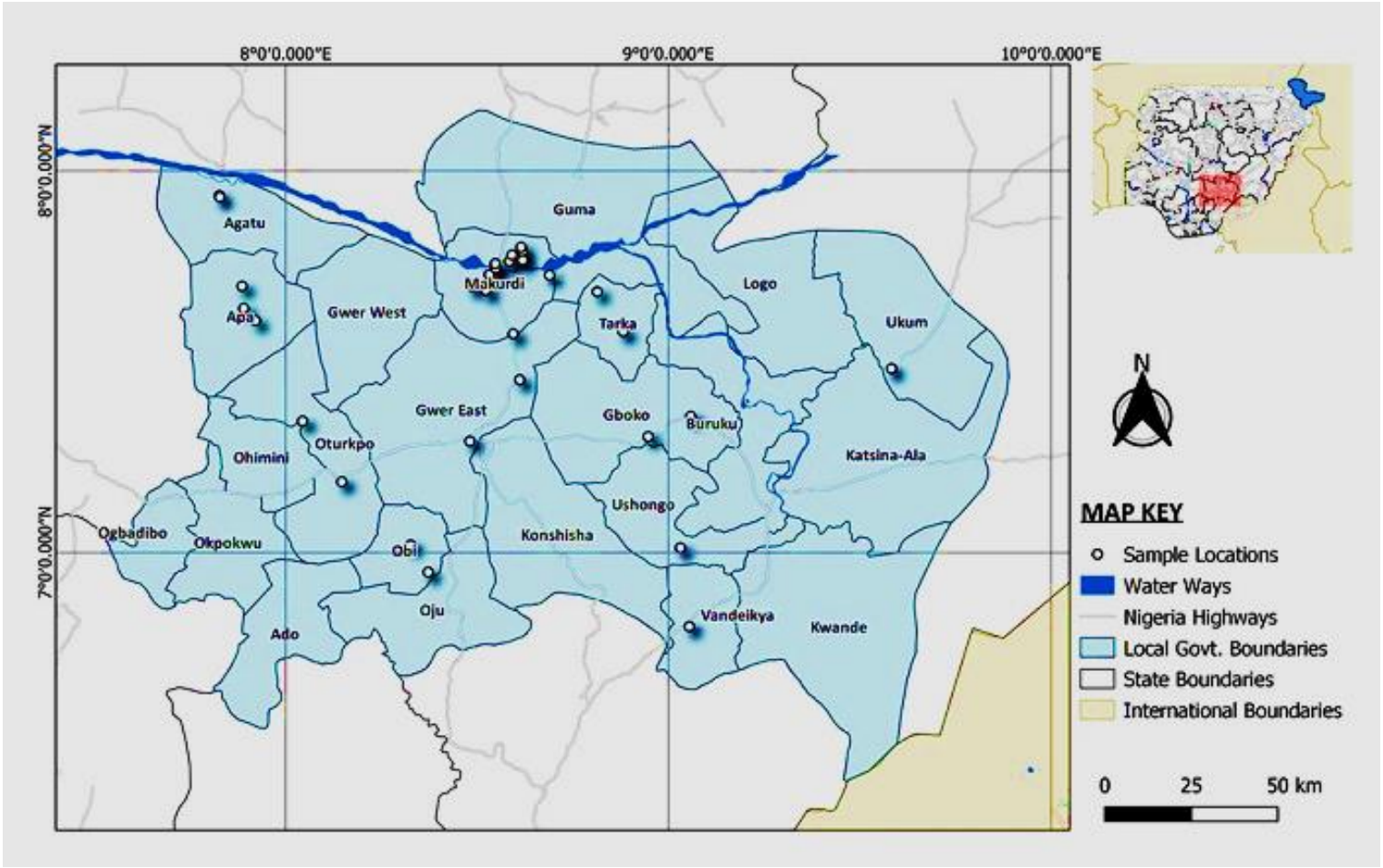

Figure 3.1: Map showing various collection sites of *Culicoides* species in Benue State, Nigeria (Oke *et al*., 2022 unpublished)

3.1.2 Sample Collection

A purposive and convenience sampling technique was adopted in this study. Adult *Culicoides* biting midges were collected from thirty different locations across the three geopolitical zones in Benue State, North Central Nigeria (Figure 3.1). The protocols of Harrup (2014) were used for the selection of sites, collection of adult biting midges, identification and storage of trapped specimens. Field collections of adult *Culicoides* species were carried out weekly for a period of twenty-four months using two CDC traps (Miniature Downdraft Black-light (UV) Trap – Model 1212 and New Jersey Standards Light Trap- Model 912).

Batteries were charged and the traps were assembled according to manufacturer's instructions, then the traps were test run to ensure proper functioning. The collecting pots were then carefully fixed into the mesh net tube and screw in place firmly over the solid fabric end of the net. The traps were attached to poles surrounding the animal pen at a height of roughly two meters at minimum distance of at least 50 metres apart to avoid interference. Insects were allowed to fall into the collecting pots containing a mixture of two drops of liquid detergent (Morning fresh®) in 150mls of water. The traps cables were connected to the batteries as per manufacturer's instruction and the traps were allowed to function from nightfall to dawn (5:00pm to 8:00am) – taking into consideration their crepuscular potentials. Furthermore, photographs of the traps *in situ*, the vicinity around the traps and the species of animals in close proximity to the traps were captured using Digital camera (Kodak EasyShare P880) – Figure 3.2. The traps were disconnected the next morning and the contents of the collecting pots were poured into the sieve and the insects were put into marked sample vials filled with 70% ethanol and were kept in a secured, cool and dark area. The samples upon retrieval were transported to the Entomology laboratory, Federal University of Agriculture, Makurdi where they were sorted out and preserved.

A total of thirty (30) locations were screened, however, only 2 sites were adopted for a whole year collection (College farm, South Core and University farm, North Core). Collections were undertaken from the many sheds where diverse hosts, including cattle, sheep, and goats, horses, dogs, chickens and human co-habit. Accuweather.com® was used

to record weather data for each collection, including temperature, humidity, wind direction and speed, rainfall, and precipitation. For other sites, random samplings near livestock were performed to enable a fair representation. The geographical coordinates were recorded using Global positioning system (GPS) unit (Garmin eTrex® 30X)

Figure 3.2: Trap suspended at one of the collection sites in Benue State, Nigeria (Oke *et al*., 2017)

Keys: A – A 6 volts battery (Source of power supply)

B – Light trap suspended on a tree

3.1.2 Stereomicroscopy and Storage of Adult *Culicoides*

In the laboratory, *Culicoides* species were then sorted based on sex. The females were afterward grouped according to their physiological status: nulliparous, parous, gravid or blood-engorged by observing the abdomen. *Culicoides* species were identified first to species groups (Imicola group; Schultzei group; Milnei group, *etc*.) using identification keys and based on distinctive wing patterns. Each group was separately preserved in sample bottle containing 70% ethyl-alcohol and were later identified into various species. Digital pictures of various characteristic features were taken for referencing and species identification. All data obtained were properly documented for easy analysis.

The following measurements were taken to aid morphological identification:

- Length of every palpal segments
- Length of flagellar segments
- Length and width of wings (from arculus to tip)
- Length and width of spermathecae
- Antennae ratio (AR): 11-15 antennal segments divided by segments 3-10)
- Palpal ratio (PR): length of segment 3 divided by greatest breath)

The following angles were taken to also aid identification:

- Arculus angle
- r-m cross vein angle
- Median angle
- Cubital angle

Measurements were made using Image-Pro Plus software while slide-mounted specimens were seen through a Nikon Alphaphot-2 YS2 compound light microscope (Nikon Instruments, Europe) equipped with a Q Imaging (QI CAM) camera attachment (Media Cybernetics Inc., Rockville, USA). Individuals had their heads (antennae and palps) wings, and abdomens (spermathecae) measured morphometrically. Six ratios were calculated from the thirteen variables, based on data that prior literature had found to be significant in terms of species discrimination. In addition, four angles were measured.

3.1.2.1 Head measurements

The third palpal segment from the maxillary palps was measured for length and width, and the palpal ratio was determined (length: width). Flagellomeres 10^{th} and 11^{th}'s lengths, as well as the sum of their five apical and eight basal flagellar segments, were measured from the antennae. The head measurements were used to compute the antenna segment ratio, flagellar ratio and palp ratio. Flagellar ratio is the length of flagellomere 11/length of flagellomere 10. The antenna1 ratio (A.R.) is calculated by dividing the lengths of the eight preceding segments by the sum of the five distal flagellar segments *i.e.*, (total length of 5 apical segments (11-15)/total length of 8 basal segments). Proportions given for flagellar segments refer to relative lengths and should not be regarded as absolute measurements. The third palpal segment's length is divided by its widest segment to determine the palpal ratio (P.R.).

3.1.2.2 Wing Measurements

Wing's lengths were measured from the arculus to the tip, costa length, and width (from the site of the second radial cell to the base of vein Cu_1). The wing ratio (wing length/width) and costa ratio (costa length/wing width) were calculated using these measurements. The costal ratio is calculated by dividing the length of the costa by the wing width. The distance between the basal arculus and the wing tip is the length of the wing.

3.1.2.3 Abdomen measurements

Both spermathecae's length and width were measured on the posterior region of the abdomen. The larger and smaller spermathecae were measured to determine the spermatheca ratio (spermatheca length/spermatheca width) separately for larger and smaller spermathecae.

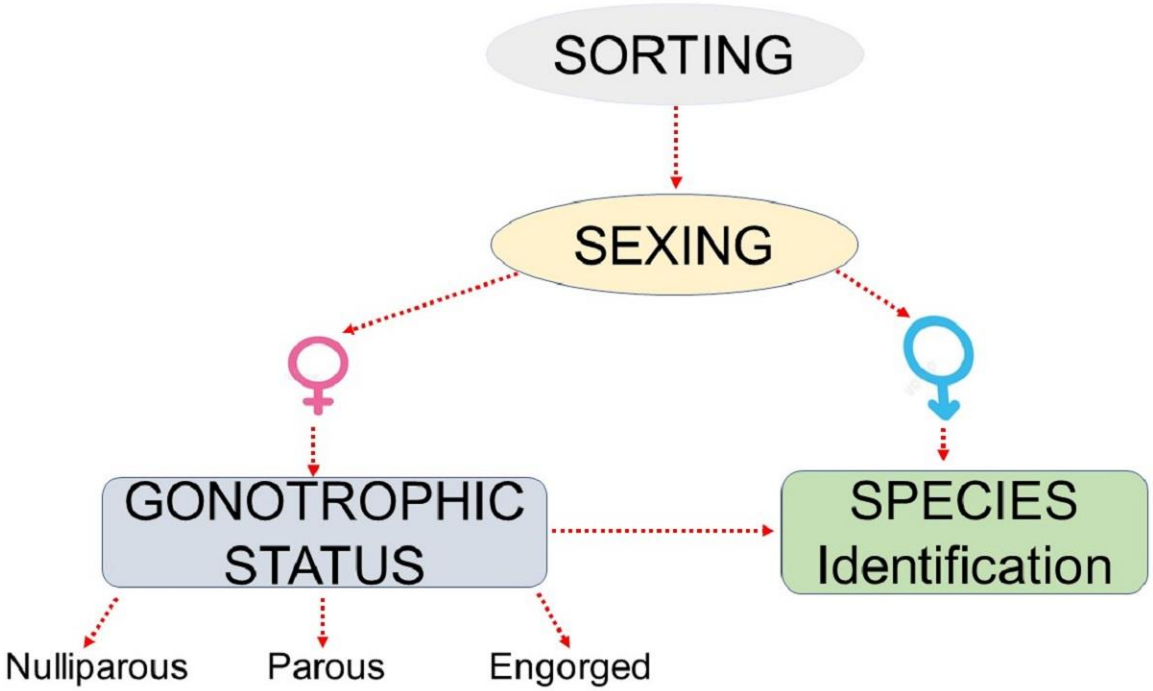

Figure 3.3: Work flow for stereomicroscopy of *Culicoides* species (Oke *et al*., 2022 Unpublished)

3.1.3 Data Analysis

Data were analysed appropriately using descriptive statistics. However, Linear regression analysis was carried-out in Microsoft Excel to compare the correlation between the numbers of *Culicoides* collected using the two light traps while Chi- square, Correlation coefficient, Odds ratio, Risk factor and Relative risk were conducted using GraphPad prism®.

3.2 Experiment Two: Identification of *Culicoides* species by Polymerase Chain Reaction

3.2.1 Extraction of DNA

Extraction of deoxyribonucleic acid (DNA) was carried out using a commercial DNA extraction kit (Quick-DNA™ Tissue/Insect Miniprep Kit, USA, 50 Preps, Catalog No: D6016). A total of 50 pools from the two most abundant species were used for DNA extraction with each pool containing 25 engorged adults female *Culicoides* species of similar morphological identities. Preliminary morphological identification of individual specimens in each pool was conducted before extraction. Slight modification was made to the producer's guidelines by manually grinding the *Culicoides* species with pestle and mortar instead of spinning them using beads as stated by the manufacturer. All other procedures in the protocols were fully followed. The specimens were spun at 10,000 revolution per minute (rpm) for 1 minute after each step using the Centrifuge - Eppendorf 5424R. The DNA products were then preserved at 4^{o}C until further processed.

3.2.2 Quantity Check

The obtained DNAs from *Culicoides* species were quantified by Nano-dropping technique using Thermoscientific NanoDrop 2000 Spectrophotometer®. The machine was returned to blank by using elution buffer to run the first step. Subsequently, individual samples were run separately to determine the DNA quantity in ng/µl.

3.2.3 Polymerase chain reaction

Culicoides species were identified using the ribosomal gene internal transcribed spacer 1(*ITS-1*). The primers PanCul-F (5′-GTA-GGT-GAA-CCT-GCG-GAA-GG-3′) and PanCul-R (5′ -TGC-GGT-CTT-CAT-CGA-CCC-AT-3′) were used. Amplification was carried out at an initial denaturation of 94^{o}C for 5 mins, then 30 cycles of denaturation at

94^{o}C for 1 minute, annealing at 58^{o}C for 1 minute and elongation step at 72^{o}C for 1 minute with a final elongation step at 72^{o}C for 10 minutes and holding temperature at 4^{o}C until processed using a total reaction volume of 25 µl comprising 2.5 µl of 10 x PCR buffer, 1 µl of 25 mM $MgCl_2$, 1 µl each of forward (PanCul-F) and reverse (PanCul-R) primers, 1 µl of DMSO, 2 µl of 2.5 mMdNTPs, 0.1 µl of 5Units/µl Taq DNA polymerase, and 5 µl of 10 ng/µl DNA in 11.4 µl nuclease free water, Former *et al*., 1994).

3.2.4 Agarose Gel Electrophoresis for *Culicoides* Amplicons

A preparation of 1.5% Agarose gel (w/v) was obtained by mixing three tablets of agarose (each table containing 0.5g agarose) into 0.1liter NaEDTA buffer. The mixture was stirred, allowed for 2 minutes in microwave until properly dissolved. This was decanted into gel casting tray after incorporating 50mg/l of ethidium bromide and brought to solidify. The amplicons were mixed (5:1) with the loading dye buffer and each gel wells were loaded with 100µl of separate amplicons. The first and the last wells were filled with molecular size 50bp plus ladder (Fermentas) to travel along with the samples for easy determination of the fragments' size. A voltage of 110 volts was used to power the electrophoresis tank for 60 minutes. The gels were visualized for presence of bands and the images taken using Biorad transilluminator.

3.3 Experiment Three: Molecular Characterization and Phylogeny of Selected *Culicoides* species

3.3.1 Sequencing of PCR amplicons

Selected amplicons that displayed positive bands sizes on gel electrophoresis were used for sequencing to confirm *Culicoides* species. The selected amplicons were sequenced in both directions. Forward and reverse primers (PanCul-F and PanCul-R) were respectively used for sequencing. The obtained sequences were trimmed using the software - Bioedit® and consensus sequences were generated which were further used. The sequences were analyzed by blasting using NCBI Basic Local Alignment Search Tool (BLAST) and then compared with sequences in the GenBank database (NCBI, 2010). The most likely species were those sequences in a given pair-wise alignment with the lowest E-value and >90% similarity.

3.3.2 Phylogeny

The phylogeny of obtained *Culicoides* species were determined using Molecular Evolutionary Genetic Analysis (MEGA) 11 software. The sequences of established *Culicoides* species were imported to MEGA-11 in Festa format along with other sequences in GenBank which established similarities. DNA sequence alignment were created after which the data were saved in MEGA format. The phylogynetic tree was constructed using neighbour joining tree (Boostrap method) 1000 Boostraps replication, (Tamura *et al*., 2021).

3.4 Experiment Four: Determination of sources of blood meals

3.4.1 Extraction of DNA and quantity Check

A total of 50 pools containing 1-50 adult engorged female *Culicoides* species per pool were used. Each pool contained morphologically identical species using wing veinations. The DNA extraction was conducted with a commercial DNA extraction kit (Quick-DNA™ Tissue/Insect Miniprep Kit, USA, 50 Preps, Catalog No: D6016). Each pool was discretely emptied into mortal and gently crushed after proper draining of the preservative (70% ethanol). This was mixed with 750 µl of bashing bead buffer and centrifuged at 10,000 rpm. 400 µl of genomic lysis buffer was added to the filtrate in collection tube and double centrifuged at 10,000 rpm/minute. 200 µl of pre-wash DNA buffer was then added and spined at 10,000 rpm. This was washed with 500 µl of g-DNA buffer and centrifuged at 10,000 rpm/minute. The DNA was eluted into 1.5 ml microcentrifuge tube using 100 µl DNA elution buffer and spined at 10,000 rpm/minute. The DNA products were then preserved at 4ºC until further processed. The obtained DNAs were quantified (ng/µl) by Nano-dropping technique using Thermoscientific NanoDrop 2000 Spectrophotometer®.

3.4.2 Polymerase Chain reaction/Amplification

For detection of sources of blood meals, the DNA products obtained from *Culicoides* species were amplified using the forward and reverse primers: *Cyt-B 1* (5'- CCA-TCC-AAC-ATC-TAC-GCA-TGA-TGA-AA 3') and *Cyt-B 2* (5'-GCC-CCT-CAG-AAT-GAT-ATT-TGT-CCT-CA-3') as described by Steuber *et al*., (2005).

Amplification was carried out at an initial denaturation of 95ºC for 5 minutes, followed by 35 cycles of denaturation at 95ºC for 45 secs, annealing at 50ºC for 30 secs and elongation

step at 72ºC for 45 secs then final elongation step at 72ºC for 7 minutes and a holding temperature at 4ºC forever using a total reaction volume of 25 µl comprising 2.5 µl of 10 x PCR buffer, 1 µl of 25 mM $MgCl_2$, 1 µl each of forward (Cyt-B 1) and reverse (Cyt-B 2) primers, 1 µl of DMSO, 2 µl of 2.5 mMdNTPs, 0.1 µl of 5Units/µl Taq DNA polymerase, and 5 µl of 10 ng/µl DNA in 11.4 µl nuclease free water.

3.4.3 Agarose Gel Electrophoresis

A preparation of 1.5% Agarose gel (w/v) was obtained by mixing three tablets of agarose (each table containing 0.5g agarose) into 0.11iter NaEDTA buffer. The mixture was stirred, allowed for 2 minutes in a microwave until properly dissolve. This was decanted into gel casting tray after incorporating 50mg/l of ethidium bromide and allowed to congeal. The amplicons were mixed with loading dye (5:1) and individual gel well was loaded with 100µl separately. The first and the last wells were filled with molecular size 50bp ladder (Fermentas) to travel sideways with the samples for easy determination of the fragments' size. A voltage 110 volts was used to power the electrophoresis tank for 60 minutes. The gels were visualized for presence of bands and the images taken using Biorad transilluminator.

3.4.4 Sequencing of Amplicons

Amplicons that displayed positive bands sizes on gel electrophoresis were selected for sequencing to confirm their sources of blood meals. The observed amplicons were sequenced in both directions. The sequencing primers were the respective forward and reverse primers (*Cyt-B 1* and *Cyt-B 2*). The obtained unknown sequences were edited using Bioedit® and the consensus sequences which were generated were blast in NCBI. The BLAST results were compared with those previously placed in the GenBank database (NCBI, 2010). Sequences of a given pair-wise alignment with the lowest E-value and 90% similarity and above were selected as the most likely species of host.

3.5 Experiment Five: Detection of Filarial parasites from adult female *Culicoides*

3.5.1 Polymerase Chain Reaction/Amplification

The primers PanFila-F (5′-TGT-GCT-GCG-CTA-CAT-CGA-TG-3′) and PanFila-R (5′-AAA-CCG-CTC-TGT-CTC-ACG-AC-3′) was employed (Kronefeld *et al.*, 2014). Amplification was carried out at an initial denaturation of 94ºC for 5 minutes, followed by

35 cycles of denaturation at 94^{o}C for 30 secs, annealing at 55^{o}C for 45 seconds and elongation step at 72^{o}C for 90 secs then final elongation step at 72^{o}C for 7 mins and hold temperature at 4^{o}C forever using a total reaction volume of 25 µl comprising 2.5 µl of 10 x PCR buffer, 1 µl of 25 mM $MgCl_2$, 1 µl each of forward (PanFila-F) and reverse (PanFila-R) primers, 1 µl of DMSO, 2 µl of 2.5 mMdNTPs, 0.1 µl of 5Units/µl Taq DNA polymerase, and 5 µl of 10 ng/µl DNA in 11.4 µl nuclease free water.

3.5.2 Gel Electrophoresis for Panfilaria Amplicons

A preparation of 1.5% Agarose gel (w/v) was obtained by mixing three tablets of agarose (each table containing 0.5g agarose) into 0.11iter NaEDTA buffer. The mixture was stirred, allowed for 2 minutes in a microwave until properly dissolve. This was emptied into gel casting tray after incorporating 50mg/l of ethidium bromide and allowed to clot. The amplicons were mixed with loading dye (5:1) and separate gel well was loaded with 100µl separately. The first and the last wells were filled with molecular size 50bp ladder (Fermentas) to travel laterally with the samples for easy determination of the fragments' size. A voltage 110 volts was used to power the electrophoresis tank for 60 minutes. The gels were visualized for presence of bands and the images taken using Biorad transilluminator.

3.5.3 Sequencing of Amplicons

Amplicons that displayed positive bands on gel electrophoresis were selected for sequencing to confirm the pathogens harboured. The observed amplicons were sequenced in both directions. The sequencing primers were the respective forward and reverse primers (PanFilaF and PanFilaR). The obtained unknown sequences were edited using Bioedit® and the consensus sequences which were generated were blast in NCBI. The BLAST results were compared with those previously placed in the GenBank database (NCBI, 2010). Sequences of a given pair-wise alignment with the lowest E-value and 90% similarity and above were selected as the most likely filarial parasites.

3.5.4 Data Analysis

Data generated from these studies were analyzed using SPSS version 21 (IBM, USA) for Chi-square. The BioRad CFX 96 software was used to process PCR results.

CHAPTER FOUR

RESULTS

4.1 Morphological Identification of *Culicoides* species from the field

A total of thirty thousand one hundred and sixty-three (n=30,163) adult specimens were collected during the fifty-two-week sampling period. This comprises twenty-nine thousand five hundred and eighty-six (n=29,586) morphologically identified species and five hundred and seventy-seven (575) unidentified specimens. Among these, female represented 87.86% while 31.37% of these had taken blood meal (parous and engorged) before being trapped. The highest number of collection (45.21%) was recorded in Benue South geopolitical zone while the lowest catches were obtained from Benue Northeast zone (23.62%) Table 4.1.

