Abundance, composition and barcoding of Tabanidae in Kruger National Park and screening for *Besnoitia besnoiti*

by

Andeliza Smit

Submitted in partial fulfilment of the requirements for the degree

Master of Veterinary Science

in the

Department of Veterinary Tropical Diseases

Faculty of Veterinary Sciences

UNIVERSITY OF PRETORIA

December 2019

Acknowledgements

I would like to thank all my colleagues that aided me during this project. There are several that I would like to mention individually. First, I would like to thank Prof Luis Neves for giving me the opportunity to complete my masters under his guidance. It was truly a vast experience that I will carry with me through my life. To Louwrens Snyman and Ayesha Hassim who took time out of their busy schedule no matter when I had questions to ask about the simplest of things. You two truly made masters enjoyable and taught me more than the basic scope required. Thank you. To Annmarie Bosman who aided in laboratory training and all technical inquiries.

I would like to truly thank all the State Veterinarians of Kruger National Park who allowed and assisted us with the tabanid sampling. Without their contribution, none of this project would have been possible.

I would also like to thank the Meat Industry Trust (MIT), AgriSETA and the University of Pretoria for their contributions to the funding of the project.

Contents

Acknowledgements ii

List of Figures vi

List of Tables x

List of abbreviations xi

Abstract xiii

Literature Review 1

Introduction 1

Tabanidae 2

Besnoitia besnoiti 10

DNA extraction methodologies 15

Molecular markers for identification and phylogenetic inference 17

Motivation 20

Aim 22

Objectives 22

Comparison of four genomic DNA extraction methods on arthropods 23

Abstract 23

Introduction 24

Material and Methods 25

Samples 25

Extraction methods 25

Quantity and Quality 27

Time per extraction 27

Cost per extraction 27

Gel Electrophoresis 27

Statistical analysis 28

Results 28

Time and Cost analysis 28

Spectrophotometer measurements 28
Gel Electrophoreses Analysis 29
Discussion 30
Contribution to the systematics of Tabanidae of South Africa, including species pages 34
Abstract 34
Introduction 35
Material and Methods 36
Study site, sampling and identification 36
DNA extraction 40
Amplification 41
Gel electrophoresis 42
Phylogenetic analysis 42
Ethic consideration 43
Results 43
Tabanid abundance and composition 43
Species pages of tabanids collected 45
Discussion 94
Detection of *Besnoitia besnoiti* from tabanids from Skukuza in Kruger National Park, South Africa 100
Abstract 100
Introduction 101
Material and Methods 102
Samples 102
Extraction method 102
Screening 102
Gel electrophoreses 103
Results 103
Discussion 104
Concluding discussion 106

References 109

Annexure A 118

Annexure B 128

Annexure C 129

List of Figures

Figure 1: Basic structure of the antennae of the subfamilies of Tabanidae. 1= scape, 2= pedicel, 3= flagellum, a=flagellomeres b=base flagellum. Obtained from Cheng (1976). 5

Figure 2*:* Summarised life cycle of Tabanidae obtained from Baldacchino *et al. (2014a)*. 9

Figure 3: *Besnoitia besnoiti* acute stage indicating merozoites (left); merogony in the endothelium (right). 12

Figure 4: Cysts with bradyzoites in the endothelium (Left); Collagen deposited around the histiocyte (Right)..... 13

Figure 5: Representative results from gel electrophoresis analysis of four different genomic DNA extraction methods from five different tabanid samples. Electrophoresis was conducted on a 1.5% agrose gel with 2% ethidium bromide for 2 hours at 50V and a 1kb base pair ladder was used. 29

Figure 6: Diagram illustrating the cluster format of the three traps at each of the sites (excluding Sabie bucket and Shishangeni watering hole sites). The dotted blue line indicates the directing of the rotation of the traps; solid black line indicates the approximate distance between the traps. 37

Figure 7: The composition and abundance of tabanids caught in Kruger National Park from 2018 to 2019. 44

Figure 8: COI phylogenetic analysis using RaXML of the Chrysopini, Rhinomyzini and Philolichini tribes by Maximum likelihood (ML) alongside reference sequences obtained from GenBank. A total of 70 taxa were evaluated with a matrix length of 676 characters. The grey lines indicate the separation between the three tribes as well as between the subgenera within the Philolichini (Subgenera abbreviations: *Phi*.: *Philoliche*; *Omm*.: *Ommatiosteres*; *Ste*.: *Stenophara*; *Sub*.: *Subpangonia*; *Dor*.: *Dorcaloemus*; *Ret*.: *Returneria*; *Pha*.: *Phara*; *Bup*.: *Buplex*). Red dots indicate new sequences obtained during this study; purple arrows are discussed in text..... 45