Twenty-one species were morphologically identified using wing pigmentation (Plates 4.1 – 4.26) and seven of these species were trapped in every collection during the entire period (Table 4.2). Seven of the twenty-one species that were morphologically identified throughout the sampling period were obtained at each location. The *Culicoides* species identified belong to seven species groups (Schultzei, Imicola, Milnei, Similis, Neavei, Grahami and Dasyops). Also, all the species identified have their females fully represented. However, males of five species were not trapped. (Table 4.2). *Culicoides imicola, C. oxystoma, C. subschultzei* and *C. enderleini,* were the most prevalent in the following respective orders 37.56%, 13.81%, 11.60% and 10.48% while *C. karaensis* (0.41%), *C. kanagai* (0.33%) and *C. indistinctus* (0.24%) were the least captured species (Table 4.2). The highest catches occurred in September (49.30%) while January had the lowest (0.91%). It was also observed that *Culicoides* species were present throughout the period of collection (Table 4.6). The species morphologically identified using characteristic features in comparison with standard keys for identification of *Culicoides* are presented below with their individual notes:

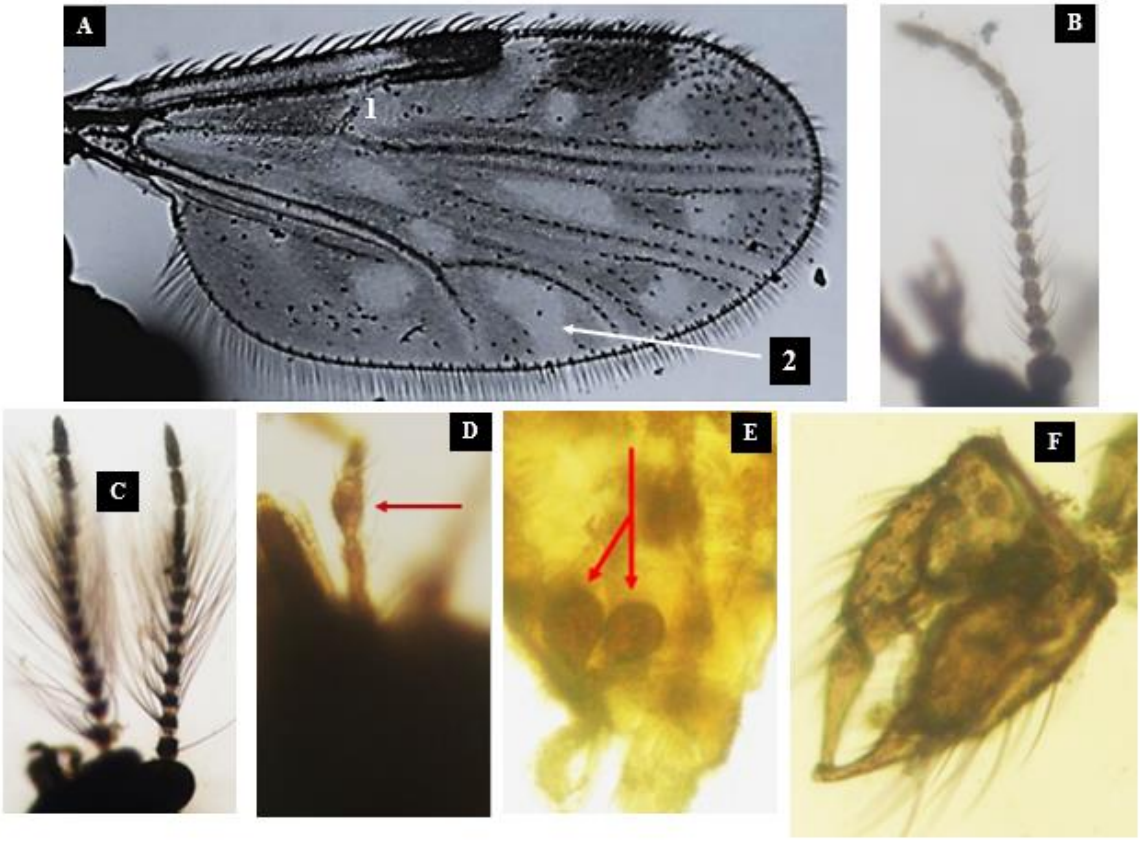

Plate 4.1: A = Wing of *Culicoides subschultzei* with distinct pale spot over r-m cross-vein (**1**); pale spot in cubital cell extending almost the entire height (**2**).

B = Pilose Female antenna

C = Plumose Male antenna

D = Third palp segment (short and moderately inflated)

E = Two functional ovoid spermathecae

F = Male genitalia

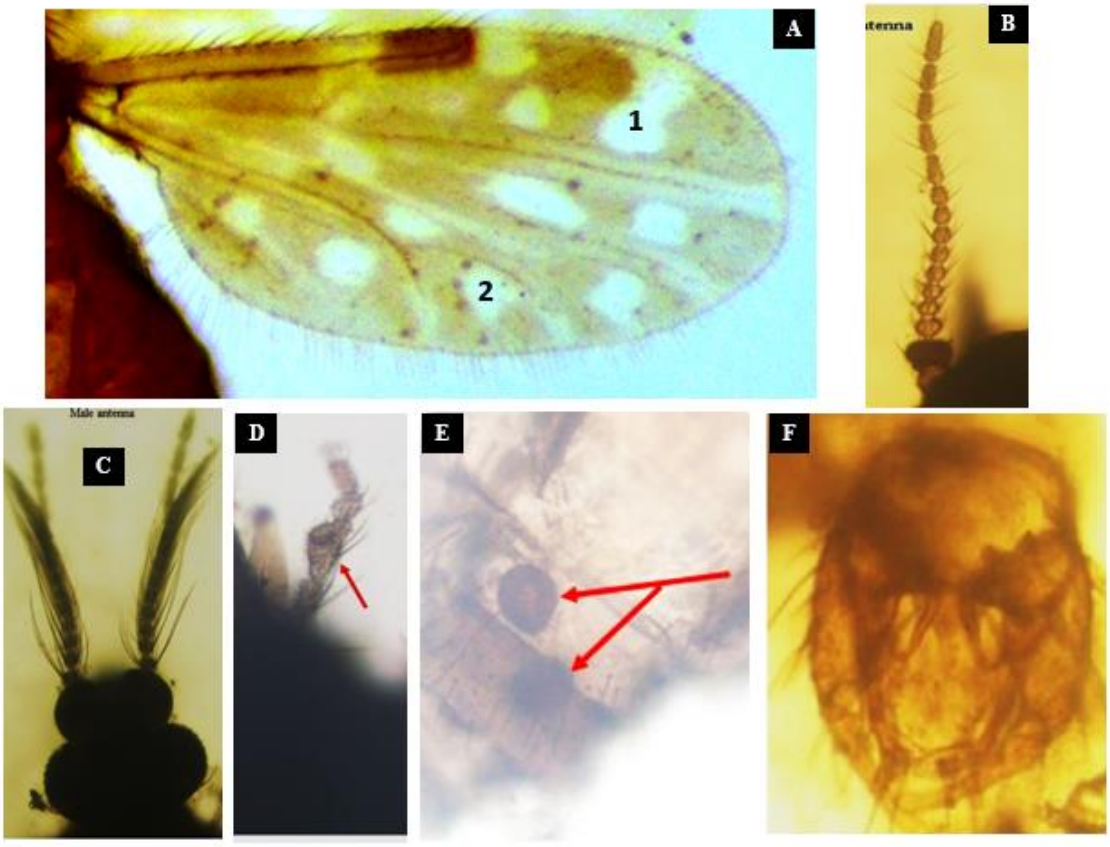

Plate 4.2: A = Wing of *Culicoides enderleini* with cell R5 having a moderately large pale spot (**1**); a single pale spot in cubital cell that abuts the cubital vein (**2**).

B = Pilose Female antenna
C = Plumose Male antenna
D = Third palp segment (short and moderately inflated)
E = Two subequal spermathecae
F = Male genitalia

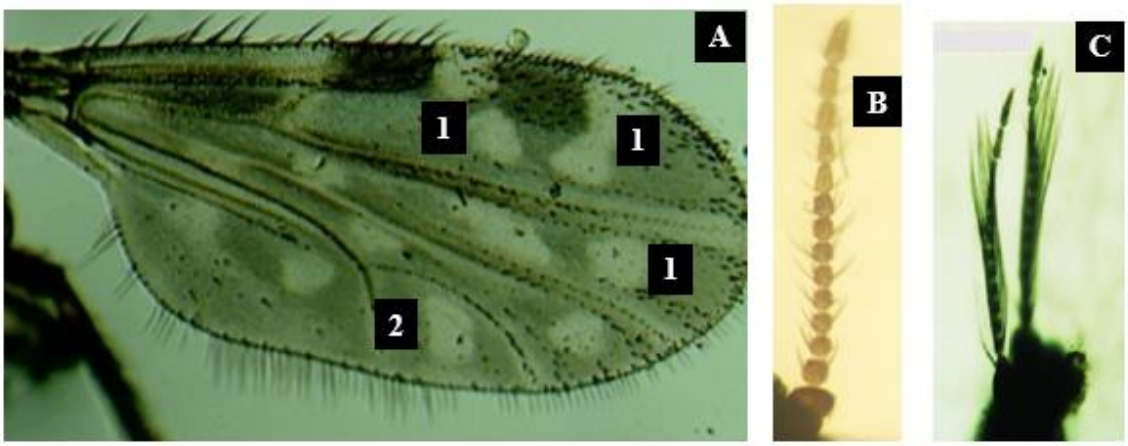

Plate 4.3: A = Wing of *Culicoides nevilli* with prominent well defined pale spots (**1**); a single large pale spot in cubital cell (**2**).

B = Pilose Female antenna

C = Plumose Male antenna

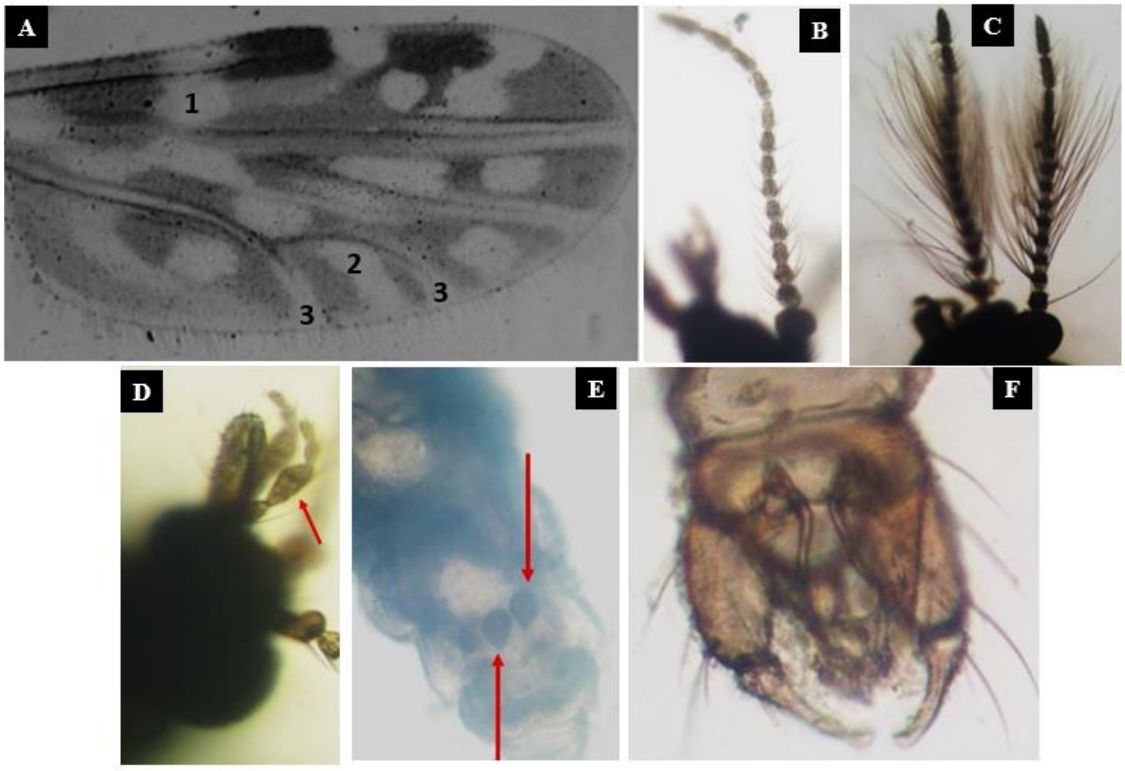

Plate 4.4: A = Wing of *Culicoides oxystoma* with a large pale spot on the r-m cross-vein (**1**); single pale spot in cubital cell that abuts the cubital vein (**2**); margins of M1 and M2 are pale (**3**).

B = Pilose Female antenna

C = Plumose Male antenna

D = Third palp segment (moderately inflated)

E = Two functional oval subequal spermathecae with third rudimentary

F = Male genitalia (aedeagus somewhat 'V' shape

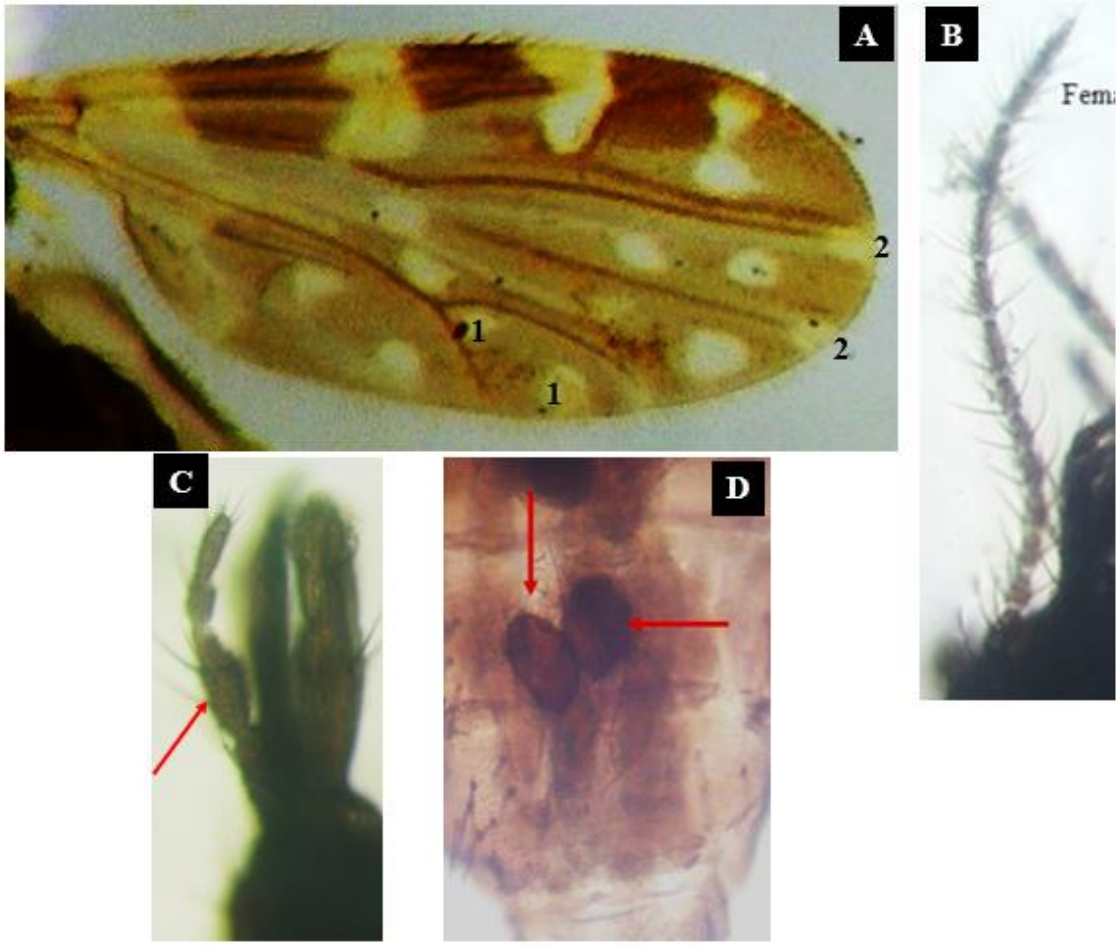

Plate 4.5: A = Wing of *Culicoides moreli* with two pale spots in cubital cell (**1**); pale spots at the end of m1 and m2 (**2**) absent in *C. milnei*.

B = Pilose Female antenna

C = Third palp segment, somewhat elongated

D = Two equally large pigmented ovoid spermathecae

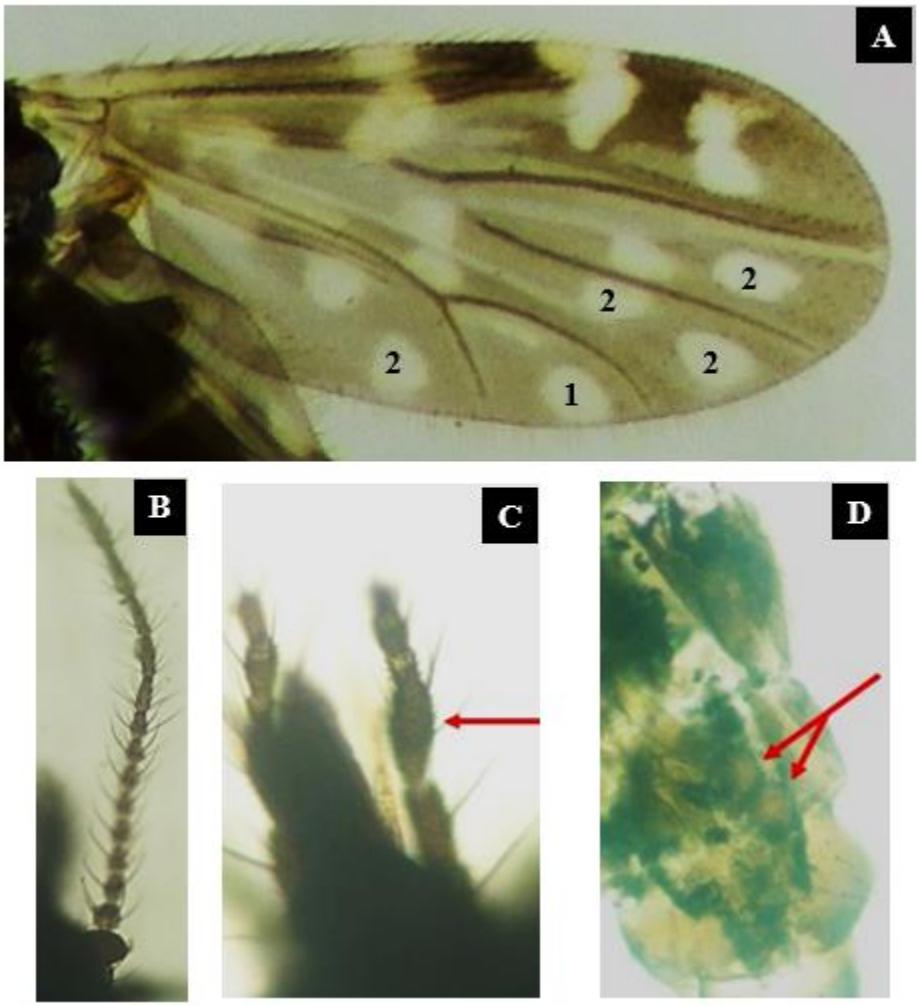

Plate 4.6: A = Wing of *Culicoides milnei* with a large pale spot in cubital cell (**1**); majority of the pale spots are large (**2**).

B = Pilose Female antenna
C = Third palp segment, somewhat elongated
D = Two round unequal spermathecae

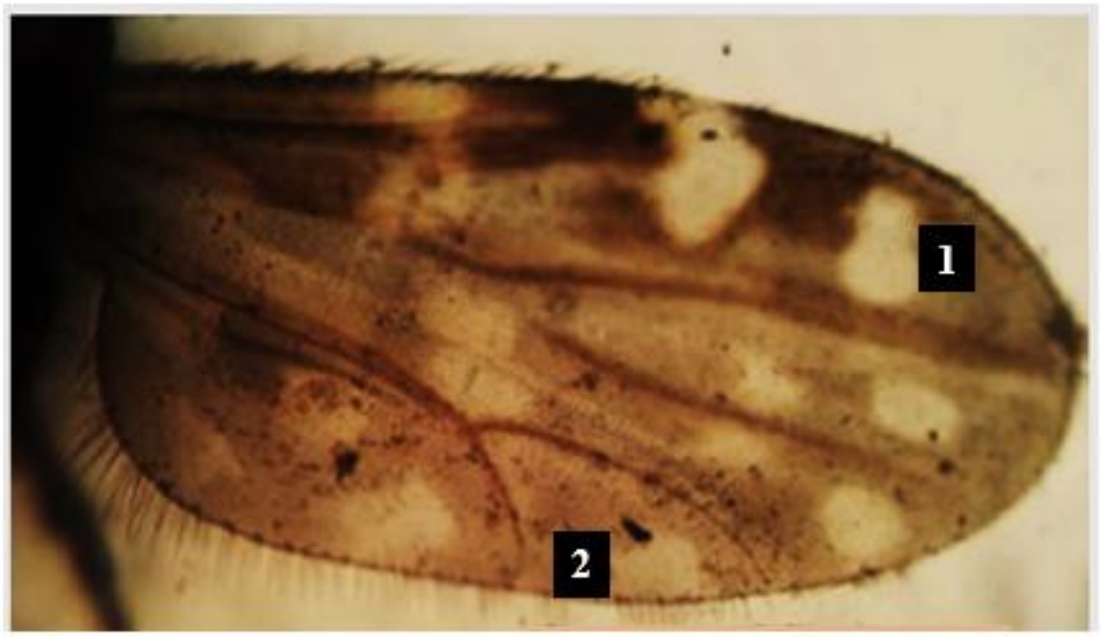

Plate 4.7: A = Wing of *Culicoides austeni* similar to *C. milnei* but with a distinct vertical pale band (**1**); a single large pale spot in cubital cell that abuts the wing margin (**2**).

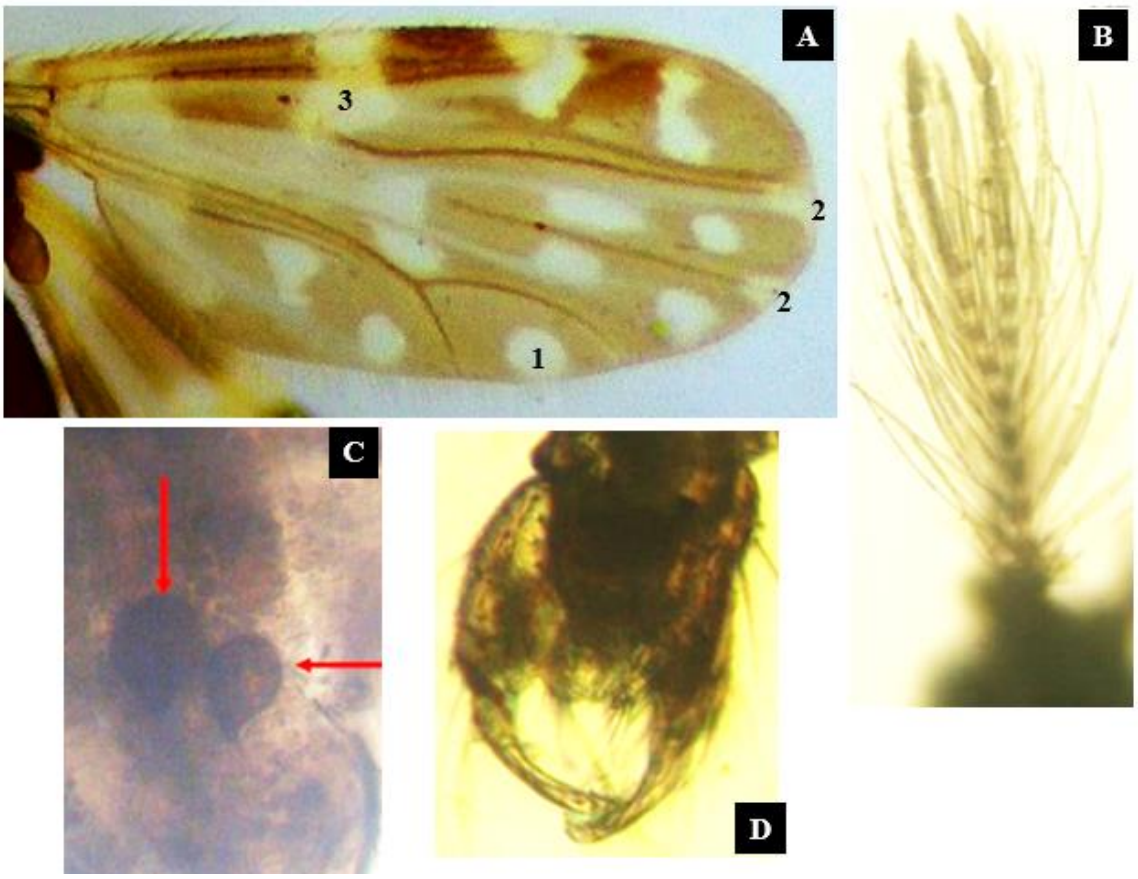

Plate 4.8: A = Wing of *Culicoides zuluensis* with a large pale spot in cubital cell (**1**); pale spots at the end of m1 and m2 which are absent in *C. milnei* (**2**); quadrangular pale spot over r-m cross-vein (**3**)

B = Plumose Male antenna

C = Two round unequal spermathecae

D = Male genitalia

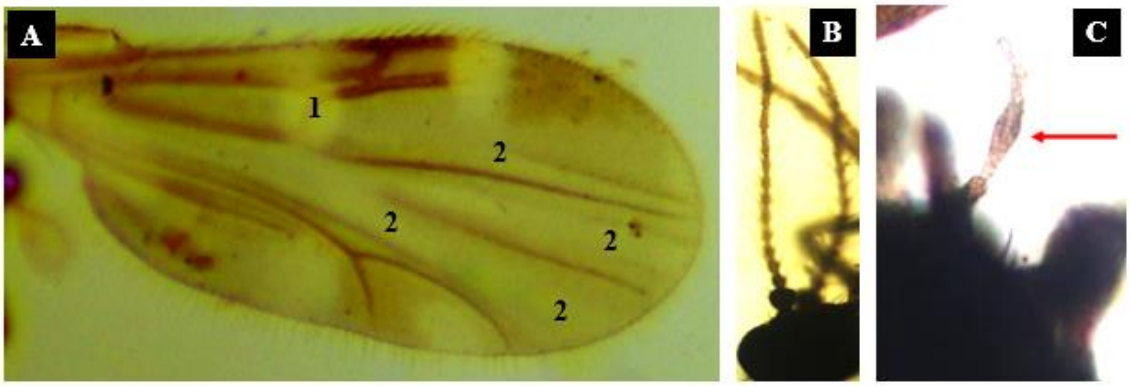

Plate 4.9: A = Wing of *Culicoides fulvithorax* with a round distinct pale spot over the r-m cross-vein (**1**); ill-defined spot over the mid-line that extend to the wing margins (**2**)

B = Pilose Female antenna

C = Elongated third palp segment

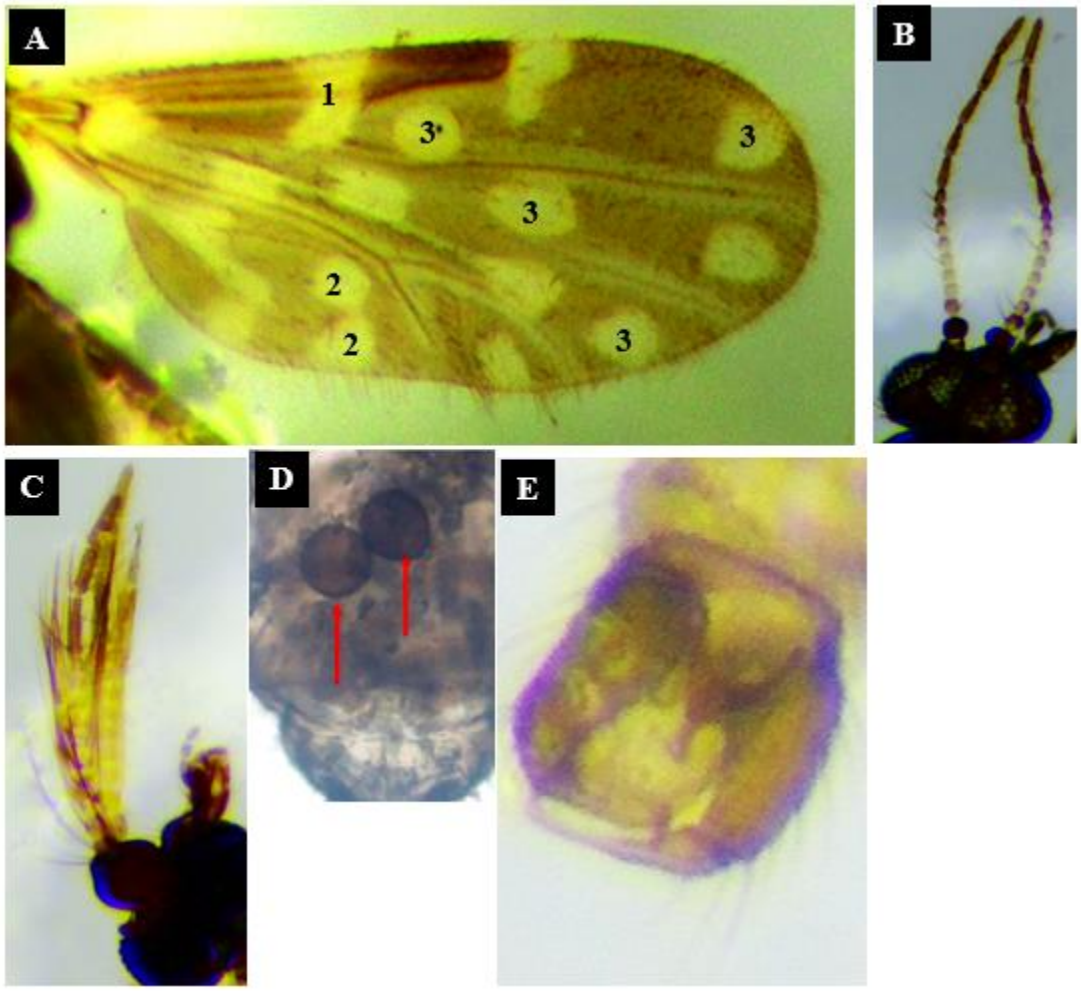

Plate 4.10: A = Wing of *Culicoides neavei* with a large pale spot over r-m cross-vein extending to costal margin (**1**); two large round pale spots in anal cell (**2**); entirely large pale spots (**3**)
B = Pilose Female antenna
C = Plumose Male antenna
D = Two equally large well pigmented round shaped spermathecae
E = Male genitalia (aedeagus triangular with curved lateral arms

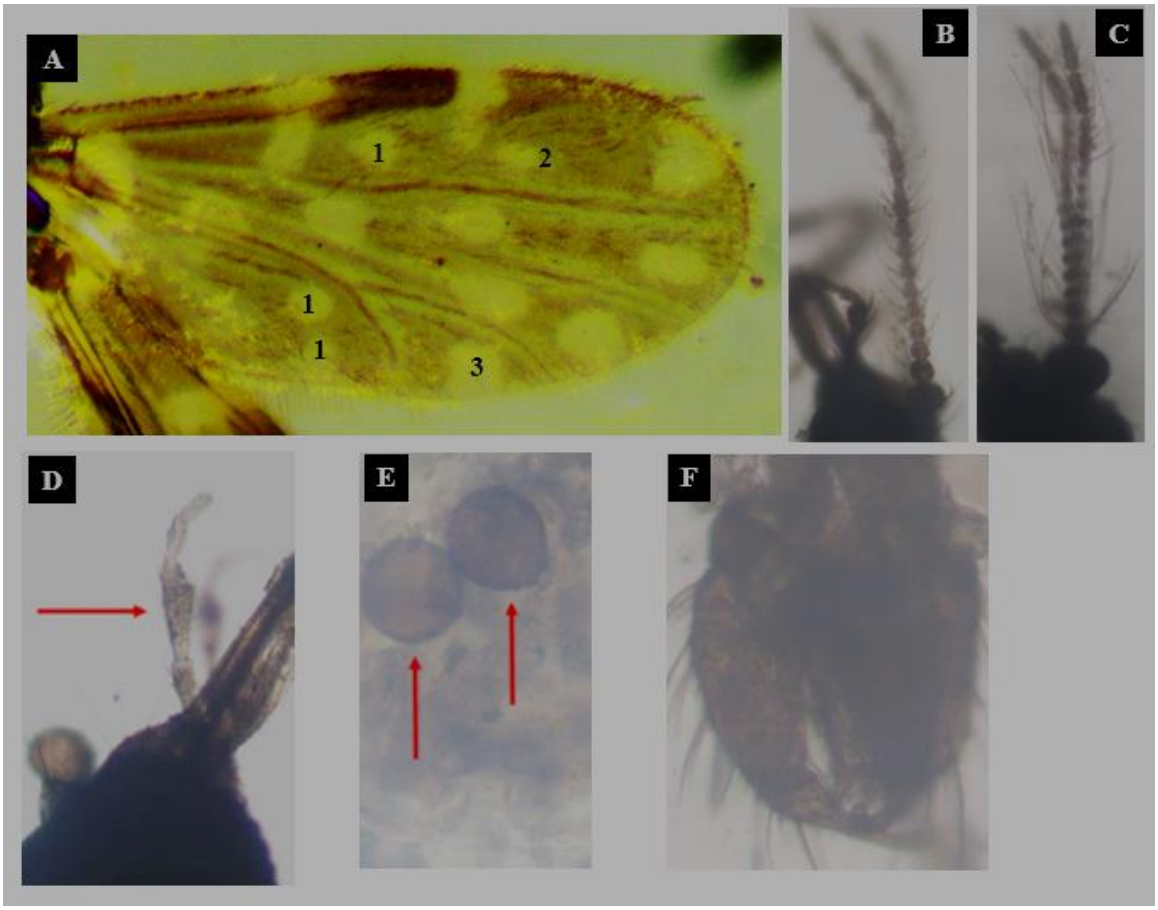

Plate 4.11: A = Wing of *Culicoides pycnosticus* with smaller and less confluent pale spots (**1**); possesses additional pale spot below and beyond the 2nd costal spot (**2**); a single large round pale spot in cubital cell that abuts cubital vein (**3**)

B = Pilose Female antenna
C = Plumose Male antenna
D = Third palp segment somewhat elongated
E = Two equally large round spermathecae
F = Male genitalia

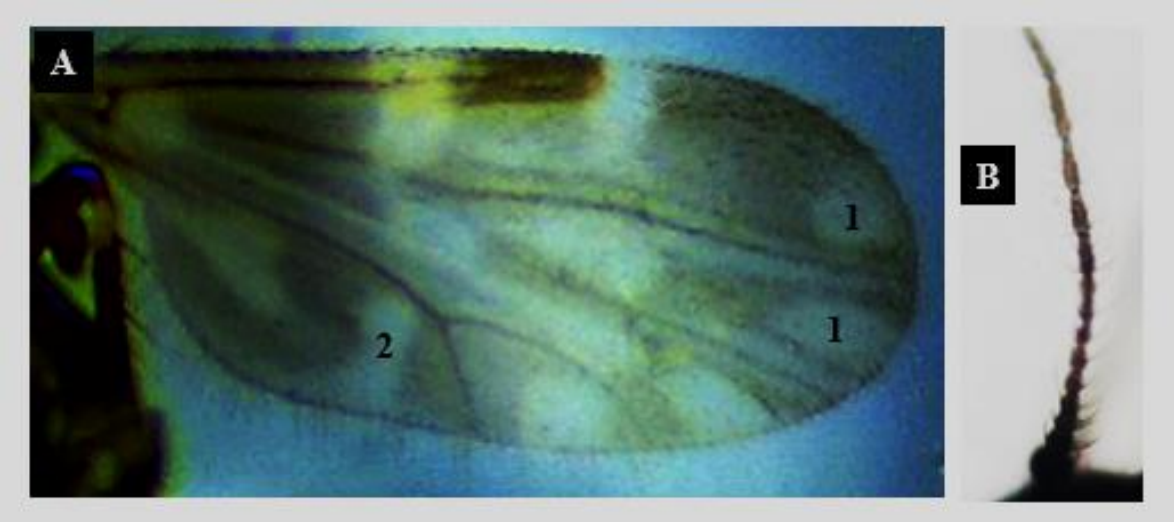

Plate 4.12: A = Wing of *Culicoides ovalis* with distal pale spots in M1 and M2 extend to the wing margin (**1**); distal pale spot in anal cell large but not double (**2**)
B = Pilose Female antenna

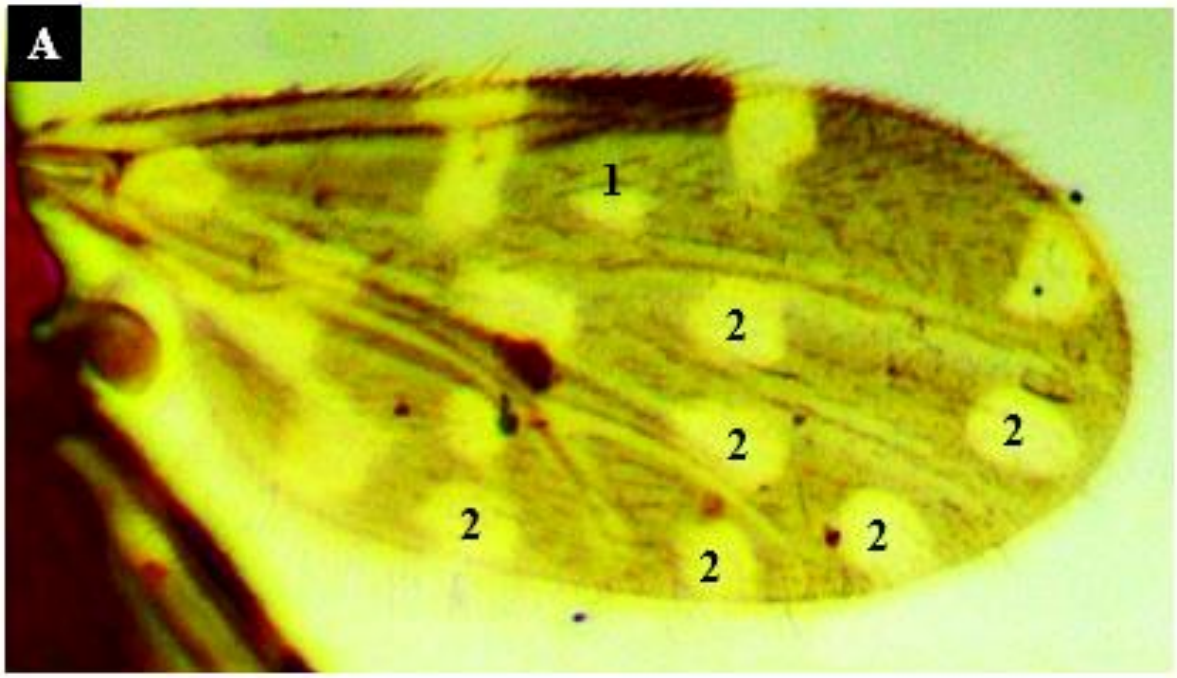

Plate 4.13: A = Wing of *Culicoides festipennis* with distinguished pale spot just below the radial cell, between the 1st and 2nd coastal spots (**1**); many large round discrete pale spots (**2**);

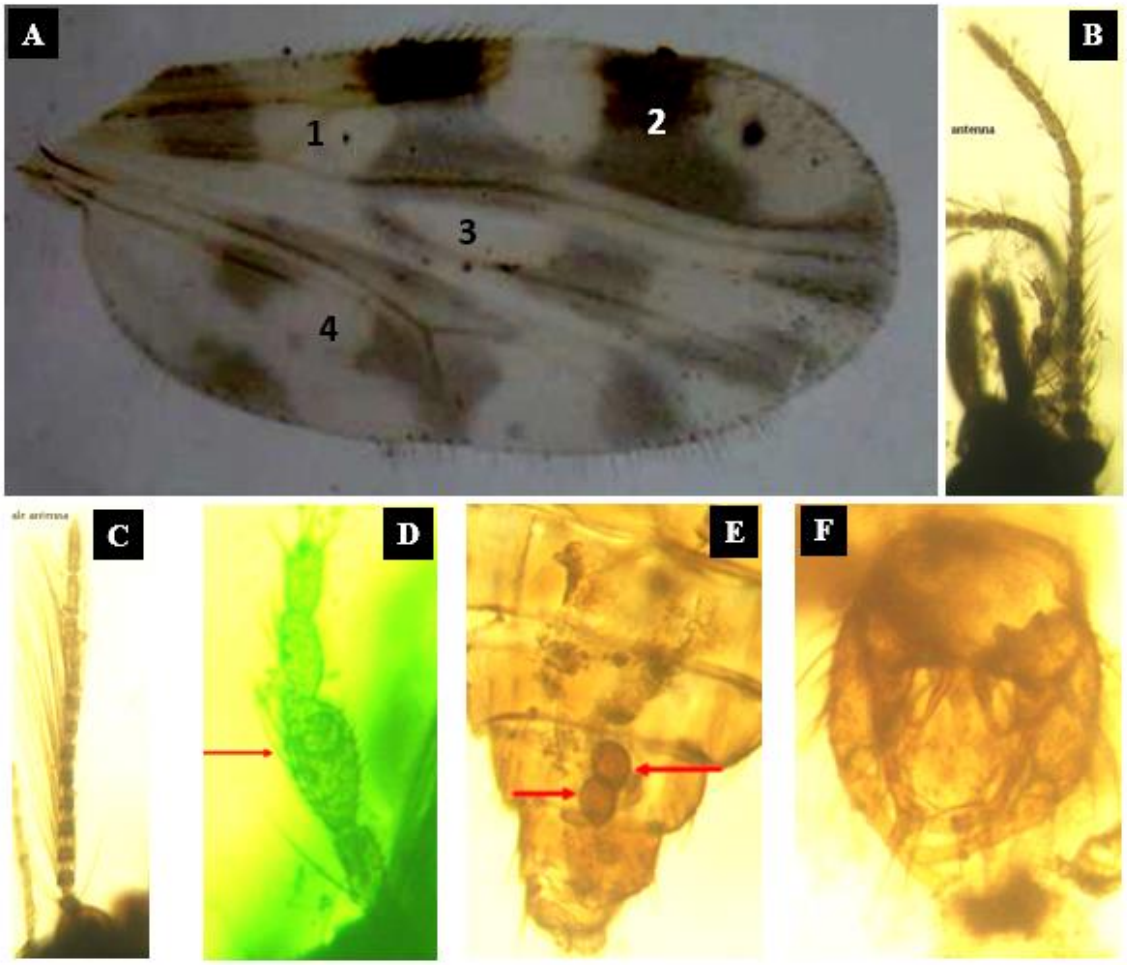

Plate 4.14: A = Wing of *Culicoides imicola* with a very large spot over r-m cross-vein (**1**); characteristic dark spot in r3 cell (**2**); a white almond shaped pattern in proximal m1 cell (**3**); a white vertical hourglass shaped pattern in the anal cell is typical of this species (**4**)

B = Pilose Female antenna

C = Plumose Male antenna

D = Third palp highly inflated and triangular

E = Two moderately pigmented subequal spherical spermathecae

F = Male genitalia with aedeagus body almost straight shoulder

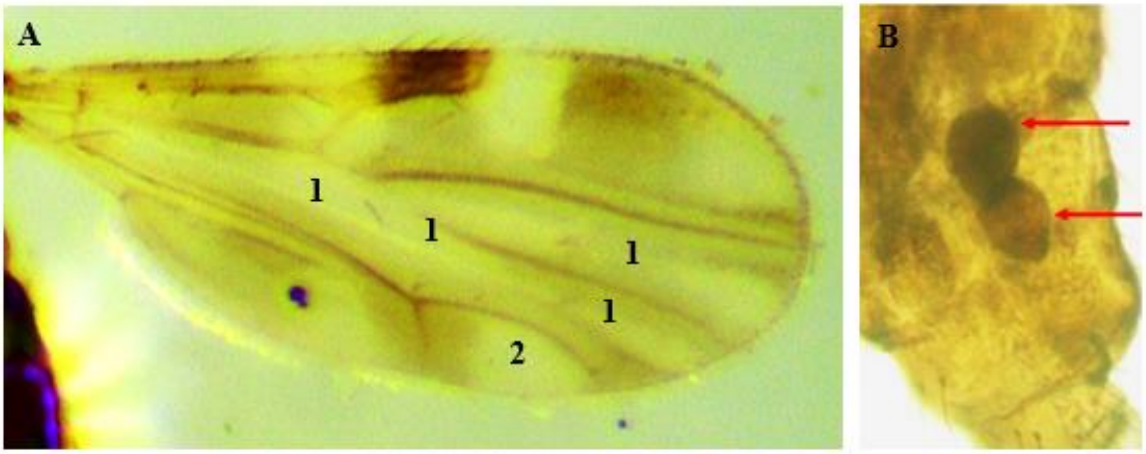

Plate 4.15: A = Wing of *Culicoides grahami* with varying number of ill-defined spots along the mid-line (**1**); single very large pale spot in cubital cell (**2**)
B = Two subequal lightly pigmented spermathecae

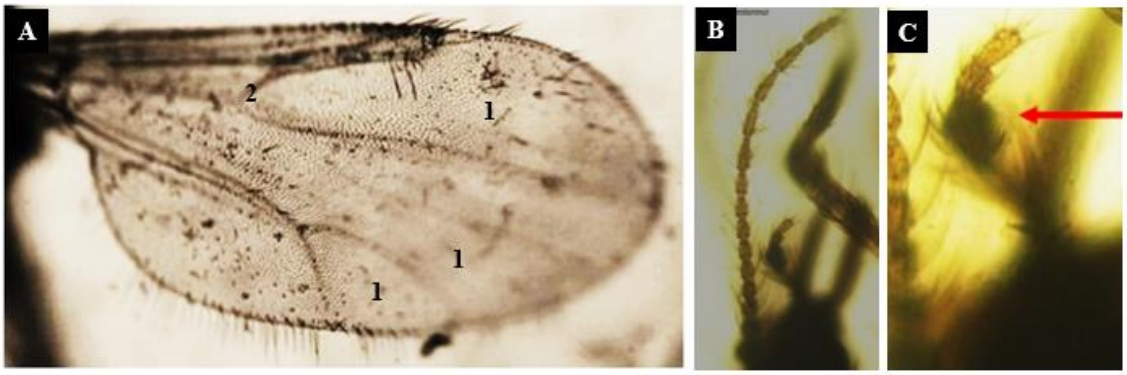

Plate 4.16: A = Wing of *Culicoides kanagai* with weak pale spots (**1**); pale spot somewhat absent over the r-m cross-vein (**2**)

B = Pilose Female antenna

C = Third segment of palp is short and robust

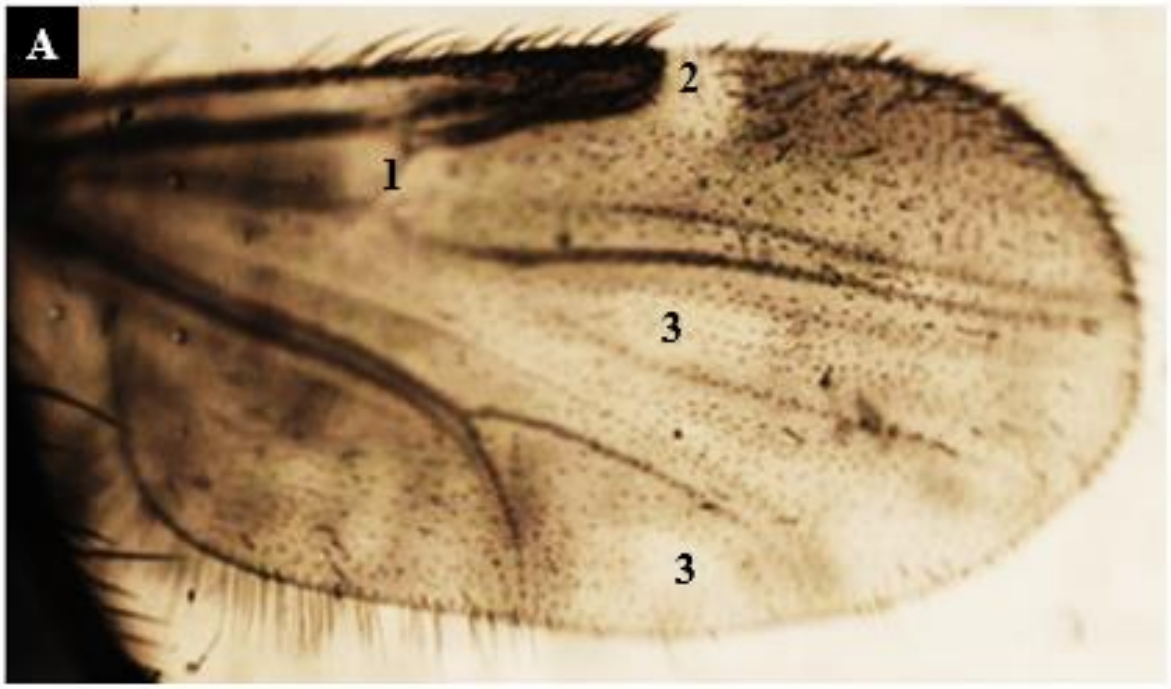

Plate 4.17: A = Wing of *Culicoides karaensis* with a weak pale spot over the r-m cross-vein (**1**); a pale spot distal to the radial cells (**2**), weak ill-defined pale spots in mid-line and cubital cell (**3**)

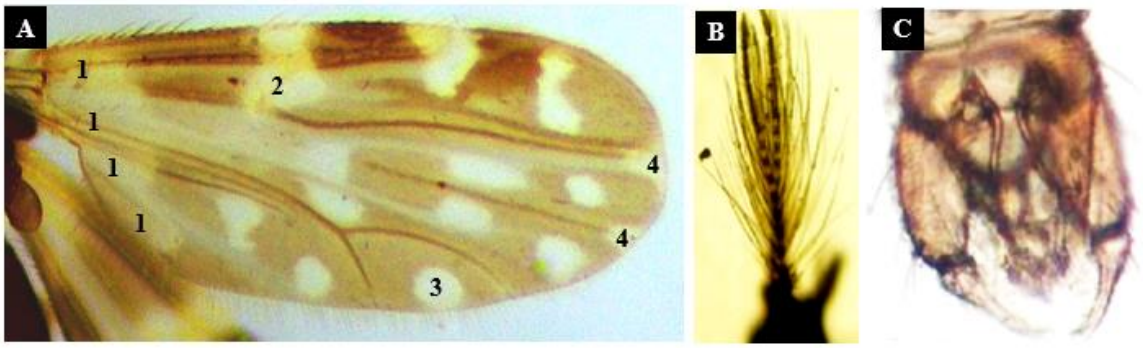

Plate 4.18: A = Wing of *Culicoides isoloensis* with extensively pale band extending from costal margin to posterior wing margins (**1**); a large pale spot over the r-m cross-vein (**2**); a round pale spot in cubital cell (**3**), pale spots at the end of M1 and M2 veins (**4**)