Figure 9: Sequential images of *Chrysops obliquefasciata* from top left going clockwise: dorsal, facial, enlarged lateral and complete lateral views. 46

Figure 10: Distribution map representing locations at which *Chrysops obliquefasciata* (red dots) has been collected in South Africa (n= 101). 47

Figure 11: Sequential images of *Philoliche suavis* from top left going clockwise: lateral, dorsal, ventral and facial views. 49

Figure 12: Distribution map representing locations at which *Philoliche suavis* (red dots) has been collected in South Africa (n= 60). 50

Figure 13: Sequential images of *Philoliche medialis* from top left going clockwise: dorsal, ventral, facial and lateral views..... 52

Figure 14: Distribution map representing locations at which *Philoliche medialis* (red dots) has been collected in South Africa (n= 5). 53
Figure 15: Sequential images of *Philoliche rondani* from top left going clockwise: dorsal, facial, lateral and ventral views. 55
Figure 16: Distribution map representing locations at which *Philoliche rondani* (red dots) has been collected in South Africa (n= 218). 56
Figure 17: COI phylogenetic analysis using RaXML of the Haematopini tribe by Maximum likelihood (ML) alongside reference sequences obtained from GenBank. In total 78 taxa, with a matrix length of 658 characters, was analysed. Red dots indicate new sequences obtained during this study; blue dots are reference sequences of species that occur in the Afrotropics. 58
Figure 18: Sequential images of *Haematopota daveyi* from top left going clockwise: ventral, lateral, facial and dorsal views. 59
Figure 19: Distribution map representing locations at which *Haematopota daveyi* (red dots) has been collected in South Africa (n= 3). 60
Figure 20: Sequential images of *Haematopota decora* from top left going clockwise: facial, lateral, dorsal and ventral views. 62
Figure 21: Distribution map representing locations at which *Haematopota decora* (red dots) has been collected in South Africa (n=27). 63
Figure 22: Sequential images of *Haematopota vittata* from top left going clockwise: facial, lateral, dorsal and ventral views. 65
Figure 23: Distribution map representing locations at which *Haematopota vitatta* (red dots) has been collected in South Africa (n=26). 66
Figure 24: COI phylogenetic analysis using RaXML of the Tabanini tribe by Maximum likelihood (ML) alongside reference sequences obtained from GenBank. In total 116 taxa, with a matrix length of 662 characters were analysed. The GTR + G model was used, with a random starting tree and using the autoMRE bootstopping function for bootstrap support calculations. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Red dots indicate new sequences obtained during this study; purple arrows indicate incongruencies observed. 68
Figure 25: Sequential images of *Atylotus agrestis* from top left going clockwise: dorsal, lateral, ventral and facial views. 69
Figure 26: Distribution map representing locations at which *Atylotus agrestis* (red dots) has been collected in South Africa (n=54). 70
Figure 27: COI Phylogenetic analysis of the *Atylotus* genus by Maximum Likelihood using RaXML alongside reference sequences obtained from GenBank. In total 35 taxa, each with 658 characters

were evaluated. The GTR + Gamma model was used with a random starting tree and using the autoMRE bootstopping function for bootstrap support calculations. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. 72
Figure 28: Sequential images of *Tabanus atrimanus* from top left going clockwise: dorsal, ventral, facial and lateral views..... 73
Figure 29: Distribution map representing locations at which *Tabanus atrimanus* (red dots) has been collected in South Africa (n=34)..... 74
Figure 30: Sequential images of *Tabanus gratus* from top left going clockwise: lateral, facial, ventral and dorsal views. 76
Figure 31: Distribution map representing locations at which *Tabanus gratus* (red dots) has been collected in South Africa (n=109)..... 77
Figure 32: Sequential images of Tabanus minuscularius from top left going clockwise: dorsal, ventral, facial, abdominal and lateral views..... 79
Figure 33: Distribution map representing locations at which *Tabanus minuscularius* (red dots) has been collected in South Africa (n=638)..... 80
Figure 34: Sequential images of *Tabanus par* from top left going clockwise: dorsal, ventral, facial and lateral views. 82
Figure 35: Distribution map representing locations at which *Tabanus par* (red dots) has been collected in South Africa (n=40)..... 83
Figure 36: Sequential images of *Tabanus pullulus* from top left going clockwise: lateral, facial, ventral and dorsal views. 85
Figure 37: Distribution map representing locations at which *Tabanus pullulus* (red dots) has been collected in South Africa (n=109)..... 86
Figure 38: Sequential images of *Tabanus sericiventris* from top left going clockwise: lateral, dorsal, ventral and facial views..... 87
Figure 39: Distribution map representing locations at which *Tabanus sericiventris* (red dots) has been collected in South Africa (n=23)..... 88
Figure 40: Sequential images of *Tabanus taeniola* from top left going clockwise: lateral, facial, ventral and dorsal views. 91
Figure 41: Distribution map representing locations at which *Tabanus taeniola* (red dots) has been collected in South Africa (n=79)..... 92
Figure 42: Gel image, where lane 1 and 13 contains 100bp Ladders, lane 11 contained the negative control, lane 12 contains the positive control and lane 3 is pool 2 that tested positive..... 104

Figure 43: Individual samples that constituted pool 2. All of which tested negative. Lane 1 and 10 contained the 100bp ladder, 8 contained the negative control and 9 contains the positive control. 104

List of Tables

Table 1: Comparison of four genomic DNA extraction methods based on the time per extraction and cost per extraction. 28

Table 2: Comparison of four genomic DNA extraction methods using three spectrophotometer measurements (ng/uL, 260/280 nm and 260/230 nm); standard errors of marginal means are aindicated in parentheses. 30

Table 3: Locations and site names of sampling area within Kruger National Park, including the dates and type of traps used for the collections. 39

Table 4: Primer information for this study, including: target region, primer name, length, sequence, expected product size and reference. 42

Table 5: Summarized information on the Besnoitia besnoiti primers used for amplification including: primer names, length, sequence, target size and reference. 103

List of abbreviations

AARS	Aminoacyl-tRNA Synthetases
AATS/ AlaRS	Alanyl-tRNA-Synthetase
ATC	Aspartate Transcarbamylase
Bup.	*Buplex*
CAD	Carbamoyl-phosphate synthase 2, Aspartate transcarbamylase, and Dihydroorotase
COI	Cytochrome Oxidase I
CPS	Carbamoylphosphate Synthase
CsCl	Cesium Chloride
DHO	Dihydroorotase
Dor.	*Dorcaloemus*
DVTD	Department of Veterinary Tropical Diseases
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELIZA	Enzyme-linked Immunosorbent Assay
EtBr	Ethidium Bromide
ETS	External Transcriber Spacer
GPS	Global Positioning System
HE	Hematoxylin and Eosin
HHWRS	Hans Hoheisen Wildlife Research Station
IFAT	Indirect Fluorescence Antibody Test
ITS	Internal Transcriber Spacer
KNP	Kruger National Park
Omm.	*Ommatiosteres*
PCI	Phenol Chloroform Isoamyl
PCI-SA	Phenol Chloroform Isoamyl Sodium Acetate
PCR	Polymerase Chain Reaction
Pha.	*Phara*
Phi.	*Philoliche*
rDNA	ribosomal DNA
Ret.	*Returneria*
SA	South Africa
SAWC	South African Wildlife Collage

SDS	Sodium Dodecyl Sulfate
Ste.	*Stenophara*
Sub.	*Subpangonia*
TAE	Tris-acetate-EDTA
TEM	Transmission Electron Microscopy
Tris-HCl	Tris hydrochloride
tRNA	transcriber RNA
US$	American Dollars
ZAR/R	South African Rands

Abundance, composition and barcoding of Tabanidae in Kruger National Park and screening for *Besnoitia besnoiti*

by Andeliza Smit (14023190)

Supervisor: Prof. Luis Neves Co-Supervisor: Dr. Louwrens Snyman

Degree in Masters of Veterinary Science Tropical Diseases

Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences

Abstract

Tabanidae (Diptera) is a diverse haematophagous fly family, known to transmit over 35 livestock pathogens both mechanically and biologically. Kruger National Park (KNP) (in Limpopo and Mpumalanga provinces), South Africa, has a high diversity of tabanids, representing 26 of the total indigenous species (n=213) present in South Africa. Little modern taxonomical work has been done on tabanids within South Africa despite their medical, veterinary and environmental importance. This study aimed to determine a cost-effective DNA extraction method as well as comparing the traditional alpha-taxonomic approach to species delimitation with molecular methods using two gene regions, mitochondrial cytochrome oxidase I (COI) and nuclear alanyl-tRNA-synthetase (AATS) of tabanids collected in KNP. Furthermore, the study aimed to elucidate the role of tabanids in the transmission of *Besnoitia besnoiti* as this pathogen has found to ciculate in impala (*Aepyceros melampus*) and blue wildebeest (*Connochaetes taurinus*). A comparative study on DNA extraction methods were conducted, of which the most effective method was selected for DNA extractions. Tabanids were captured in three locations within KNP. The flies were morphologically identified then homogenized. DNA was pooled for the *B. besnoiti* screening, followed by sequencing and phylogenetic analysis. In total, 856 flies were captured belonging to 16 species under five genera. The COI barcode indicated that some species are genetically uniform while others formed co-occurring haplotypes. This study found that COI alone was not adequate in distinguishing between all the species of the South African Tabanidae. It is apparent that the classification of Tabanidae should be placed under scrutiny. A larger sample size, especially with regards to the *Tabanus* genus, or the use of several markers will aid in clarifying their relationships. No *B. besnoiti* positives were detected in the screened tabanids. Furthermore, in-depth research should also be conducted in other regions of South Africa; not only on tabanid ecology and composition but their role as pathogen vectors.