B = Plumose Male antenna

C = Male genitalia

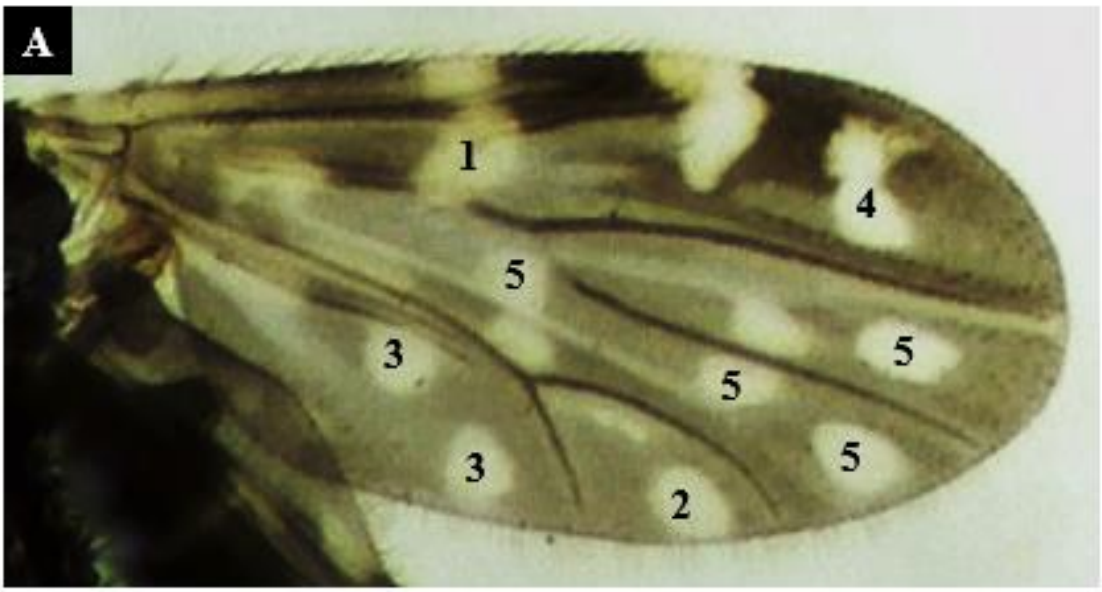

Plate 4.19: A = Wing of *Culicoides krameri* with a transverse pale over the -m cross-vein which extends to costal margin (**1**); single round pale spot in cubital cell (**2**); two round pale spots in anal cell (**3**), cell R5 with a transverse pale spot, often with a mesal constriction and appearing as 2 spots (**4**); cell M2 with numerous large pale spots (**5**)

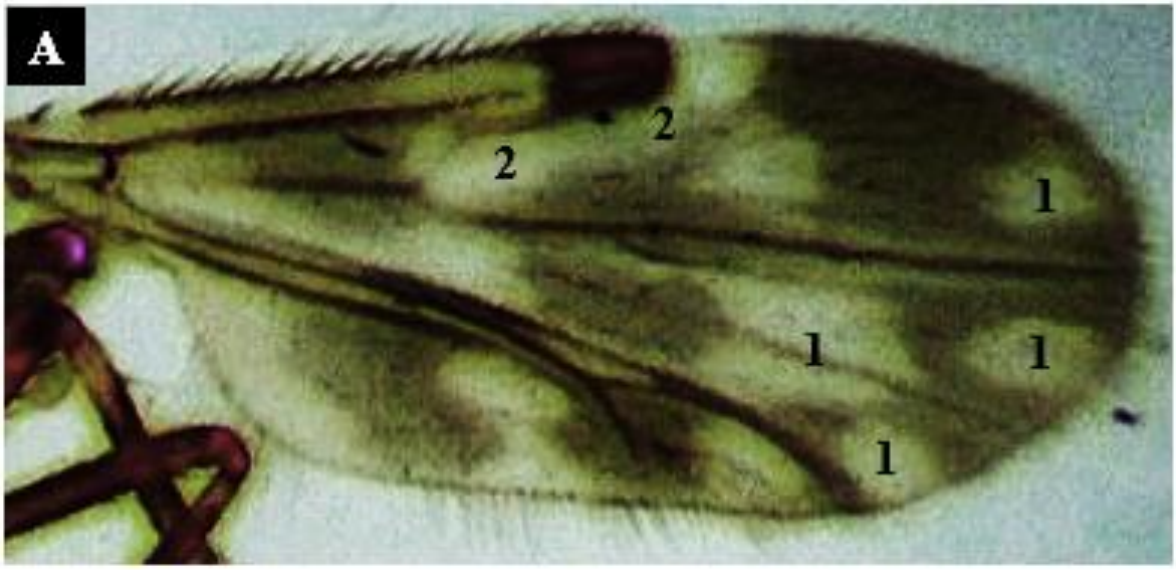

Plate 4.20: A = Wing of *Culicoides similis* with fairly conspicuous pale spots (**1**); a pale spot just distad to r-m cross-vein at base of cell R5, extending anteriorly to the costal margin just distad to 2nd radial cell (**2**)

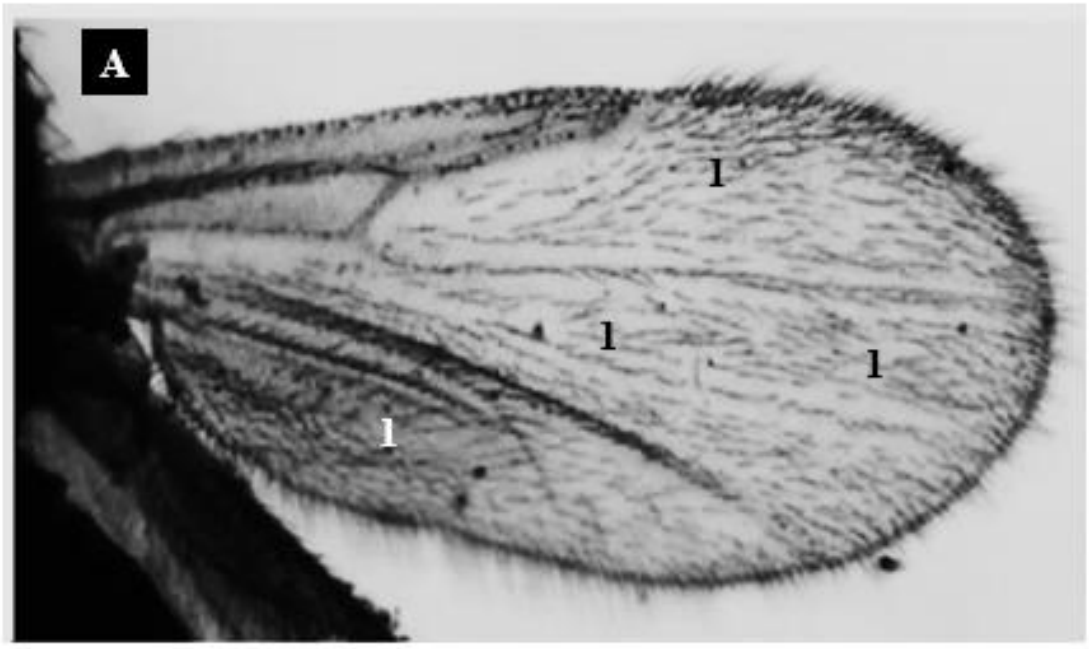

Plate 4.21: A = Wing of *Culicoides indistinctus* without any conspicuous pale spot but with numerous macrotrichia and scales covering the cells (**1**)

According to season, the highest number of collections were made during rainy seasons (87.96%) while 12.04% were collected in dry season. Benue South geopolitical zone had the highest number for both the dry and rainy seasons, with 37.9% (n = 1,376) and 46.45% respectively (Table 4.3). Also, the highest number of nulliparous and parous females were recorded in Benue South geopolitical zones (47% and 43.65%), respectively Table 4.2.

Miniature downdraft light suction trap – Model 1212 was more efficient with an overall 86.65% catch (Tables 4.1). There was a statistically significant difference (P-value <0.0005) between the two traps used. Miniature downdraft black light (UV) trap showed a higher degree of efficiency when compared with New Jersey Standard light trap.

The degree of correlation varied between monthly collection and weather conditions (temperature, humidity, rain, wind speed and precipitation). This ranges from very weak as noticed between wind speed and total number collected (P-value = 0.16), moderately correlated as observed between precipitation and humidity and total number collected (P-values 0.50, P value = 0.11, r = 0.48) respectively, to very strong correlation between rain and total number collected (r = 0.96, @95% C.I). However, there was a negative correlation between temperature and number collected (P value = 0.2398, r = -0.3676 @ 95% C.I) Figures 4.1 to 4.5.

Table 4.1: Distribution of *Culicoides* species from Benue State according to Sex

Geopolitical Zones	Male	Female	TOTAL
Benue northwest	859	8,841	30.97%
Benue northeast	1,027	6,096	23.62%
Benue south	1,775	11,925	45.42%
TOTAL	**12.14%**	**87.86%**	

Table 4.2: Parity Distribution of *Culicoides* species identified in Benue State Nigeria

Geopolitical Zones	Nulliparous	Parous	Engorged
Benue northwest	2,728	1,698	270
Benue northeast	6,911	2,363	354
Benue south	8,549	3,322	307
TOTAL	**68.69%**	**27.86%**	**3.51%**

Table 4.3: Seasonal Variations in the Distribution of *Culicoides* species in Benue State, Nigeria.

Geopolitical Zones	Dry Season	Rainy Season	TOTAL
Benue northwest	952	8,388	9,340
Benue northeast	1,303	5,820	7,123
Benue south	1,376	12,324	13.700
TOTAL	**12.04%**	**87.96%**	

Table 4.4: Composition of *Culicoides* species in Benue State Nigeria and their distribution

S/No	*Culicoides* Species	Period (months) of Collection												Prevalence		Sex	
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	Female	Male
1	*C. imicola*	86	207	566	78	960	882	762	79	7,302	216	102	89	11,329	37.56	9,974	1,355
2	*C. oxystoma*	81	154	439	56	411	577	286	121	1,801	86	74	79	4,165	13.81	3,384	781
3	*C. subschultzei*	15	86	472	91	216	628	120	100	1,589	83	15	83	3,498	11.60	2,705	793
4	*C. enderleini*	37	21	322	13	294	250	84	103	1,863	99	18	57	3,161	10.48	2,744	417
5	*C. moreli*	04	86	260	01	48	173	96	96	566	216	05	11	1,562	5.18	1,464	98
6	*C. milnei*	28	102	276	33	202	118	91	23	412	151	27	11	1,474	4.89	1,402	72
7	*C. similis*	05	55	45	00	50	101	41	31	214	34	00	03	579	1.92	570	09
8	*C. pycnosticus*	05	13	89	01	102	63	38	96	97	00	02	16	522	1.73	516	06
9	*C. neavi*	10	11	101	05	66	51	27	43	135	28	06	18	501	1.66	452	49
10	*C. zuluensis*	00	15	75	03	40	62	37	30	143	63	09	02	479	1.59	479	00
11	*C. krameri*	00	22	33	02	20	76	57	45	91	10	05	04	371	1.23	371	00
12	*C. fulvithorax*	00	13	03	01	08	44	45	89	110	42	08	01	364	1.21	360	04
13	*C. nevilli*	03	27	05	11	02	53	30	51	120	16	00	07	325	1.08	303	22
14	*C. ovalis*	00	18	13	00	32	48	17	13	43	48	00	12	244	0.81	244	00
15	*C. austeni*	00	28	00	02	00	27	15	12	59	40	01	03	187	0.62	184	03
16	*C. isoloensis*	00	31	00	00	05	19	01	29	51	42	02	03	183	0.61	183	00
17	*C. grahami*	00	19	21	02	33	12	11	10	53	07	03	04	175	0.58	175	00
18	*C. festipennis*	00	05	12	00	44	16	30	04	44	17	00	00	172	0.57	170	02
19	*C. karaensis*	00	08	00	00	15	32	28	14	26	01	00	00	124	0.41	119	05
20	*C. kanagai*	00	04	05	01	11	01	25	23	14	10	00	05	99	0.33	92	07
21	*C. indistinctus*	00	03	12	01	22	14	07	03	04	03	00	03	72	0.24	69	03
22	Unidentified sp.	00	29	51	08	61	56	49	58	132	108	14	11	577	1.91	542	35
TOTAL		**274**	**957**	**2,800**	**309**	**2,648**	**3,303**	**1,897**	**1,073**	**14,869**	**1,320**	**291**	**422**	**30,163**	**100**	**26,502**	**3,661**

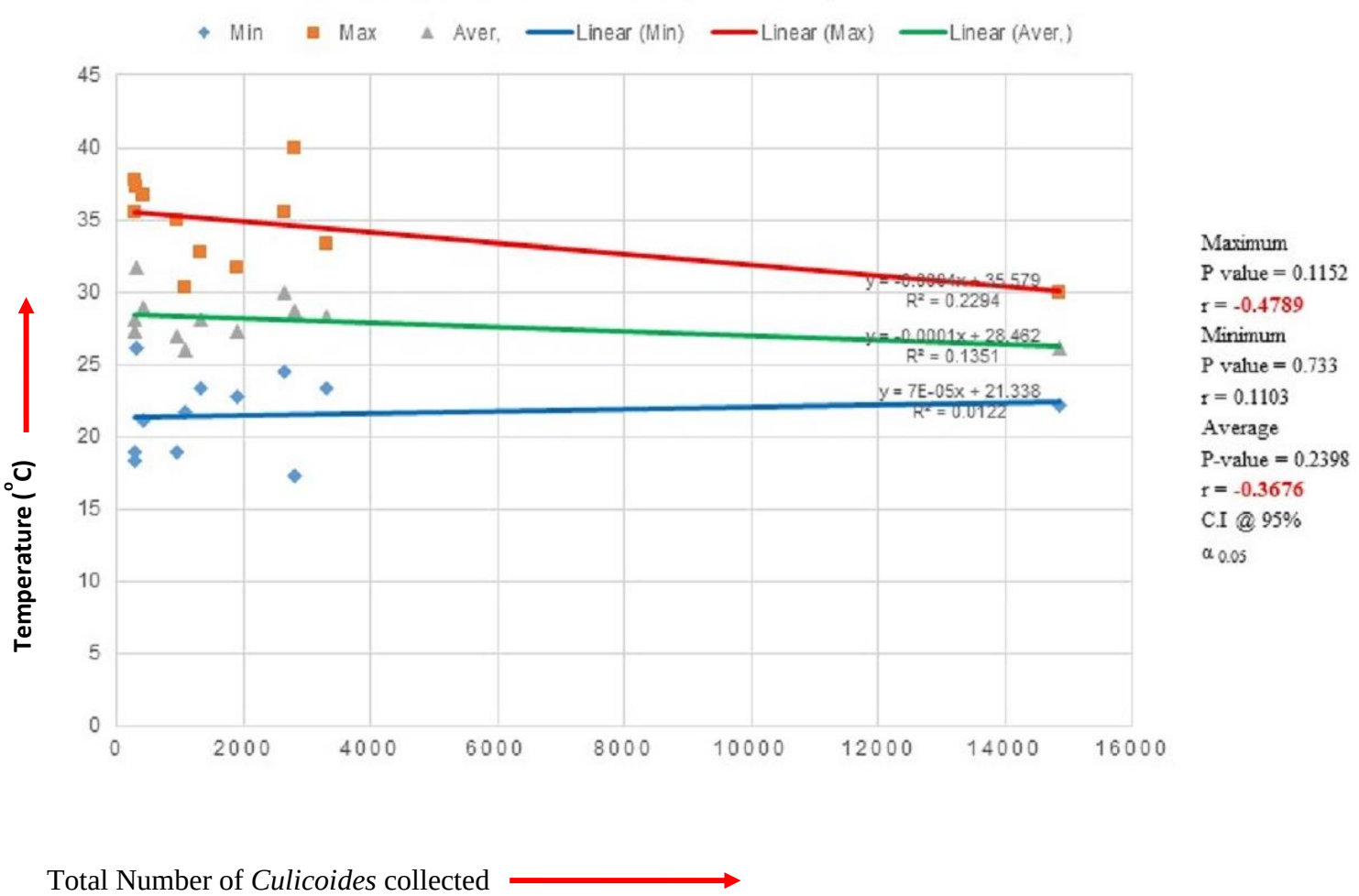

Figure 4.1: Correlation between number of *Culicoides* collected and temperature (°C)

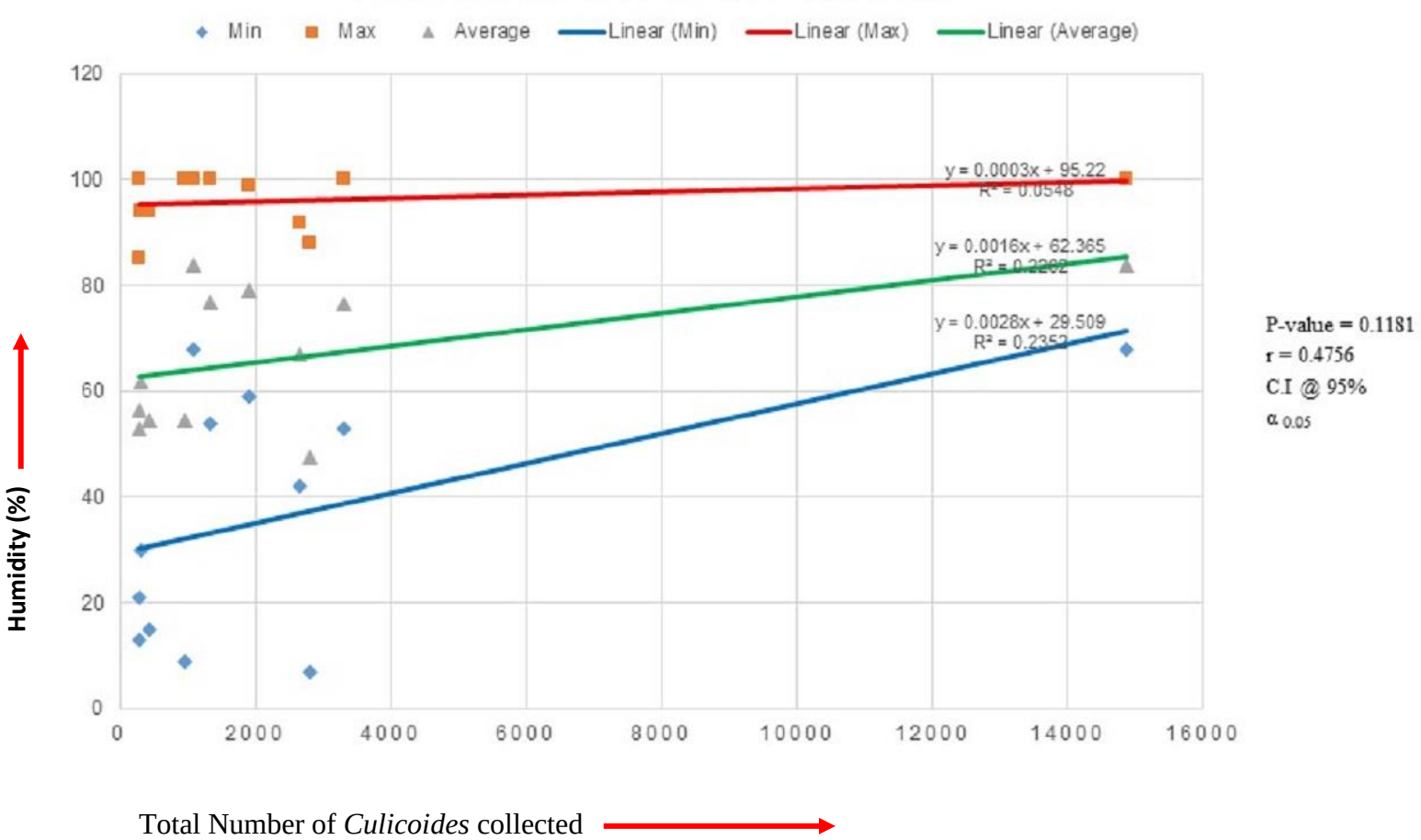

Figure 4.2: Correlation between number of *Culicoides* collected and humidity (%)

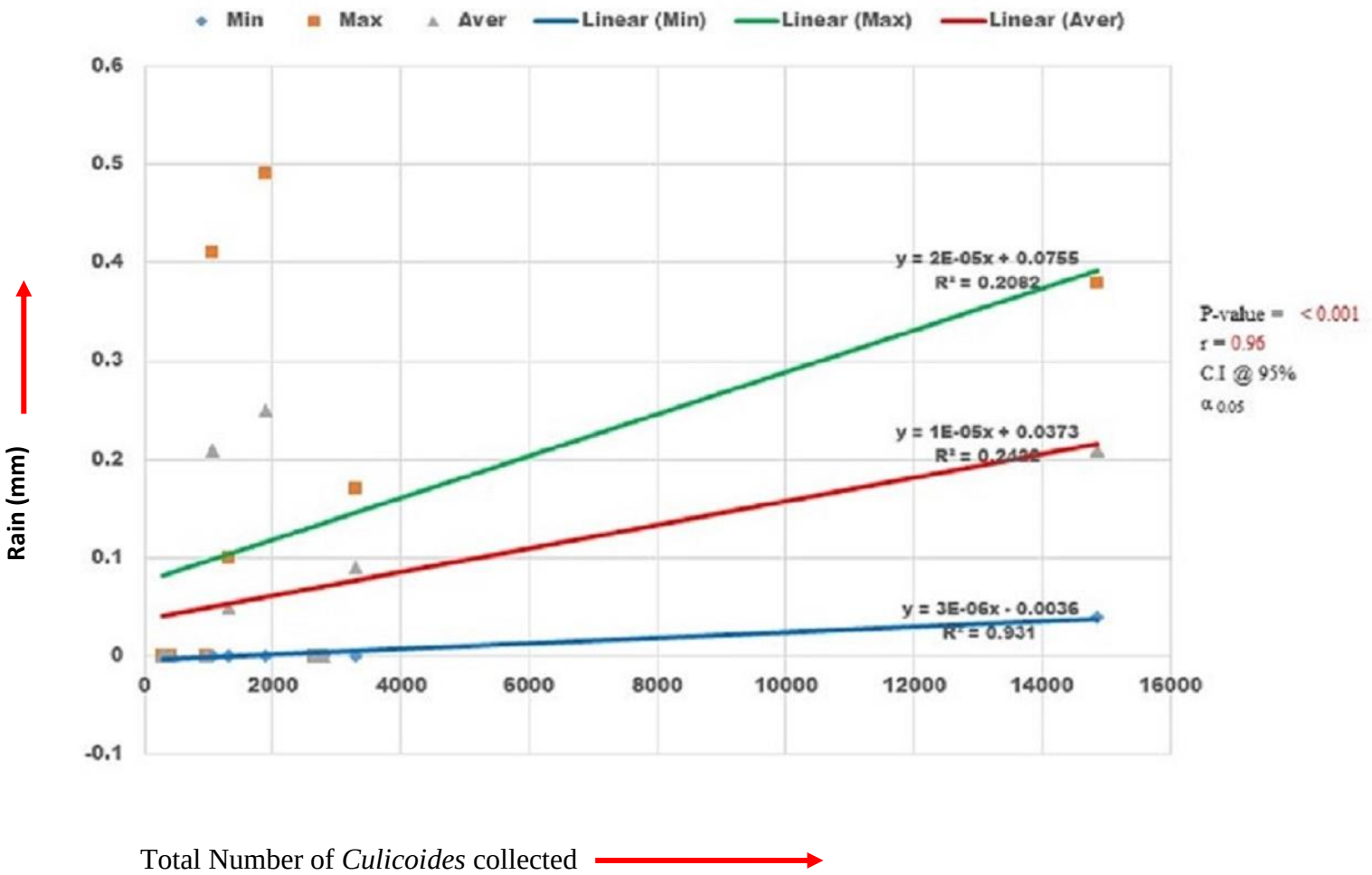

Figure 4.3: Correlation between number of *Culicoides* collected and rain (mm)

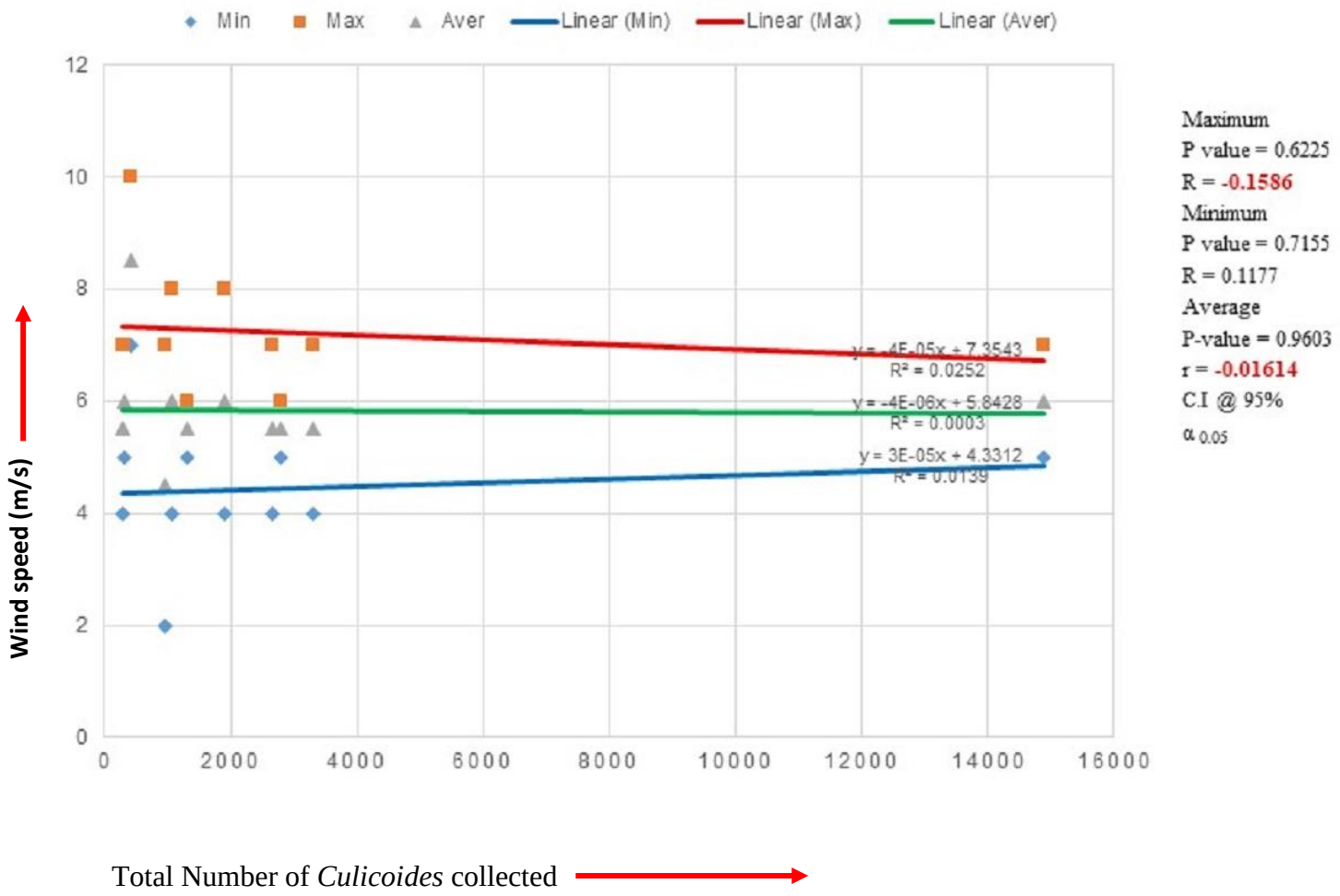

Figure 4.4: Correlation between number of *Culicoides* collected and wind speed (m/s)

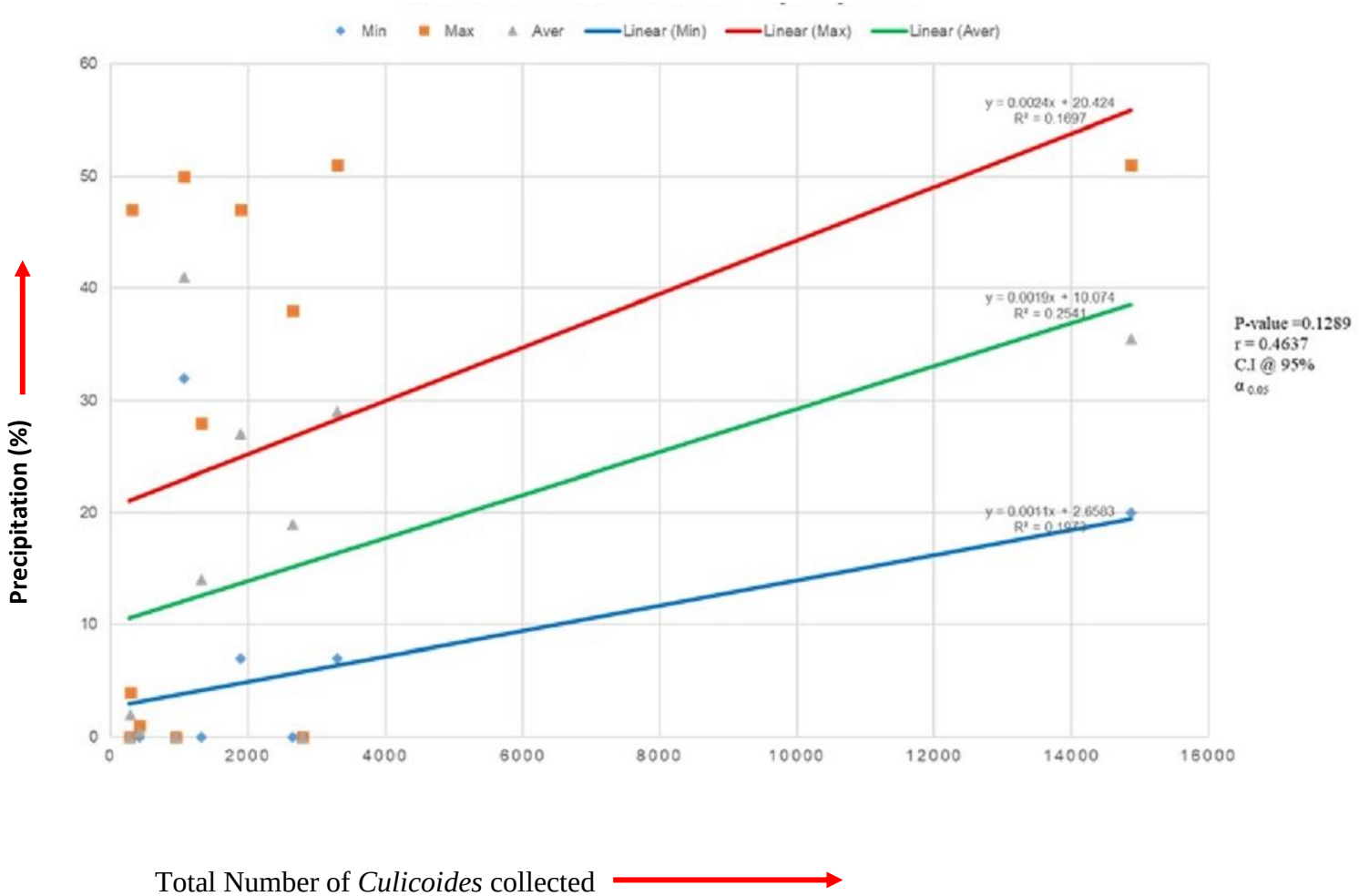

Figure 4.5: Correlation between number of *Culicoides* collected and precipitation (%)

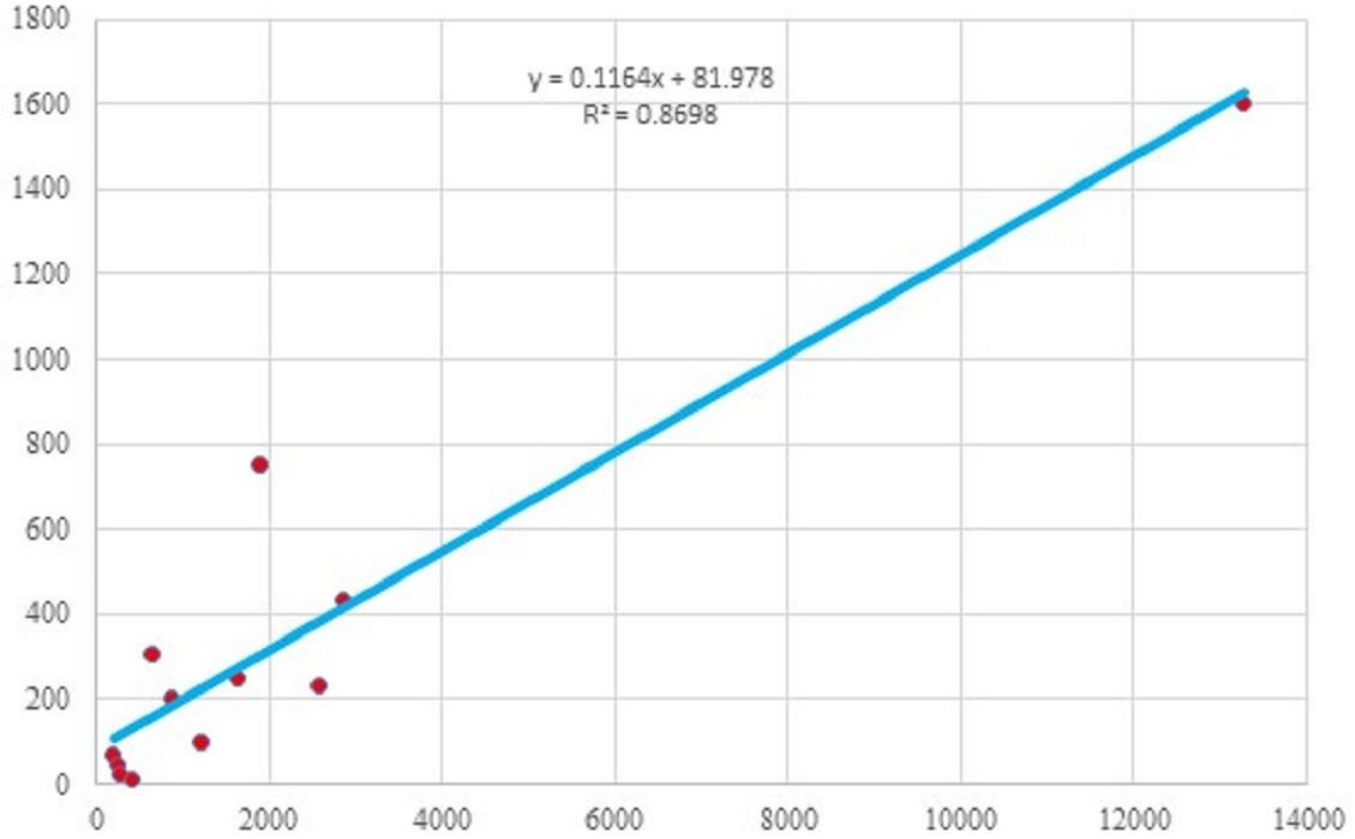

Figure 4.6: Correlation of number of catches between the two traps

The percentage parous comprising both females with fresh blood meal and those with digested blood was 31.37% (Figure 4.8) and the risk of possible disease transmission according to Odds ratio (OR) analysis appeared very high in October, November, August and September (12.21, 6.47, 5.65 and 5.02), respectively Table 4.4. These were statistically significant for all the months stated while March and June were statistically not-significant with Odds ratio of 0.94 and 0.89 respectively.

Stereomicroscopy also revealed the presence of morphologically abnormal specimens and ectoparasitism. The abnormalities observed were multiple abdomens attaching to the thorax (Plate 4.27). Two of such abnormalities were recorded in female specimens, one each in *Culicoides imicola* and *Culicoides subschultzei*. One of the specimens clearly displayed bi-abdomen while the second showed evidence of the third rudimentary abdomen between the dorsal and ventral abdominal divisions. One of the specimens showed evidence of previous blood meal as revealed by occurrence of burgundy red pigments in the abdomen. This same specimen also displayed three spermathecae in each of the abdominal divisions making a total of six. Another abnormality reported was a case of gynandromorphism (Plate 4.28) in which the specimen displayed an anterior portion typical of female structure (pilose antennae/dichoptic eyes) and a posterior male structure (male genitalia). A species of unidentified arthropod suspected to be mite was found feeding on *Culicoides subschultzei* at the ventral thorax (Plate 4.29).

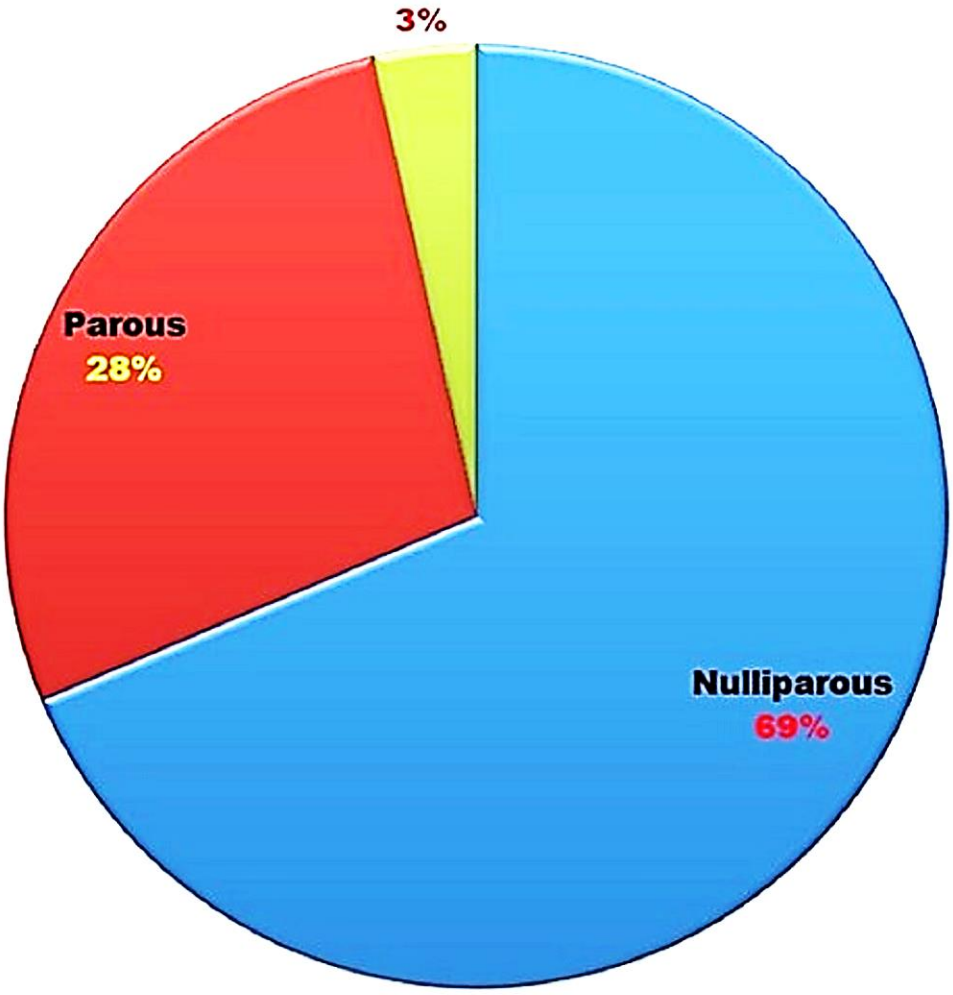

Figure 4.7: Parity status of collected female *Culicoides* species from Benue State, Nigeria

Figure 4.8: Comparison between number of catch and parity status of collected *Culicoides* species

Table 4.5: Distribution pattern of *Culicoides* species in Benue State, Nigeria

Month	TOTAL	Female	Male	Nulliparous	Parous	Engorged	912	1212
January	274	11	263	122 (46.39%)	115 (43.72%)	26 (9.89%)	71	203
February	957	34	923	555 (60.13%)	286 (30.99%)	82 (8.88%)	307	650
March	2,800	23	2,777	1,245 (44.83%)	1,307 (47.07%)	225 (8.10%)	230	2,570
April	309	60	249	174 (69.88%)	55 (22.09%)	20 (8.03%)	47	262
May	2,648	151	2,497	1,450(58.07%)	960 (38.45%)	87 (3.48%)	751	1,897
June	3,303	335	2,968	1,291(43.50%)	1,499 (50.50%)	178 (6.00%)	434	2,869
July	1,897	238	1,659	997 (60.10%)	591 (35.62%)	71 (4.28%)	249	1,648
August	1,073	308	765	635 (83.01%)	104 (13.59%)	26 (3.40%)	200	873
September	14,869	2,312	12,557	10,206 (81.28%)	2,182 (17.38%)	169 (1.34%)	1,601	13,268
October	1,320	129	1,191	1,088(91.35%)	101 (08.48%)	02 (00.17%)	99	1,221
November	291	27	264	224(84.85%)	39 (14.77%)	01 (0.38%)	23	268
December	422	33	389	201(51.67%)	144 (37.02%)	44 (11.31%)	14	408
Percentage (%)		**12.14**	**87.86**	**68.63**	**27.86**	**03.51**	**13.35**	**86.65**

Table 4.6: Morphometric analysis of *Culicoides* species from Benue State, Nigeria

S/No	*Culicoides* species	Veins Measurement							Angle Measurement			
		WR	CR	FR	AR	PR	SR 1	SR 2	Arculus angle	r-m cross	Median angle	Cubital angle
1	*C. imicola*	2.34	1.40	1.37	1.00	4.03	1.18	1.13	114.58	142.82	35.31	66.80
2	*C. oxystoma*	2.28	1.16	1.19	1.06	2.50	1.24	1.02	107.36	131.08	61.46	62.54
3	*C. subschultzei*	2.66	1.54	1.09	0.89	1.84	0.87	1.07	135.54	131.69	47.12	62.53
4	*C. milnei*	2.32	1.44	1.37	1.9	1.78	1.28	1.15	124.12	138.50	49.14	72.83
5	*C. moreli*	2.15	1.22	1.56	1.21	2.96	1.64	1.37	127.58	128.23	30.32	68.46
6	*C. pycnosticus*	2.08	1.27	1.88	1.29	2.98	1.36	1.26	122.75	113.05	43.55	51.05

Keys: WR – Wing ratio
CR – Costal ratio
FR – Flagellar ratio
AR – Antennal ratio
PR – Palp ratio
SR 1 – Large spermathecal ratio
SR 2 – Small spermathecal ratio

Table 4.7: Odds ratio and relative risk of monthly distribution of Culicoides species in Benue State, Nigeria

Months	Sf	RR	OR
January	Ns	1.00	1.00
February	Yes***	1.35	1.74
March	Ns	0.97	0.94
April	Yes***	1.78	2.68
May	Yes***	1.28	1.60
June	Ns	0.95	0.89
July	Yes***	1.34	1.74
August	Yes***	3.16	5.65
September	Yes***	2.86	5.02
October	Yes***	6.20	12.21
November	Yes***	3.54	6.47
December	Ns	1.11	1.24

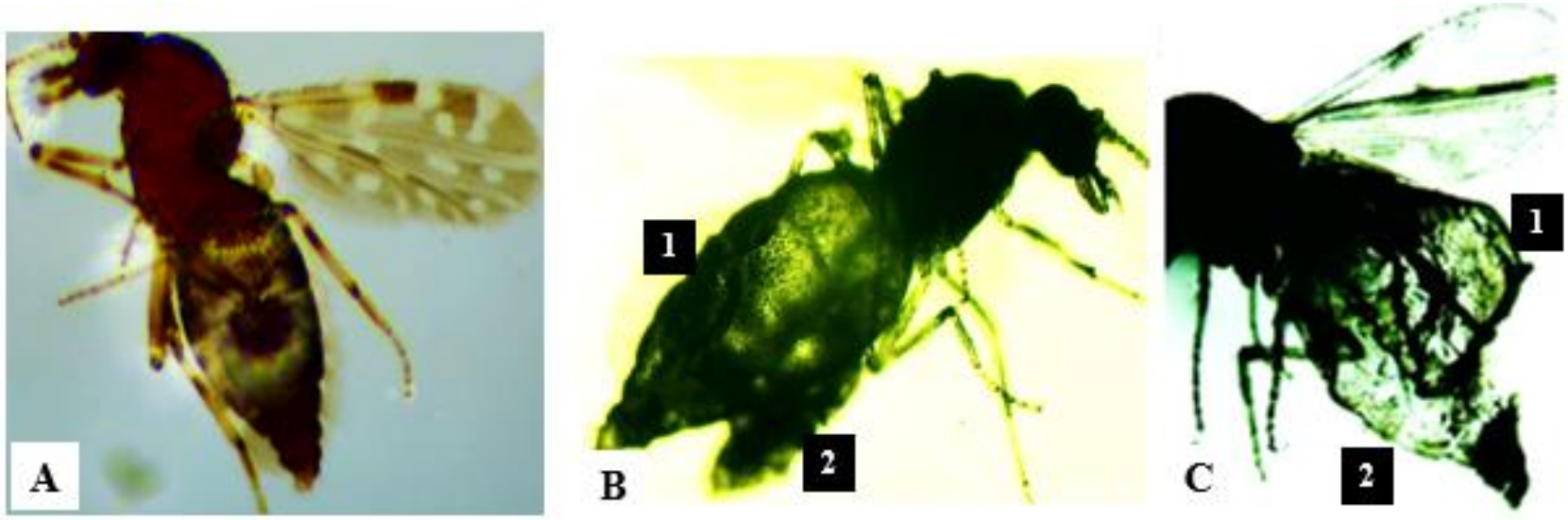

Plate 4.27: **A** - *Culicoides* species showing normal (single) abdomen

B - *Culicoides* species with double abdomens

C - *Culicoides* species with multiple abdominal attachments

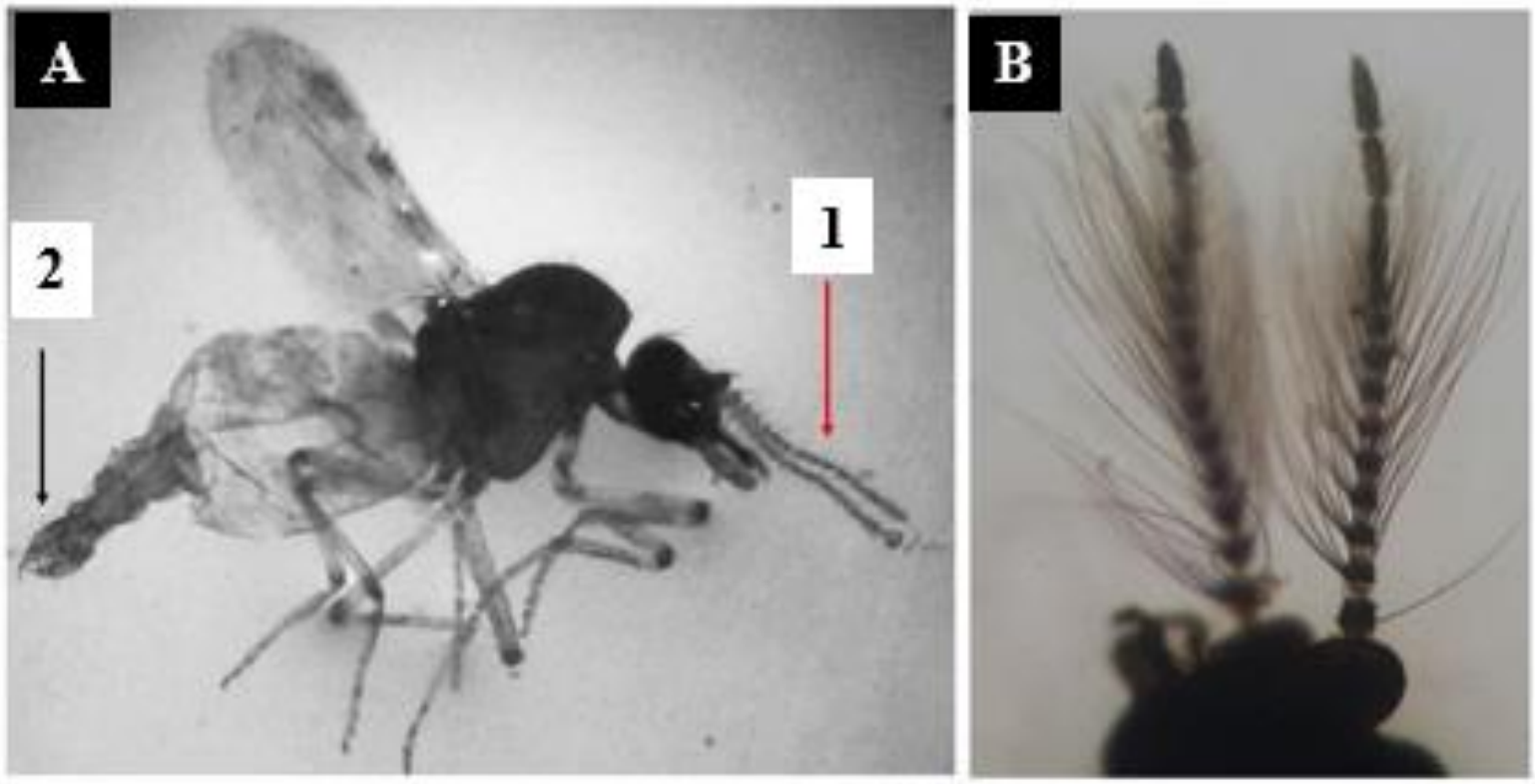

Plate 4.28: **A - Gynandromorphism with a female anterior (1) and male posterior (2)**