Key words: Tabanids, Afrotropical, Phylogenetics, Classification

Literature Review

Introduction

Tabanidae, more commonly referred to as horseflies, deerflies or clegs, is thought to be the most diverse haematophagous arthropod family (Guimaraes *et al.*, 2017). The family is represented by more than 4400 species with a high abundance and diversity in sub-tropical regions (McKeever and French, 1997, Guimaraes *et al.*, 2017). This family has several cryptic species, with the main form of identification still being heavily reliant on morphological features (Banerjee *et al.*, 2015).

Morphological identification, however, has several pitfalls which may lead to incorrect identification of species (Hebert *et al.*, 2003). A solution to this is the use of molecular barcoding to identify various species. Several gene regions are available for such barcoding, ranging from mitochondrial and ribosomal genes to nuclear genes. Several drawbacks in widely used genes, such as the protein-coding mitochondrial marker, cytochrome oxidase 1 (COI) gene, and the 28S rDNA region, has led to the search of new markers, including Alanyl-tRNA synthetase (AATS) and carbamoyl-phosphate synthase 2, aspartate transcarbamylase, and dihydroorotase (CAD) (Meiklejohn *et al.*, 2013). Another way to resolve some of the pitfalls is the use of several different makers when barcoding (Meiklejohn *et al.*, 2013). However, for successful barcoding, high quantity and quality of DNA, is required.

Through the years several different DNA extraction methods have risen, each with advantages and disadvantages. With the wide selection available, choosing a suitable method has become a crucial step in molecular research. Decisions are based on optimizing DNA yield, minimize DNA degradation, and the efficiency in terms of cost, time, labour, and consumables (Chen *et al.*, 2010).

Tabanids occur worldwide in a variety of habitats which range from water-rich areas such as salt marches and rainforests to oases in deserts (McKeever and French, 1997). Tabanids adults are generally known to be diurnal feeders, however, some species have crepuscular or nocturnal behaviours (Burnett and Hays, 1977, Krcmar *et al.*, 2005). Tabanid males are solely reliant on carbohydrate feeding, whereas tabanid females feed either on carbohydrates or, during reproduction, on a blood meal. Blood meals can be obtained from a great diversity of animals including humans, domestic and wild animals; as well as amphibians, reptiles, and birds (Kniepert, 1980).

Horseflies are renowned globally for their persistent and painful biting behaviour and are subsequently effective vectors of pathogens. They are known to transmit at least 35 pathogens either mechanically or biologically to livestock (Mullen and Durden, 2002, Al Dhafer *et al.*, 2009, Guimaraes *et al.*, 2017). One of these pathogens is *Besnoitia besnoiti*, an apicomplexan protozoan parasite, which is the cause of elephant skin disease in susceptible ruminants (Basson *et al.*, 1970). Currently, no definitive host for *B. besnoiti* is known, resulting in little knowledge about the lifecycle of the disease (Basson *et al.*, 1970, Cortes *et al.*, 2014). *B. besnoiti* results in great economic losses due to the damage to the hide of the cattle, a substantial decrease in general health and subsequent decrease in production-potential of the affected animal due to a loss of fertility in bulls (Dubey *et al.*, 2013).

Tabanidae

Systematics

The family Tabanidae (Diptera: Brachycera), forms part of the lower Brachycera and comprises more than 4400 species worldwide (Baldacchino *et al.*, 2014b). They are cosmopolitan (excluding Antarctica) and have great diversity and abundance in tropical areas (Sasaki, 2001). While the temperate fauna has been documented extensively, the tropical fauna has been less studied, with most studies from the region being taxonomic in nature (Mullen and Durden, 2002, Taioe *et al.*, 2017). All tabanids are divided into five subfamilies: Adersiinae, Scepsidinae, Chrysopsinae, Pangoniinae, and Tabaninae, based on the morphological characteristics (Mackerras, 1954).