B - Antenna typical of male *Culicoides* species

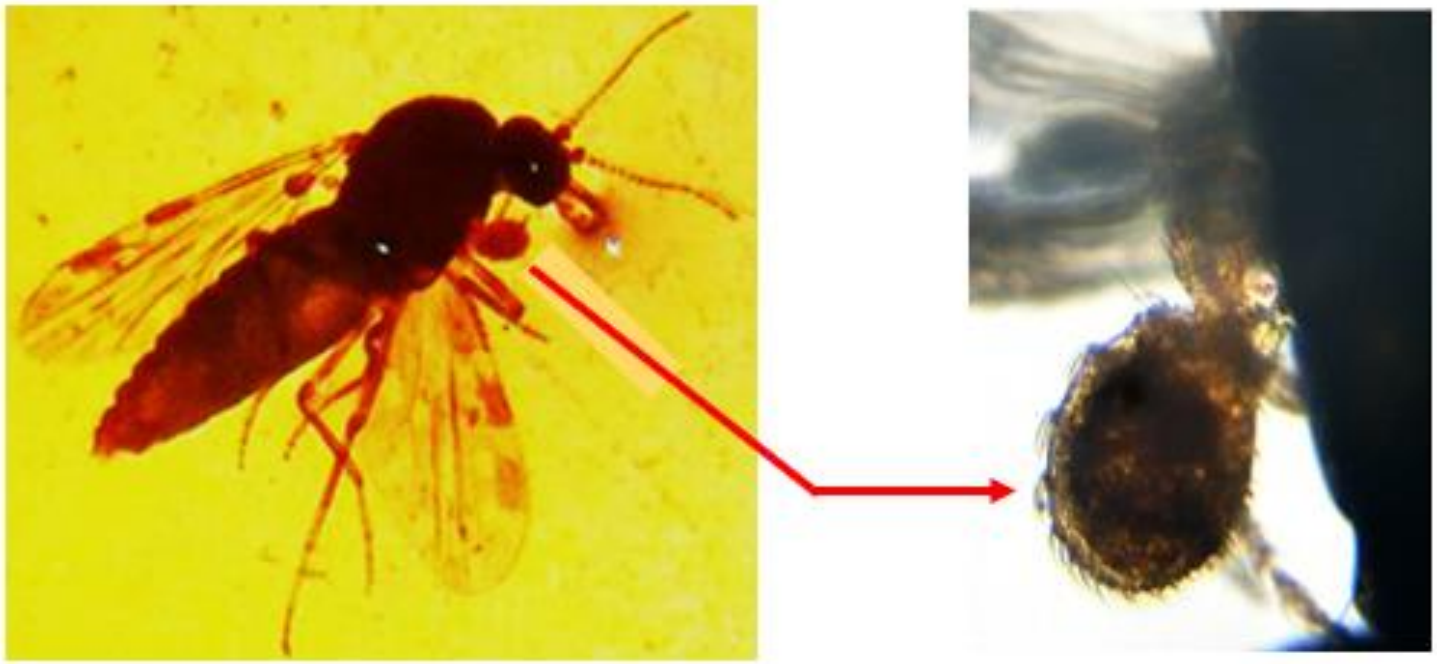

Plate 4.29: *Culicoides* species with an attachment of ectoparasite (Mite) on the thorax

4.2 Molecular Characterisation and Phylogeny of selected *Culicoides* species

Polymerase chain reactions as confirmed by gel electrophoresis established the presence of diverse *Culicoides* species in Benue State with bands of varying sizes ranging from 300 to 400 base pairs (Plates 4.30a and 4.30b). Molecular identification through sequencing of some selected positive amplicons confirmed three distinct species (*Culicoides oxystoma, C. imicola* and *C. indistinctus*) and their accession number are presented in Table 4.8 below:

Phylogenetic tree using neighbour joining unrooted with 1000 bootstrap value showed that *Culicoides imicola* has similarities with some existing *Culicoides imicola* from different regions although degrees of similarities vary. All the three *C. imicola* sequences obtained during this study had 100% similarities with those from Israel and Scotland and to some *Culicoides imicola* from France (Figure 4.10). This is however different for *C. oxystoma* and *C. indistinctus* in that they have similarities of varying degrees with many other *Culicoides* species from different regions (Figures 4.11 and 4.12).

Table 4.8: Accession number and the corresponding *Culicoides* species from the study area

Accession number	*Culicoides* species
OM459830	
OM459831	*Culicoides imicola*
OM459832	
OM459833	
OM459834	*Culicoides oxystoma*
OM459855	
OM459836	*Culicoides indistinctus*

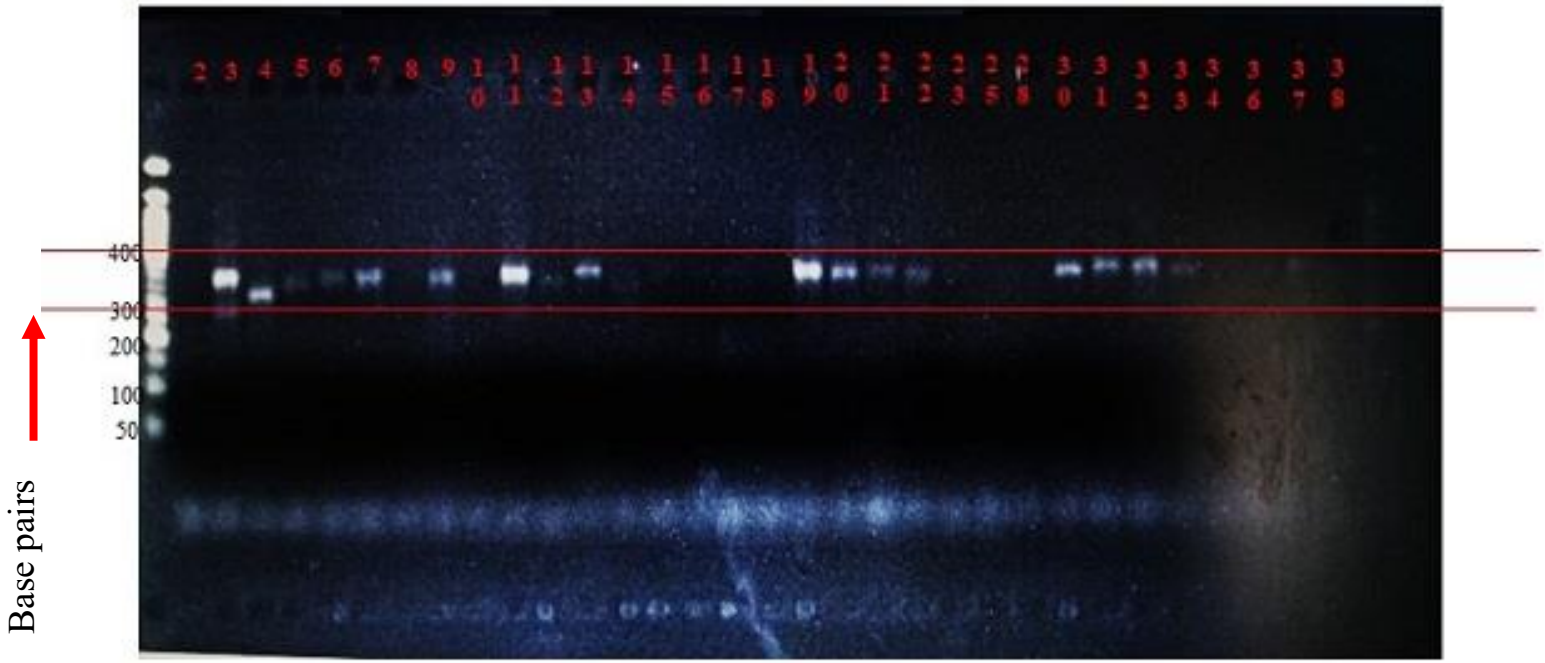

Plate 4.30a: Gel electrophoresis showing positive bands for molecular identification of *Culicoides* species from Benue State, Nigeria

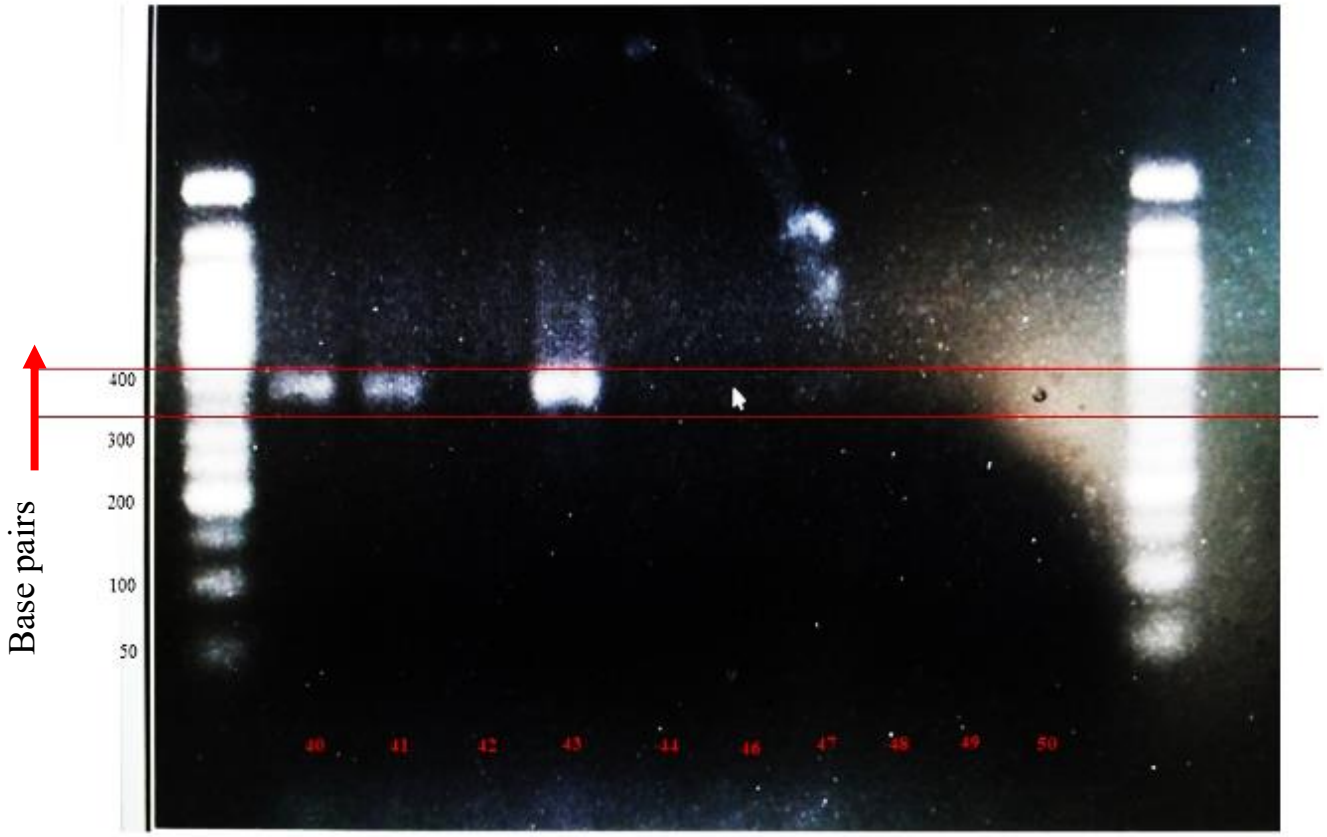

Plate 4.30b: Gel electrophoresis showing positive bands for molecular identification of *Culicoides* species from Benue State, Nigeria

Table 4.9: *Culicoides imicola* showing genetic relatedness with *Culicoides imicola* from other countries

***Culicoides* sp.**	**Percentage similarity**	**Base pair**	**Country**	**Accession Number**	**Reference**
C. imicola	100	352	Nigeria	OM459830.1	Oke *et al*., 2022 Unpublished
C. imicola	96.60	353	Nigeria	OM459831.1	Oke *et al*., 2022 Unpublished
C. imicola	94.90	353	Nigeria	OM459832.1	Oke *et al*., 2022 Unpublished
C. imicola	95.79	394	France	AY861144.1	Perrin *et al*., 2006
C. imicola	95.78	971	Scotland	AF074019.1	Unpublished
C. imicola	95.18	814	France	MK893016.1	Mathieu *et al*., 2020
C. imicola	95.42	391	Israel	JN408480.1	Morag *et al*., 2012
C. imicola	95.15	393	Israel	JN408479.1	Morag *et al*., 2012
C. imicola	95.08	829	France	MK893014.1	Mathieu *et al*., 2020
C. imicola	94.79	807	France	MK893015.1	Mathieu *et al*., 2020
C. imicola	93.77	341	India	KY491655.1	Maheshwari and Mahore,
C. imicola	92.39	337	India	KY967713.1	2017 (Unpublished)

Below are the sequences obtained following BLAST search in National Center for Biotechnology Information (NCBI) of *Culicoides imicola* from Benue State, Nigeria. Their unique Accession Number as evidence of their deposition in GenBank are displayed above each sequence:

>OM459830.1

TTGGACTTTTTTTTATATTTTTTTTTGGGTACCTTATACCTCACTCCAAAGTAT
CTTGATAAAAAATATCAATGCATATGGCATTACACTATATGCATACTAATGAC
ACGAAGTCACTAGTTTTACGGGGCCTTGGGCAAACAAGTCTTTAAAGCTCTTC
TATAAAGCTACCCAAGAAATTTTTTTTGGTGTGTATTACAGCACTCGCTACAG
ATATAATAAACTACACCTTTATAGTGAGCTTCGCGAAGCCACTGTAATAAAC
AACGTTACAACCCATACCTTAAGAATACATCATTATATTTTTTTTTTTAAAAA
AAAAAAAGGGCCCCCCCGGAAAAACAAAAAAAAA

>OM459831.1

TTGGACTTTTTTTTATATTTTTTTTTGGGTACCTTATACCTCACTCCAAAGTAT
CTTGATAAAAAATATCAATGCATATGGCATTACACTATATGCTTACTAATGAC
ACGAAGTCACTAGTTTTACGGGGCCTTGGGCAAACAAGTCTTTAAAGCTCTTC
TATAAAGCTACCCAAGAAATTTTTTTTGGGGTTGTATTACAGCCCCCGCCACC
AAAAAAAAAAACTACACCTTTATAGGGGGCTTCGCGAAGCCACTGTAATAAA
CAACGTTACAACCCATCCCTAAAGAAAACATCCTTATTTTTTTTTTTTAAAA
AAAAAAAAGGGCCCCCCCGGAAAAACAAAAAAAAA

>OM459832.1

TTGGACTTTTTTTTATATTTTTTTTTGGGTACCTTATACCTCACTCCAAAGTAT
CTTGATAAAAAATATCAATGCATATGGCATTACACTATATGCATACTAATGAC
ACGAAGTCACTAGTTTTACGGGGCCTTGGGCAAACAAGTCTTTAAAGCTCTTC
TATAAAGCTACCCAAGAAATTTTTTTTGGGGTTGTATTACAGCCCCCGCCACC
AAAAAAAAAAACTACACCTTTATAGGGGGCTTCGCGAAGCCACTGTAATAAA
CAACGTTACAACCCATACCTTAAGAATACATCATTATATTTTTTTTTTTAAAA
AAAAAAAAGGGCCCCCCCGGAAAAACAAAAAAAAA

Figure 4.10: The locations of genetic differences between the three *Culicoides imicola* obtained during this study in Benue State, Nigeria

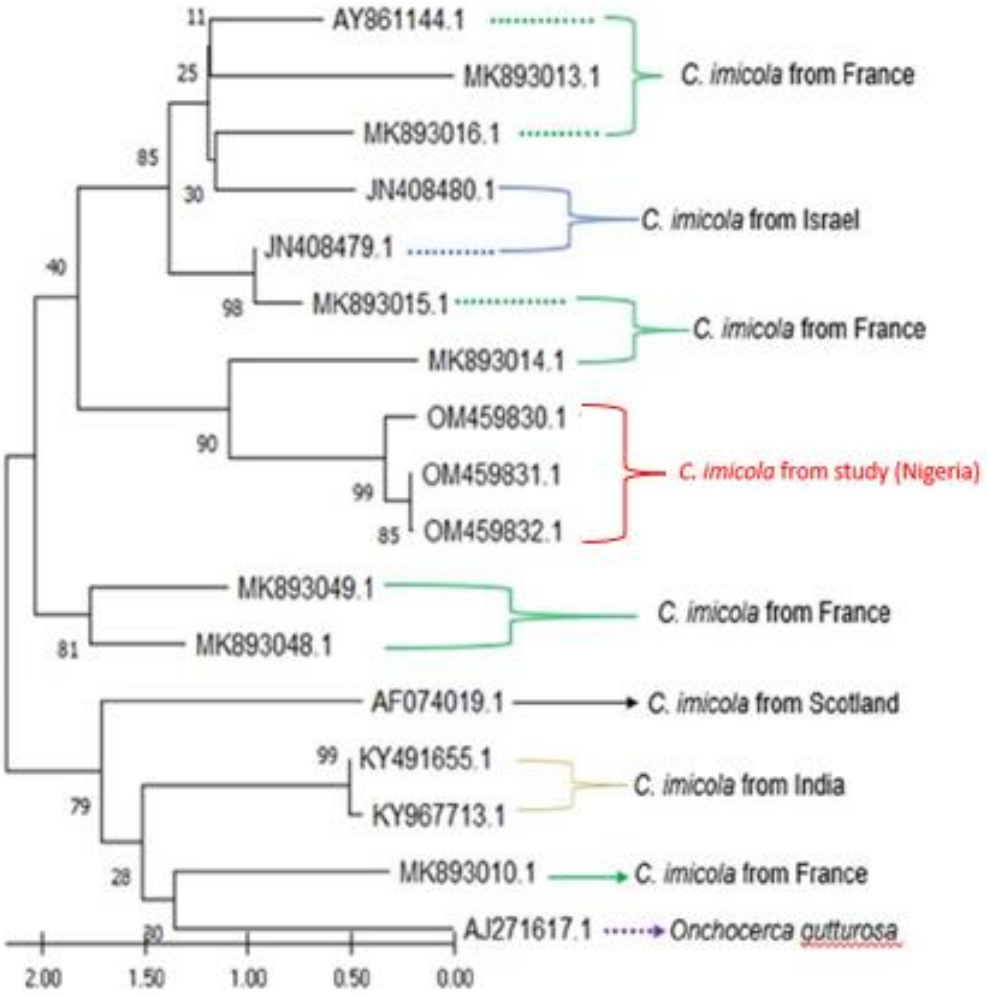

Figure 4.9: Phylogeny of *Culicoides imicola* ITS1 NJ unrooted tree. Bootstrap values >50% are indicated.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA11.

Table 4.10: *Culicoides oxystoma* showing genetic relatedness with other *Culicoides* species from various locations

***Culicoides* sp.**	**Percentage similarity**	**Base pair**	**Country**	**Accession Number**	**Reference**
C. oxystoma	100	369	Nigeria	OM459835.1	Oke *et al*., 2022 Unpublished
C. oxystoma	98.37	369	Nigeria	OM459834.1	Oke *et al*., 2022 Unpublished
C. oxystoma	97.29	369	Nigeria	OM459833.1	Oke *et al*., 2022 Unpublished
C. oxystoma	95.18	408	Israel	JN408476.1	Morag *et al*., 2012
C. oxystoma	94.19	408	Israel	JN408472.1	Morag *et al*., 2012
C. oxystoma	93.23	402	Israel	JN408469.1	Morag *et al*., 2012
C. oxystoma	92.90	400	Israel	JN408471.1	Morag *et al*., 2012
C. oxystoma	92.58	400	Israel	JN408473.1	Morag *et al*., 2012
C. oxystoma	91.94	376	Japan	AB462279.1	Matsumoto *et al*., 2008
C. imicola	95.70	305	India	KY491653.1	Maheshwari and Maheshwari, 2017
C. kibunensis	95.70	817	Japan	AB462280.1	Matsumoto *et al*., 2008
C. cylindratus	95.70	820	Japan	AB462269.1	Matsumoto *et al*., 2008
C. aterinervis	95.65	891	Japan	AB462267.1	Matsumoto *et al*., 2008
C. charadraeus	95.60	913	Japan	AB462277.1	Matsumoto *et al*., 2008
C. verbosus	94.62	805	Japan	AB462281.1	Matsumoto *et al*., 2008
C. kibunensis	94.62	419	France	AY861146.1	Perrin *et al*., 2006
C. huffi	95.51	262	China	MH809966.1	Cen, 2018
C. huffi	95.51	263	China	MH809965.1	Cen, 2018
C. pulicaris	91.26	339	Germany	FN263299.1	

C. pulicaris	91.26	338	Germany	FN263297.1	
C. punctatus	91.35	787	Japan	AB462275.1	Matsumoto *et al*., 2008
C. punctatus	91.26	400	France	AY861157.1	Perrin *et al*., 2006
C. picturatus	93.65	528	France	AY861155.1	Perrin *et al*., 2006
C cataneii.	93.62	438	France	AY861139.1	Perrin *et al*., 2006
C. impunctatus	93.62	401	England	AJ417986.1	Ritchie, 2001
C. impunctatus	93.62	415	England	AJ417985.1	Ritchie, 2001
C. arakawai	93.55	308	China	MH809952.1	Cen, 2018
C. arakawae	93.55	907	Japan	AB462265.1	Matsumoto *et al*., 2008
C. arakawae	93.55	2624	China	AJ489503.1	Lin & Hu, 2004
C. pictipennis	93.55	500	France	AY861154.1	Perrin *et al*., 2006
C. heteroclitus	93.55	421	France	AY861143.1	Perrin *et al*., 2006
C. humeralis	93.48	305	China	MH809957.1	Cen, 2018
C. humeralis	93.48	305	China	MH809956.1	Cen, 2018
C. humeralis	93.48	305	China	MH809955.1	Cen, 2018
C. variipennis	93.48	434	India	KY491654.1	Maheshwari and Maheshwari, 2017
C. paraflavescens	93.48	342	Japan	AB462284.1	Matsumoto *et al*., 2008
C. matsuzawai	93.48	908	Japan	AB462283.1	Matsumoto *et al*., 2008
C. humeralis	93.48	912	Japan	AB462282.1	Matsumoto *et al*., 2008
C. subfagineus	93.48	496	France	AY861161.1	Perrin *et al*., 2006
C. newsteadi	93.48	502	France	AY861151.1	Perrin *et al*., 2006
C. albicans	93.48	533	England	AJ417980.1	Ritchie, 2001

C. pulicaris	90.29	340	Germany	FN263298.1	
C. pulicaris	90.29	339	Germany	FN263296.1	
C. sumatrae	92.55	784	Japan	AB462276.1	Matsumoto *et al*., 2008
C. ohmorii	92.55	925	Japan	AB462273.1	Matsumoto *et al*., 2008
C. nipponensis	92.55	794	Japan	AB462272.1	Matsumoto *et al*., 2008
C. maritimus	90.29	476	France	AY861150.1	Perrin *et al*., 2006
C. malerillei	92.47	548	France	AY861149.1	Perrin *et al*., 2006
C. lupicaris	92.39	533	France	AY861148.1	Perrin *et al*., 2006
C. species	91.49	461	India	KY620882.1	Maheshwari & Maheshwari, 2017
C. pulicaris	92.31	512	France	AY861156.1	Perrin *et al*., 2006
C. grisescens	90.62	417	England	AJ417987.1	Ritchie, 2001
C. pulicaris	90.22	544	England	AJ417983.1	Ritchie, 2001

Below are the sequences obtained following BLAST search in National Center for Biotechnology Information (NCBI) of *Culicoides oxystoma* from Benue State, Nigeria. Their unique Accession Number as evidence of their deposition in GenBank are displayed above each sequence:

>OM459833.1

TGATGTTTCATCAGGTATGGTGTTGTAAGCCGTTAATATGGTAGTGTCGTCTT
GTCACACGACAGCTTGTTATAAAGATGTAGTTTATTATGTCTTAAACAAGTCA
ATATCATATATTAACGCAAAACAATCTTAGGTAGCTTTTATGTAAGAGCTTTA
AAGACTTTTTTGCCTAAGGCCCCGTAAAACTAGTTACCCATTGCGAGGGGGCT
AGTATGCATATAAAACCTTTTGTGTATTGTATGCTTTGATTTTTCGGGATAAT
AATGTGTGATGTTGTTTTATGAAAAAACAAAAAATAAAAAAAAAAAAATTCC
CGTATATCTTTTTTGGGGGATCACTTGGCCCCCGGCCCAAAAAAACCAAAAA

>OM459834.1

TGATGTTTCATCAGGTATGGTGTTGTAAGCCGTTAATATGGTAGTGTCGTCTT
GTCACACGACAGCTTGTTATAAAGATGTAGTTTATTATGTCTTAAACAAGTCA
ATATCATATATTAACGCAAAACAATCTTAGGTAGCTTTTATGTAAGAGCTTTA
AAGACTTTTTTGCCTAAGGCCCCGTAAAACTAGTAACCCATTGCGAGGTGGCT
AGTATGCATATAATACCTTTTGCGTATTGTATGCTTTGATTTTTCGGGATAATA
ATGTGTGATGTTGTTTTATGAAAAAACAAAAAATAAAAAAAAAAAAATTCCC
GTATATCTTTTTTGGGGGATCACTTGGCCCCCGGCCCAAAAAAACCAAAAA

>OM459835.1

TGATGTTTCATCAGGTATGGTGTTGTAAGCCGTTAATATGGTAGTGTCGTCTT
GTCACACGACAGCTTGTTATAAAGATGTAGTTTATTATGTCTTAAACAAGTCA
ATATCATATATTAACGCAAAACAATCTTAGGTAGCTTTTATGTAAGAGCTTTA
AAGACTTTTTTGCCTAAGGCCCCGTAAAACTAGTAACCCATTGCGAGGTGGCT
AGTATGCATATAATACCTTTTGCGTATTGTATGCTTTGATTTTTCATGATATTG
TGTGATGTTGTTTTATGCAAAAACAAAAAATAAAAAAAAAAAAATTCCCGTA
TATCTTTTTTGGGGGATCACTTGGCCCCCGGCCCAAAAAAACCAAAAA

Figure 4.11: The locations of genetic differences between the three *Culicoides oxystoma* obtained during this study in Benue State, Nigeria

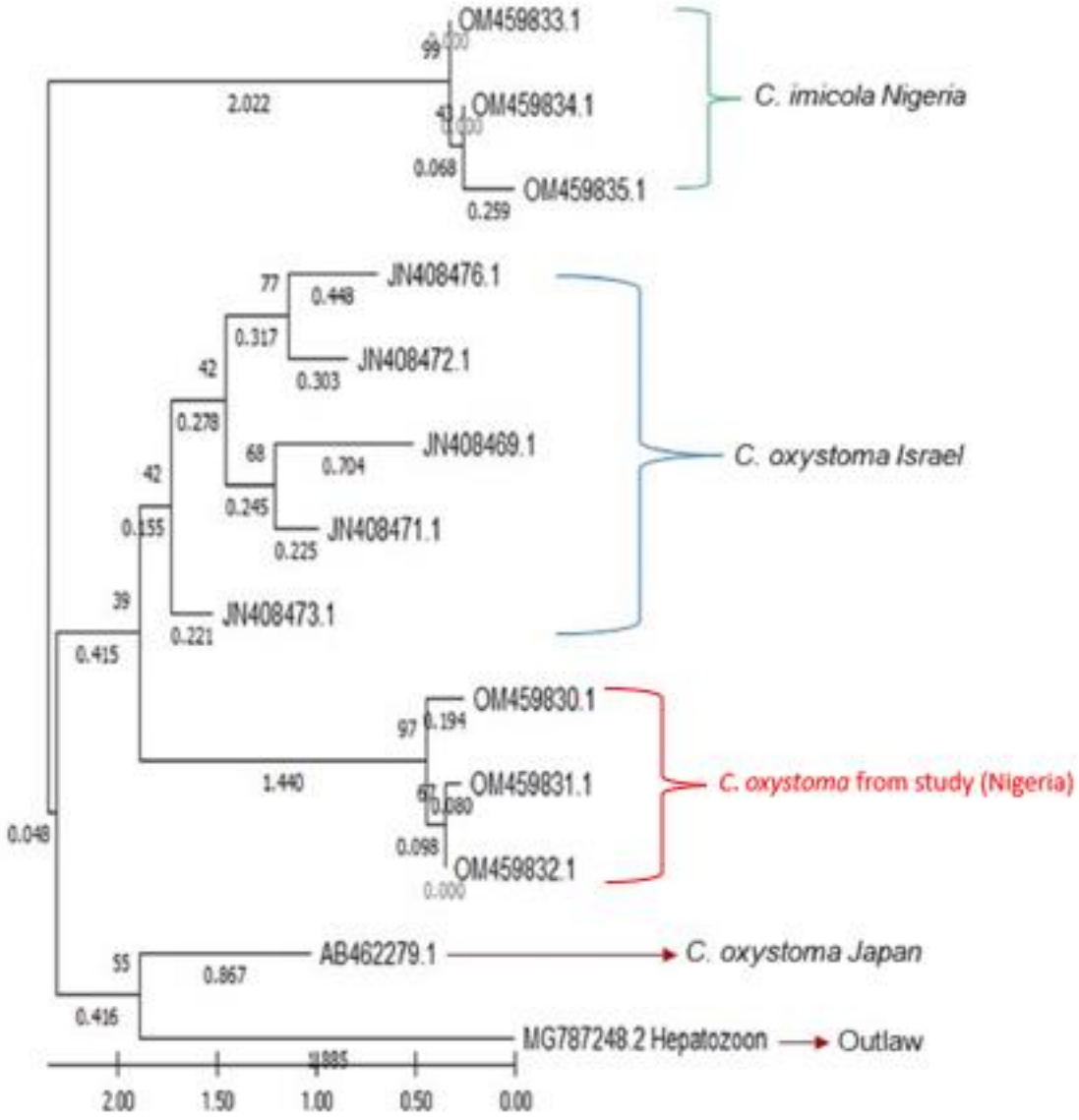

Figure 4.12: Phylogeny of *Culicoides oxystoma* ITS1 NJ unrooted tree. Bootstrap values >50% are indicated

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA11.

Table 4.11: *Culicoides indistinctus* showing genetic relatedness with *Culicoides* species from other locations.

Culicoides sp.	Percentage similarity	Base pair	Country	Accession number	Reference
C. indistinctus	100	376	Nigeria	OM459836.1	Oke *et al*., 2022 Unpublished
C. charadraeus	92.31	913	Japan	AB462277.1	Matsumoto *et al*., 2008
C. picturatus	91.49	528	France	AY861155.1	Perrin *et al*., 2006
C. indistinctus	97.33	534	France	AY861145.1	Perrin *et al*., 2006
C. impuctatus	91.49	401	England	AJ417986.1	Ritchie, 2001
C. impuctatus	91.49	415	England	AJ417985.1	Ritchie, 2001
C. imicola	91.40	305	India	KY491653.1	Maheshwari & Maheshwari, 2017
C. kibunensis	91.40	817	Japan	AB462280.1	Matsumoto *et al*., 2008
C. cylindratus	91.40	820	Japan	AB462269.1	Matsumoto *et al*., 2008
C. cylindratus	91.40	748	Japan	AB462268.1	Matsumoto *et al*., 2008
C. humeralis	91.30	305	China	MH809957.1	Cen, 2018
C. humeralis	91.30	305	China	MH809956.1	Cen, 2018
C. humeralis	91.30	305	China	MH809955.1	Cen, 2018
C. matsuzawai	91.30	908	Japan	AB462283.1	Matsumoto *et al*., 2008
C. humeralis	91.30	912	Japan	AB462282.1	Matsumoto *et al*., 2008
C. nipponensis	90.43	794	Japan	AB462272.1	Matsumoto *et al*., 2008
C. lumgchiensis	96.00	776	Japan	AB462271.1	Matsumoto *et al*., 2008
C. huffi	91.01	262	China	MH809966.1	Cen, 2018
C. huffi	91.01	263	China	MH809965.1	Cen, 2018

C. variipennis	90.22	434	India	KY491654.1	Maheshwari & Maheshwari, 2017
C. paraflarescens	90.22	842	Japan	AB462284.1	Matsumoto *et al*., 2008
C. pulicaris	95.89	512	France	AY861156.1	Perrin *et al*., 2006
C. lupicaris	90.22	533	France	AY861148.1	Perrin *et al*., 2006
C. punctatus	94.52	533	England	AJ417984.1	Ritchie, 2001
C. pulicaris	94.59	544	England	AJ417983.1	Ritchie, 2001
C. maritimus	93.33	476	France	AY861150.1	Perrin *et al*., 2006
C. dubius	91.43	1096	Japan	AB462270.1	Matsumoto *et al*., 2008
C. obscurus	100	780	France	MK893031.1	Mathieu *et al*., 2020
C. obscurus	100	787	France	MK893030.1	Mathieu *et al*., 2020
C. miombo	90.48	802	France	MK893024.1	Mathieu *et al*., 2020
C. miombo	90.48	808	France	MK893023.1	Mathieu *et al*., 2020
C. jacobsini	90.48	779	France	MK893017.1	Mathieu *et al*., 2020
C. imicola	90.48	353	Nigeria	OM459832.1	Oke *et al*., 2022 Unpublished
C. imicola	90.48	353	Nigeria	OM459831.1	Oke *et al*., 2022 Unpublished
C. imicola	90.48	352	Nigeria	OM459830.1	Oke *et al*., 2022 Unpublished

Below are the sequences obtained following BLAST search in National Center for Biotechnology Information (NCBI) of *Culicoides indistinctus* from Benue State, Nigeria. Their unique Accession Number as evidence of their deposition in GenBank are displayed above each sequence:

>OM459836.1

TTTGTTTTGTGTTCCTGTGGAGGGGGCATTATGGTAATTAAAAAAACACATAA
AGAAGTTTCTTATGGTATGGGGTGGTAAGTCGTTAATATGATAGTGTCGTCTT
TTCCCCCGCCAGCTTATTATAAGGATGTAGTTTATTTTTTTTTAAATAAGTCAA
TATCATATATTATCGCAAAACAATCTTAGGTGGCTTTCACGTAAGAGCTTTAA
AGACTTGTTTGCCCAAGGCCCCGTAAAACTAGTAACCCATTGCGAGGGGGCT
GGTATGCATATAATACCATTGGTGTATTGTATGCATTGATTTTTCATGATAAA
AATGTGGGAGGTTGTTTAATGTAAAAACAATAATTAATAAAAAAAAATGACC
AAAACC

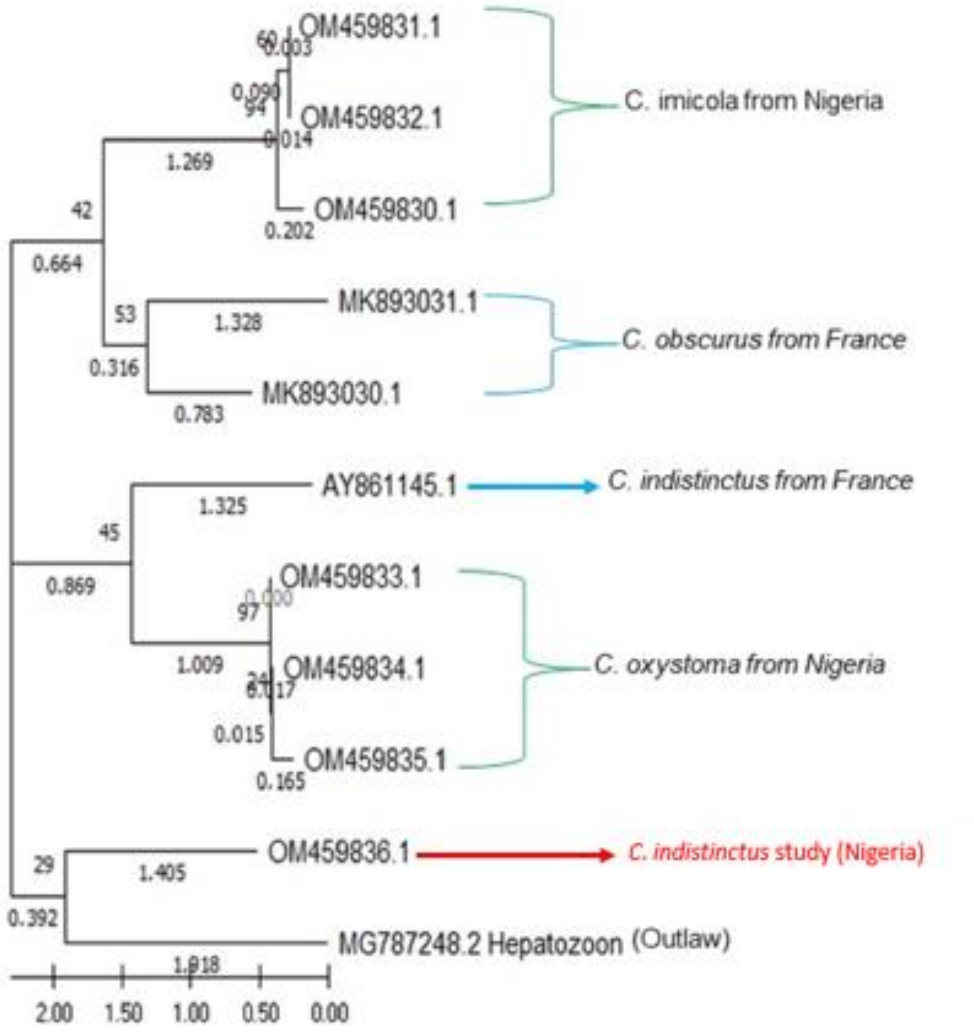

Figure 4.13: Phylogeny of *Culicoides indisticntus* ITS1 NJ unrooted tree. Bootstrap values >50% are indicated.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA11.

4.3 Molecular Detection of sources of blood meals of engorged *Culicoides* species

Determination of blood meal sources by polymerase chain reaction indicated that most of the *Culicoides* species have taken blood meals from hosts and the displayed bands on gel electrophoresis showed that only three hosts were parasitized. Sequencing to confirm this established that their engorgements were from cattle (*Bos taurus*), humans (*Homo sapiens*) and dogs (*Canis familiaris*) in the following order 60%, 37% and 3% respectively (Figure 4.13). The two vector species (*Culicoides imicola* and *C. oxystoma*) obtained blood from both *Bos taurus* and *Homo sapiens*. 74% of the blood from *Bos taurus* were taken by *Culicoides imicola* while for humans *C. oxystoma* took 85%. However, there was no case of multiple blood feeding among the species.

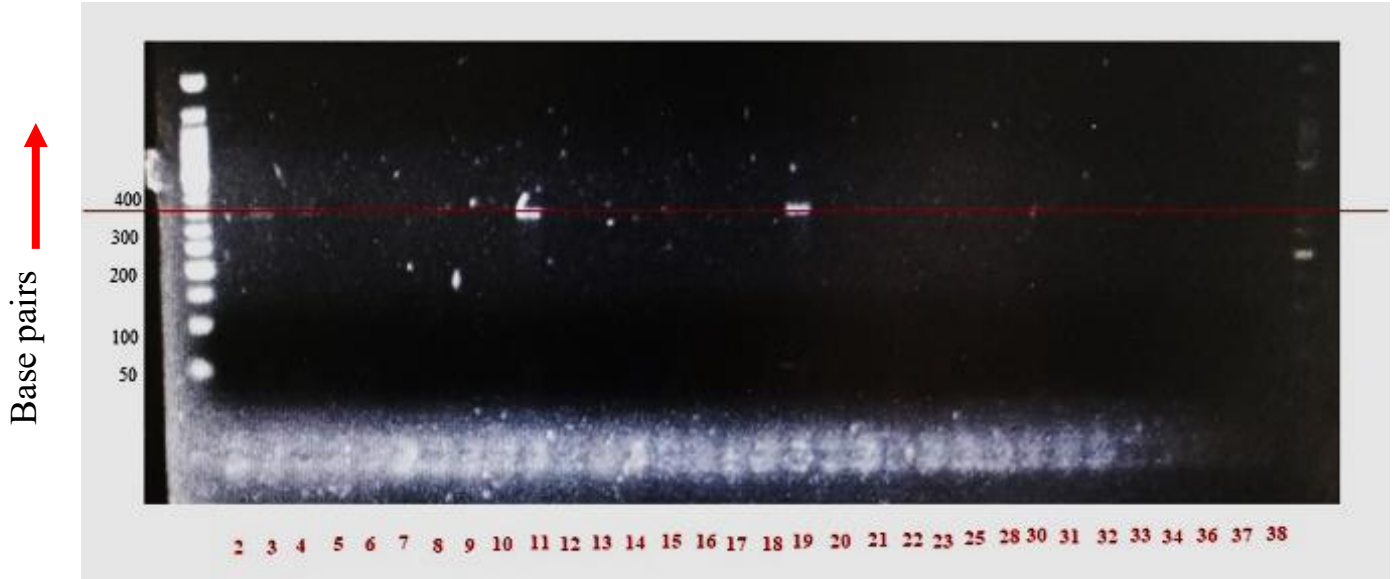

Plate 4.31: Gel electrophoresis for detection of sources of blood meals of *Culicoides* species from Benue State, Nigeria

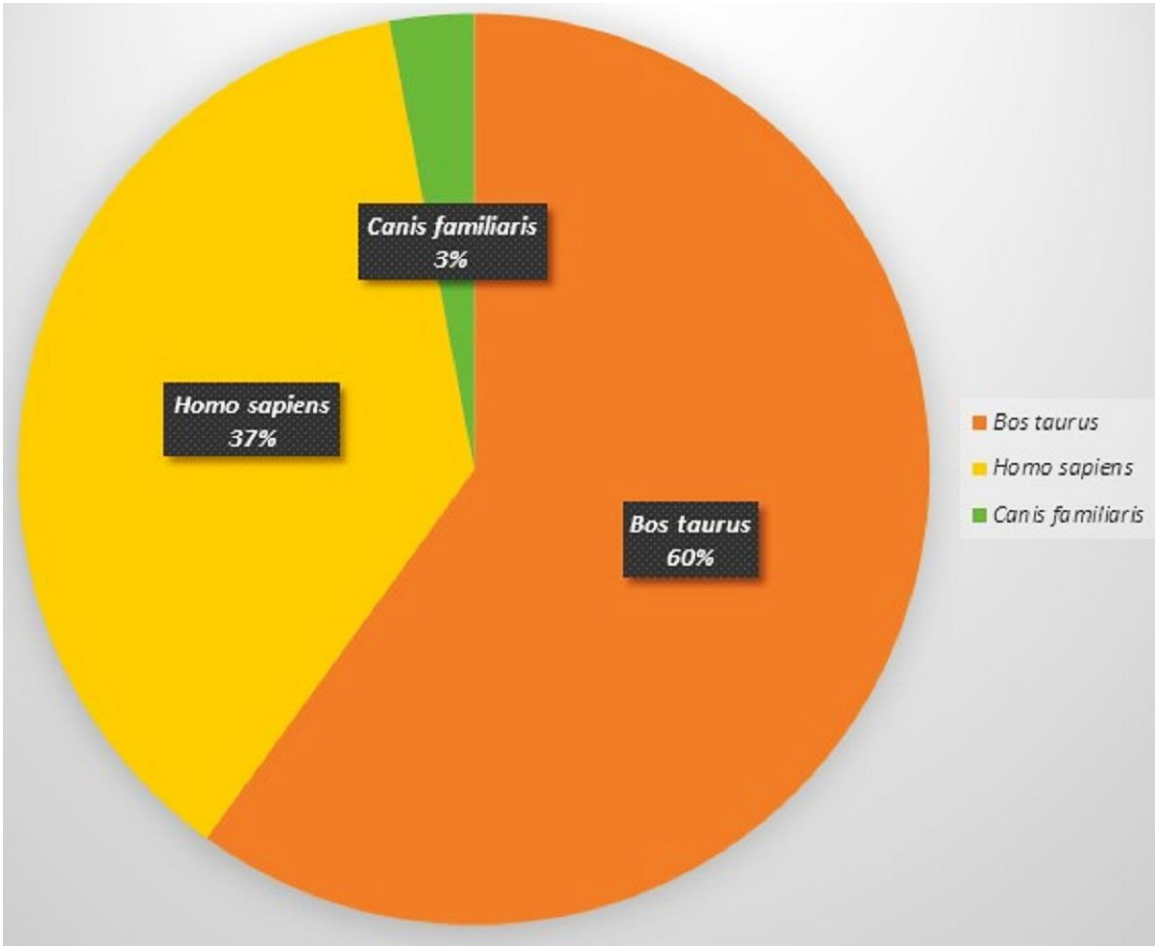

Figure 4.14: Pie chart representing the proportion and diversity of hosts from *Culicoides* species from Benue State, Nigeria

4.4 Molecular Detection of Filarial nematodes from Pools of *Culicoides* species

Detection of filarial nematodes by polymerase chain reaction showed that some of the *Culicoides* species harboured filarial parasites as observed from the bands on gel electrophoresis (Plate 4.32). Sequencing gave a total of eight (17.02%) positive results. These were five *Onchocerca* species (62.5%) from *Bos taurus* fed *Culicoides imicola* and three *Mansonella* species from *Homo sapiens* fed *Culicoides oxystoma* represents (37.5%). All the *Onchocerca* species detected were obtained from *Culicoides* captured between September and November while the *Mansonella* species were recovered from *Culicoides* species in March.

Benue South had the highest filariae obtained recording 50% (n = 4). Both filarial species (*Onchocerca gutturosa* and *Mansonella perstans*) were present in Benue South – Table 4.12. Only *Onchocerca gutturosa* were found in Benue Northwest whereas for Benue Northeast, only *Mansonella perstans* were found – Table 4.12. were in the Five of the eight filarial species obtained were *Onchocerca gutturosa*. The results further revealed that *Culicoides imicola* and *C. oxystoma* could serve as vectors of *Onchocerca gutturosa* while *C. oxystoma* could be linked to *Mansonella perstans* – Table 4.13.

The sequences were deposited in GenBank with Accession Numbers **OP859021**, **OP859022** and **OP859023** for *Onchocerca* species while OP859024 for *Mansonella* species.

Plate 4.32: Gel electrophoresis showing detection of filarial nematodes in *Culicoides* species

Table 4.12: Distribution of Filarial nematodes by geopolitical zones of Benue State, Nigeria

Geopolitical Zones	Filarial Nematodes		TOTAL
	Onchocerca gutturosa	*Mansonella perstans*	
Benue Northwest	2	0	**2**
Benue Northeast	0	2	**2**
Benue South	3	1	**4**
TOTAL	**5**	**3**	**8**

Table 4.13: Distribution of Filarial nematodes as found in *Culicoides* vectors from Benue State, Nigeria

	***Culicoides* species**		
Filarial species	*C. imicola*	*C. oxystoma*	**TOTAL**
Onchocerca gutturosa	4	1	**5**
Mansonella perstans	0	3	**3**
TOTAL	**4**	**4**	**8**

CHAPTER FIVE
DISCUSSION

5.1 Morphological Identification of *Culicoides* species from the field

The total number (30,163) of adult *Culicoides* species trapped during this study varied greatly when compared with previous works conducted within Nigeria. In separate studies carried out by Oke *et al*., 2017; Adeyefa and Dipeolu, 1993 and Dipeolu and Ogunrinade 1977, the total number of catches reported were lower. However, number collected were significantly higher as reported by Dipeolu, 1976 and Dipeolu and Sellers, 1976. The female specimens collected represents 87.86% of the total population. The higher females to males' ratio as recorded consistently agrees with reports from various authors within and outside the African continents [Oke *et al*., (2016), Dipeolu (1976), Maryam *et al*., (2014), Takken *et al*., (2008) and Arcana *et al*., 2016]. The concentration of females around the hosts is an indication of their continuous search for blood meals as a requirement for development of ovaries. Invariably, it could translate to extreme hosts discomfort, interference with farming activities in human hosts and likelihood of disease transmission. The disparities observed in terms of species distribution and abundance could possibly be due to: (i) deforestation and urbanization which could lead to extinction of certain species and reduction of suitable breeding habitats (Mellor and Boorman, 2000); (ii) variation in geographical locations coupled with changes in weather conditions have effects on *Culicoides* activities and distribution; (iii) methods and duration and frequency of collection - some methods are more efficient than others and certain *Culicoides* species are better attracted to various light colours. Also, a longer period of sample collection may produce more samples; (iv) Geographical coverage during sampling could play a very important role; (v) The type of hosts at the vicinity of collection – insects are attracted to some hosts more than others.

The species identified during this research showed a higher level of similarity when compared with the study carried out in Ibadan by Oke *et al*., (2016). However, some species (*Culicoides ovalis*, *C. isoloensis*, *C. festipennis*, *C. austeni*, *C. karaensis* and *C.*

indistinctus) reported during this research were absent in their studies, although they also reported four species (*C. expectator, C. brucei, C. bedfordi* and *C. distinctipennis*) which were not captured during this present study. Twenty of the identified species have been previously reported by Dipeolu (1976) except *Culicoides indistinctus* which is being identified in this report for the first time. Two among the four numerous species (*C. enderleini* and *C. imicola* – the second and third most abundant respectively) during the collection were also reported to be of high prevalent (occupying first and second position in terms of prevalence) as documented by Oke *et al*., (2016) while and *C. imicola* was the most abundant to be observed by Dipeolu (1976). *Culicoides fulvithorax* that was solely reported in western Nigeria during human bait study was also collected during the course of this research. Other authors outside Nigeria have also documented species that were partially in agreement with this study. For instance, Maryam *et al*., (2014) in Senegal reported *Culicoides enderleini*, *C. oxystoma, C. nivosus, C. pycnosticus*, and *C. imicola,* Mame *et al*., (2013 and 2018) also in Senegal established the presence of *C. oxystoma* along with all other species reported in this study. This further established that there are species similarities within the West African countries. Belkharchouche *et al*., 2020 reported *C. imicola* in Algeria, in Saudi Arabia (*C. imicola, C. oxystoma* and *C. neavei*) as well as Israel (*C. imicola*, *C. oxystoma* and *C. distinctipennis*) as identified by Bravaman (1988). Most of the species identified were reported to be present in South Africa. *Culicoides imicola* was reported the predominant species in Zimbabwe followed by *C. enderleini* this was however, the other way round in our findings. Other than the two species mentioned, we have some species in common. *Culicoides oxystoma* was also reported to be present in abundance both in Japan and China (Di *et al*., 2021). All the species identified were previously described in Kenya. Among the twenty-one (21) species identified, only seven (7) species were collected throughout the year while some were absent at certain month. All the species identified have their females fully represented while five species did not have male representation. The availability of *Culicoides* species all through the period of collection across the collection sites is an indication that biting midges are available in the study area all year round. Also, the presence of blood or burgundy pigments in the abdomen of all the females imply that all the species trapped were bitters, hence, they could serve as sources of annoyance to their various hosts.

The total number of catches during the dry and rainy seasons differed statistically significantly from one another. The total collection in dry season was 12.04% of the entire collection. The decrease in number collected in dry season could be attributed to low moisture due to lack of rainfall, hence, unavailable breeding sites. For the species breeding in animals' manure, the ability to retain moisture is also reduced due to rise in temperature caused by intense sunlight. We had the highest catches in September (49.30%) perhaps because it serves a break in rainfall after a continuous period of rains observed in June to August. This little break as observed in September created a conducive environment for breeding to take place, resulting in high emergence. January has the lowest (0.91%) probably as a result of heavy wind blowing with increasing wind speed which could cause long distance dispersal. The highest number recorded in Benue South geopolitical zone could be attributed to high animal density due to availability of pasture for grazing animals. This in turns provide suitable habitats for reproduction and a readily available source of blood meals for the biting midges

Trap efficiency could be affected by many factors such as types of traps, prevailing climatic conditions, distance between trap and livestock and internal factors peculiar to each trap. Among these, the most likely to influence number of catches during the study was the inbuilt factor. It was observed that the Miniature downdraft light suction trap – Model 1212 was more efficient for collection of biting midges probably due to absence of the gating system which was present in New Jersey Standard light trap. The gating system consume consumes more power thereby reduces the ability of the suction fan thus reduced number of catches.

Culicoides can vary in range according on the climate. As a determining factor of distribution, low temperature often has a greater impact than high temperature (Gates, 1993). Precipitation can influence the distribution of *Culicoides* at suitable temperatures by impacting the availability of breeding grounds. The dispersal of *Culicoides* can also be impacted by wind speed and direction (Wittman & Baylis 2000). By altering the proportion of adults who can transmit the virus within the population as well as the size of the adult population as a whole, climatic conditions may also have an impact on the ability of *Culicoides*

populations to vector diseases (Wittman & Baylis, 2000). The recent emergence of African equine virus and bluetongue in Europe has been linked to climate change (Summer 2009).

Weather variables (temperature, humidity, rain, wind speed, and other factors) and the number of *Culicoides* species collected each month showed varying degrees of correlation. Temperature affects flight activity of *Culicoides* biting midges and this in turn determine trap efficiency. The heavy wind speed serves as hindrance to light traps with subsequent decrease in number of catches. Furthermore, this could also be attributed to absence of rain throughout December causing dryness and unsuitable environment for breeding. However, Heung *et al*., (2015) in different locations reported that the month of May has the highest number of collections. Humidity, precipitation and rainfall showed positive correlation with number of catches. The positive correlation was moderate for humidity and precipitation (P = 0.12, r = 0.48 and P = 0.13, r = 0.46, respectively at 95% C.I). However, for rainfall, there is a high statistically significant difference with r = 0.96. Thus, increase in rainfall leads to increase number of catches. This probably explains the reason while *Culicoides* species are more predominant during rainy season because there are more breeding places available. On the other hand, wind speed and temperature showed negative correlation with the number of catches (P = 0.96. r = -0.02 and P -0.23 and r = -0.36, respectively). This is in agreement with several authors who established that the higher the wind speed the lower the number of catches due to dispersal and possibility of influence of wind on light traps. Also, the higher the temperature, the lower the number of catches hence, the reason while it is difficult to trapped biting midges during the day when temperature is at optimal except during raining season when the temperature is moderate even in the day time to permit midges activities (Figures 4.2 to 4.6). Numerous writers have noted the significant impact of climatic conditions on *Culicoides* populations, which has an impact on the epidemiology of *Culicoides*-borne illnesses. In Morocco, Israel, and the South African region that receives winter rainfall, a discernible rise in abundance occurs around the end of summer, corresponding with or just after the year's hottest phase but several months after the last significant rainfall. During the coldest season of the year, when it rains the most, adult *C. imicola* are missing or uncommon. Yee and Juliano (2012)

and Ptatscheck and Traunspurger (2015) also established that intensity and frequency of rainfall have influence on *Culicoides* species.

Some outbreaks are strongly seasonal, occurring in the late summer and fall, and they are tightly correlated with climatic and biological conditions that affect the vectors' survival and abundance. These variables include temperature (Wittman, *et al*., 2002), vegetation index (Carpenter *et al*., 2009; Kumar *et al*., 2018), relative humidity (Carpenter *et al*., 2009; Baylis *et al*. 2010), wind speeds (Ducheyne *et al*., 2007; Carpenter *et al*., 2008; Baylis *et al*., 2010), higher rainfall in the month before the occurrence of the disease (Purse *et al*. 2004). Numerous authors have looked into the connections between *Culicoides* species and the environmental factors causing the seasonal variations in the species. In research done in the state of Rio de Janeiro, Maia-Herzog *et al*., (1988) discovered an antagonistic association between *Culicoides* abundance and rainfall. The authors found no relationship between temperature and humidity during the formation of new species. According to Silva *et al*., (2001), *Culicoides* abundance is higher during dry spells that are followed by torrential downpours. In contrast, higher abundance was noted during the colder and rainier months De Barros *et al*., (2007) in Maranho state.

Breidenbaugh *et al*. (2009) noticed a larger link between the presence of some species and high rainfall, while other species were present during dry seasons in their study of Ceratopogonidae assemblies in salt marshes from South Carolina, USA. These investigations show that *Culicoides* species are sensitive to changes in precipitation rates, typically timing the emergency period with periods of heavy rain.

On the other hand, Nigeria experiences an annual high in abundance Insects are few or nonexistent during the hot, dry season, which is immediately following the rainy season and when it is the coldest of the year. The climax occurs toward the conclusion of these seasons in Sudan and the summer rainfall region of South Africa, when the hot season and rains coincide; however, the pattern in Kenya is once again distinct. *Culicoides* distribution is drastically affected by change in temperature since they require semi-aquatic habitats for development. This was in agreement with the result obtained on the influence of temperature. Higher temperature will cause dryness of habitats thus reduces reproduction and consequently number of catches. Temperature also reduces and host-seeking activities.

Higher percentage of parous females obtained during the study coincided with peak of rainy season and months with highest number of female specimens. The rainfall is a key factor. It caused saturation of the ground thus providing suitable breeding habitats, greener pastures for animal to feed and reduced environmental temperature thereby making it conducive for biting midges activities. The period of the day which ordinarily were not conducive for biting midges' activities were temporarily altered during rainy season, thus biting midges become active all day.

Stereomicroscopy revealed the presence of morphologically abnormal specimens and ectoparasitism. The abnormalities observed were multiple abdominal segments attaching to the thorax. Two of these were recorded in female specimens, one each in *Culicoides imicola* and *Culicoides subschultzei*. One of the specimens clearly displayed bi-abdomen while the second showed evidence of the third rudimentary abdomen between the dorsal and ventral abdominal divisions. One of the specimens showed evidence of previous blood meal as revealed by the deposit of burgundy red pigments in the abdomen. This same specimen also displayed three spermathecae in each of the abdominal divisions making a total of six. This is an extremely rare occurrence and possibly the highest number of spermathecae so far reported. Majority of *Culicoides* have two functional spermathecae while some have one. However, three spermathecae have been reported in very few specimens.

Another abnormality reported was a case of gynandromorphism in which the specimen displayed an anterior portion typical of female structure (pilose antennae/dichoptic eyes) and a posterior male structure (male genitalia). The fact that this is the first report in Nigeria has established its rareness in occurrence and thus in agreement with Williams (2010), Sample (2011) and Skvarla and Dowling 2014. Also, some authors have reported similar cases elsewhere in different *Culicoides* species and these include, Curtis (1962) and Kamel (1965) in which the gynander showed absence of spermathecae. Gynandromorphism was also reported in Israel. Other abnormalities that have been reported in *Culicoides* species include abnormalities in number and size of spermathecae (*C. alatus* – Das Gupta, 1962 and 1963), antennal, palpal and genital abnormalities (Braverman *et al*., 1993).

A species of unidentified microscopic ectoparasite (suspected to be mite) was found feeding on *Culicoides subschultzei* at the ventral thorax has established the possibility of hyper-parasitism which have been reported in other species of Diptera. However, *Culicoides* species at various times have been reported to serve as hyperparasites obtaining their blood bloodmeals from about nineteen species of mosquitoes in different genera (Chu, 1959; Chhilar, 2010).

5.2 Molecular Characterisation and Phylogeny of selected *Culicoides* species

Polymerase chain reactions (PCR) confirmed the presence of diverse *Culicoides* species in Benue State showing bands of varying sizes ranging from 300 to 400 base pairs. These have established the authenticity of the genus and confirmation of the available species collected. Two among the species molecularly reported (*C. imicola and C. oxystoma*) have always been consistently reported in different countries by various authors and have been established to be worldwide in distribution. These have been recognized in Morocco, Tunisia and Senegal (Maryam *et al*., 2014). However, the results of selected amplicons revealed the availability of vector species which are *Culicoides imicola* and *C. oxystoma*. *Culicoides imicola*, the major vector of most of the *Culicoides*-borne pathogens in Africa, Asia and Europe (Mellor *et al*., 2000), while this same role has just have been recently established for *C. oxystoma*. Miura *et al*., (1988) in Japan reported the transmission of Akabane, Aino, Chuzan and Ibaraki viruses by *C. oxystoma* while in India the same species was confirmed to transmit bluetongue virus (Dadawala *et al*., (2012). Also, in Israel it was incriminated as vector of epizootic haemorrhagic disease as reported by Morag *et al*., (2012). Among the species molecularly characterized, *Culicoides imicola* is the widest spread in terms of distribution, occurring in most part of the world possibly due to its ability to adapt to varying weather conditions.

Maximum likelihood, neighbour-joining phylogenic trees were computed using Mega 11 (Tamura *et al*., 2021). The most appropriate evolutionary model was chosen using Bayesian data. The trees were subjected to 1000 bootstrap replications to assess topological reliability. The phylogenetic tree of the three *Culicoides imicola* obtained from Nigeria established varying degree of relationship with other *C. imicola*. They were observed to be very closely related with each other (within Nigeria). Also, they were closely related with

same species from Israel (Morag *et al*., 2012), and Scotland as well as certain *C. imicola* from France (Perrin *et al*., 2006). This shows the possibility of their evolution from Israel. They also exhibit certain level of relationship with *C. imicola* from India (Maheshwari and Mahore, 2017) while having a distant relationship with some *C. imicola* from France (Mathieu *et al*., 2020).

The phylogeny of the three *Culicoides oxystoma* obtained also established varying level of association with other *Culicoides* species from different regions. They were observed to be very closely related with each other. Also, they were observed to be closely related with *C. oxystoma* from Israel (Morag *et al*., 2012), *C. paraflavesces* and *C. arakawae* from Japan (Matsumoto *et al*., 2008), *C. variipennis* and *C. imicola* from India (Maheshwari and Maheshwari, 2017), *C. newsteadi, C. subfagineus* and *C. lupicaris* from France (Perrin *et al*., 2006), *C. grisescens* and *C. impuctatus* from England (Ritchie, 2001) and *C. arakawae* from China (Cen, 2018). Other than these close relationships, the species exhibited varying degree of associations with several other species.

Culicoides indistinctus also exhibited varying level of relationships across species. However, those with close relationship include *C. oxystoma* from Nigeria, *C. obscurus* and *C. miombo* from France (Mathieu *et al*., 2020), *C. impuntatus, C, punctatus* and *C. pulicaris* from England (Ritchie, 2001), *C. huffi* from China (Cen, 2018), *C. imicola* and *C. variipennis* from India (Maheshwari and Maheshwari, 2017) and *C. cylindratus, C. humeralis* and *C. paraflarescens* from Japan (Matsumoto *et al*., 2018). Other far distant relationships with various species also existed.

5.3 Molecular Detection of sources of blood meals of engorged *Culicoides* species

Detection of bloodmeal sources of *Culicoides* biting midges is gradually gaining attention when compared with other significant arthropod vectors, such mosquitoes and ticks (Borstler *et al*., 2016; Shahhosseini *et al.*, 2018) which have been widely studied. Several studies on bloodmeals of biting midges revealed that ruminants are the principal hosts (Hadj-Henni *et al*., 2015) while humans and pigs are less frequently parasitized. Polymerase chain reaction conducted in this study indicated that most of the species obtained their blood meals from three hosts. This was confirmed by the results of the sequences that the principal hosts were cattle and humans and less frequently, dogs.

Culicoides oxystoma and *C. imicola* were observed to have fed from both cattle (*Bos taurus*) and humans (*Homo sapiens*) while only *C. imicola* was observed to have taken blood meals from dogs (*Canis familiaris*).

The detection of human blood from *Culicoides oxystoma* has further confirmed the report of Oke *et al*., (2017) who found *C. oxystoma* from human dwellings. Similar reports were documented where it was established that *C. oxystoma* were always attracted to residential areas as well as pig and cattle sheds. Several authors world-over have reported the diversities of sources of blood meals. However, this is the first established report on sources of blood meals of engorged female *Culicoides* species in Nigeria. It was partially in support of the reports by Hopken *et al*., (2017) in the United State where they stated that cattle and dogs as part of the hosts from which biting midges obtained their blood meals. Furthermore, Zuzana *et al*., (2021) reported humans, zebra and cattle as the frequent host of *Culicoides* species in their locality. Other authors have reported that large mammals, precisely ruminants, are the ideal hosts. There was no report of multiple hosts involving animals and humans observed in this study. However, their zoonotic potential is not in doubt because the same *Culicoides* species that fed from human was also reported to have taken blood meals from cattle and dogs.

5.4 Molecular Detection of Filarial nematodes from Pools of *Culicoides* species

Polymerase chain reaction showed that some of the *Culicoides* species harboured filarial parasites as observed from the different band sizes on gel electrophoresis. Confirmation of this revealed *Onchocerca* and *Mansonella* species. *Culicoides* species have been reported to be responsible for transmission of filarial nematodes both in humans and animals. In humans, they transmit *Mansonella ozzardi, M. perstans, M. streptocerca* and have been reported to be of high prevalence in west and central African regions of which Nigeria belong. Several authors have reported varying prevalence of perstans filariasis in different regions of Nigeria (Wijeyaratne *et al*., 1982; Anosike, 1994; Arene and Atu, 1986; Udonsi, 1988; Ufomadu *et al*., 1990; Oyerinde *et al*., 1988; Agbolade and Akinboye, 2001). In animals, they are vectors of bovine and equine onchocercosis (Borkent, 2004).

Some of the *Culicoides* species that were either morphologically or molecularly identified have been established in the transmission of one or more pathogens at different

geographical areas. Few are recognized vectors within Nigeria and Africa while others have been of great significance outside Africa continent. The presence of zoonotic pathogens in any of these animals could predispose humans to zoonosis (Zuzana *et al*., 2021), more importantly when it was reported that zoonotic *Onchocerca* species were obtained in human tissues (Beaver *et al*., (1974); Orihel and Eberhard (1998); Sallo *et al*., (2005); Koehsler *et al*., (2007). The details of pathogens transmitted, hosts and locations are provided thus: Generally, *Culicoides* species were linked to the transmission of Dugbe and Kotonkan viruses (humans and cattle) in Nigeria, Sango and Shuni viruses affecting cattle in Nigeria as well as *Leishmania donovani* (Africa) and *L. infantum* (worldwide). *Culicoides austeni*, *C. grahami*, and members of the Milnei groups are involved in the transmission of *Mansonella streptocerca* affecting humans in Africa while *C. grahami* and *C. fulvithorax* are vectors of *M. perstans* in Nigeria. *Culicoides oxystoma* is responsible for the transmission of *Onchocerca gibsoni* in Africa, Aino virus affecting ruminants, pigs and humans in Asia, Kasba virus of ruminants in African countries. Furthermore, Bunyip Creek and D'Anguilar in Australia and Japan affecting ruminants are also vectored by *Culicoides oxystoma*. African horse sickness, Sabo and Shamonda viruses of cattle in Nigeria are transmitted by *Culicoides imicola*. However, *C. oxystoma* have been incriminated to also play roles. The following pathogens are vectored by multiple species Akabane by *C. imicola, C. oxystoma* and *C. milnei* in ruminants (Africa, Asia), bluetongue and bovine ephemeral viruses by *C. imicola*, *C. oxystoma* both in Africa and Nyabira by *C. imicola* and *C. zuluensis* in South Africa.

The dangerous ungulate pest *Culicoides oxystoma* affects large ungulates, such as cattle. It is a potential carrier of bovine ephemeral fever on the Arabian Peninsula and has been linked to the transmission of several filariae to livestock in the Sudan, according to Borman, (1989). (El-Sinnary and Hussein, 1980). Japanese researchers Kurogi *et al*., (1987) isolated the Akabane virus from *C. oxystoma*. Pathogens transmitted by fast reproducing, highly mobile, habitat-generalist vectors that exhibit some combination of these traits, such as those that are promiscuous and rapidly changing, will likely react quickly to improved climatic appropriateness. For instance, Rift Valley fever virus (RVFV), a member of the Bunyaviridae's Phlebovirus genus, is extremely promiscuous across vertebrate hosts, including rats and hippopotamuses, yet

only ruminants and humans experience clinical symptoms. A variety of possible vector species, including 23 different mosquito species, a *Simulium* sp., and a *Rhipicephalus* tick, have had this virus recovered from them.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Culicoides species are known biological vectors of numerous economically important pathogens. However, despite their importance, there is limited information on morphology and molecular identification of *Culicoides* species in Nigeria, especially Benue State. Therefore, this study was conducted to identify *Culicoides* species, their host preference and their possible involvement in filarial worm transmission in Benue State, Nigeria.

In a purposive sampling technique, adult *Culicoides* species were collected in thirty locations across the three geopolitical zones in Benue State, Nigeria. In weekly overnight collections using two CDC black-light suction traps, *Culicoides* species were trapped between January and December, 2018 and corresponding environmental data recorded. The trapped *Culicoides* species were morphologically identified using a stereomicroscope to determine their sex and parity status. Polymerase chain reactions were carried out and characterisation of dominant species was achieved using sequence analysis targeting the *ITS1* gene. Their sources of blood meals were investigated using mitochondrial *MT-cyt b* gene and their role in the transmission of filarial parasites was probed using *Cox-1* gene. Data were analysed using descriptive statistics and correlation coefficient at $\alpha_{0.05}$.

A total of 30,163 *Culicoides* species were trapped during the period of collection with the highest overall collection of 13,700 (45.4%) recorded for Benue South geopolitical zone. There was positive association between rainfall and the number of *Culicoides* species trapped (r = 0.96), while the number of catches correlated negatively with wind speed and temperature (r = -0.1586 and r = -0.4789, respectively). Twenty-one species were morphologically identified. Females represents 87.9% (n = 26,502) of the total collection

of which 31.4% (n = 8,314) were parous. Two dominant species were *Culicoides imicola* (37.6%) and *C. oxystoma* (13.8%). *Culicoides indistinctus* (0.2%) identified in this study area is a new species reported in Nigeria. The Nigerian *C. imicola* and *C. indistinctus* strains were 95.8% and 97.3% related to French strains respectively, while *C. oxystoma* was (95.2%) related to Israeli strains. Cattle (60%), humans (37%) and dogs (3%) were their preferred hosts. The filaria, *Onchocerca gutturosa* was found in *C. imicola* and *C. oxystoma* that fed on cattle, while *Mansonella perstans* was obtained only from *C. oxystoma* that fed on humans.

6.2 Conclusion

This research established diverse *Culicoides* species and among these are recognized vectors of arboviruses of veterinary importance (*Culicoides imicola* and *Culicoides oxystoma*). They were observed to obtain their bloodmeals from *Bos taurus* (cattle), *Homo sapiens* (humans) and *Canis familiaris* (dogs), hence, considered as sources of threat to their hosts due to *Onchocerca* and *Mansonella* species detected in them. The study further established that abnormalities of any kind can affect *Culicoides* species.

6.3 Recommendations

The followings were the recommendations made:

1. Increasing the scope of pathogens to include both viruses and protozoa parasites such as *Leishmania* species.
2. Continuous monitoring of *Culicoides* species to prevent outbreak of diseases.
3. Study on *Culicoides* species using their larvae be considered to enable further detection of some unknown species.

6.4 Contributions to Knowledge

1. The study brings an end to nearly 40 years period of inactivity in *Culicoides* research in Nigeria.
2. This study characteries vector *Culicoides* species (*C. imicola* and *C. oxystoma*) in Benue State, Nigeria.
3. The study deposited to the GenBank the first sets of sequences of Nigeria *Culicoides* and obtained accession numbers.
4. The study also established the sources of bloodmeals of characterized species and the associated threats.

5. The study further established the phylogenetic relationship between obtained biting midges and species other from regions.
6. This study reported, perhaps for the first-time various abnormalities and ectoparasites that could affect *Culicoides* species.

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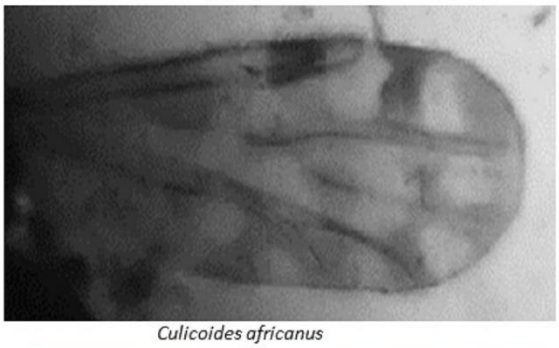
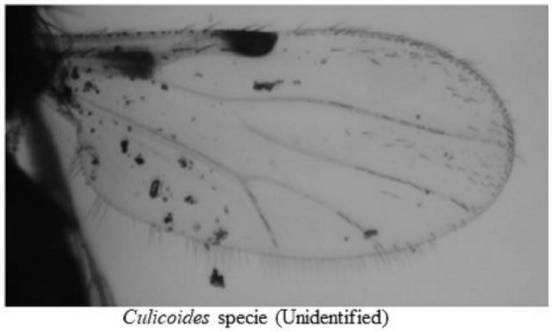
APPENDIX



Culicoides africanus

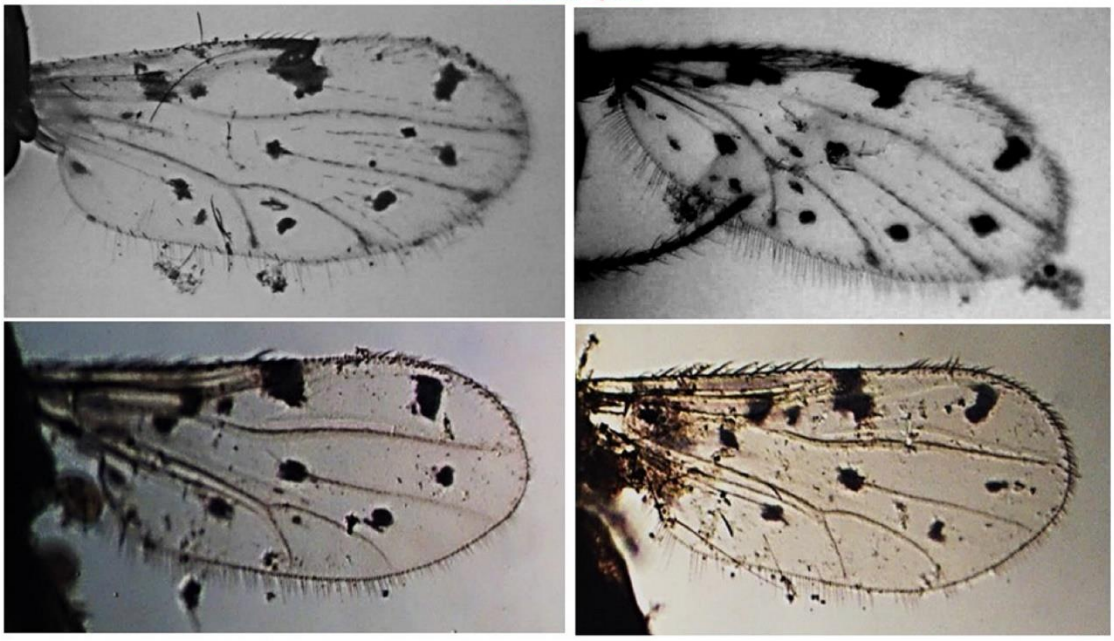
Culicoides fulvithorax

Culicoides indistinctus



Culicoides specie (Unidentified)

Undescribed species



Undescribed species