The subfamily, Scepsidinae, includes only two genera, characterized by unique genitalia and reduced mouthparts (Mackerras, 1954, Dias, 1960, Service and Service, 1980). They have the unusual characteristic in which their proboscis had atrophied and are lacking mandibles, however, it is still functional (Dias, 1960). They only occur along coastal regions where the larval stages occur in sandy areas along the beaches (Dias, 1960). According to Service and Service (1980) this subfamily has no medical importance.

The subfamily Adersiinae, with only one genus *Adersia* is under great debate. Mackerras (1955) classified *Adersia* as part of the Chrysopsinae, whereas Dias (1960) believes that *Adersia* should be in its own subfamily (Turcatel, 2014). They were formerly classified with the Scepsidinae, since they share several morphological characteristics (Dias, 1960).

Pangoniinae comprise three tribes (Pangoniini, Philolochini, and Scionini) and is commonly known as long-tongue horse flies (Morita *et al.*, 2016). Pangoniinae is regarded as the most primitive subfamily, with several genera in need of scientific scrutiny (Mullen and Durden, 2002). They have an elongated proboscis which is mainly used for carbohydrate feeding in both sexes and they are thought to be important pollinators (Karolyi *et al.*, 2014).

The subfamily, Chrysopsinae, also referred to as deerflies, encompassed three tribes (Bouvieromyiini, Chrysopsini, and Rhinomyzini) (Morita *et al.*, 2016). Species of this subfamily are persistent feeders, and all have brightly, intricate coloured eyes (Turcatel, 2014).

Lastly, the most diverse subfamily, Tabaninae, comprises approximately 3200 species. Tabaninae are commonly known as horseflies, and are divided into three tribes (Diachlorini, Haematopotini, and Tabanini) (Turcatel, 2014, Morita *et al.*, 2016). The high diversity in Tabaninae and the presence of several cryptic species results in difficulty in identification and classification (Mackerras, 1954, Turcatel, 2014). In spite of Tabanidae being ubiquitous, their taxonomy has been difficult to clarify, until recently (Morita *et al.*, 2016). The greatest concern being that nearly a quarter of the tabanid diversity is delimited to a single genus, emphasising the need for systematic revision (Morita *et al.*, 2016).

Morita *et al.* (2016) aimed to provide evidence for monophyletic lineages by means of analysing multiple genes across a broad sample of Tabanidae diversity. This was to renew phylogenetic hypotheses as context for revisory taxonomic studies. Morita *et al.* (2016) found that the higher classification of Tabanomorpha is well supported, where monophyly for Tabanidae has also been confirmed. The higher classification within Tabanidae, however, has been shown to lack a supported evolutionary framework and thus have been placed under evaluation several times (Lessard *et al.*, 2013, Morita *et al.*, 2016). According to Morita *et al.* (2016), monophyly for the subfamilies Tabaninae and Pangoniinae are supported; whereas the subfamily Chrysopsinae are found to be paraphyletic, leaving the status and classification of Scepcidinae (including Adersinae which Morita grouped together as one subfamily), which was recovered within Chrysopsidae, in doubt. These uncertainties in some of the subfamilies are extended to the description and division of the majority of the tribes.

The Chrysopsinae tribe, Bouvieromyiini, was recovered as polyphyletic, while the tribe Rhinomyzini was supported as a monophyletic one, sister to Tabaninae (Morita *et al.*, 2016). Morita *et al.* (2016)

did find that Diachlonini (Tabaninae) can be separated into five major lineages. The Pangoniini and Tabanini are paraphyletic, however, support for this is very weak and require further investigation. The Chrysopsini was rendered paraphyletic by *Adersia* (Scepsidini) and Rhinomyzini. The only supported monophyletic lineages include the Haematopotini (Tabanini) and possibly the Philolochini (Pangoniinae) (Lessard *et al.*, 2013, Morita *et al.*, 2016). The larger genus, *Tabanus,* is not supported as a monophyletic group, where a split has been observed between *Tabanus* species from different habitual ecozones (Taioe *et al.*, 2017).

Morphology and lifecycle

Morphology

Head:

Tabanids have heads that are generally as wide as the mesonotum with large brilliant coloured eyes that are either dichoptic (separated) in females or holoptic (touching) in males (Axtell, 1976, Baldacchino *et al.*, 2014a, Chainey, 2017). The frons extends from the vertex (the top of the head) to the subcallus (the lower inner angle of the eye) (Baldacchino *et al.*, 2014a). This area has distinct patterns that are formed by one or more raise bare areas distinguished as the frontal calli that can be used for morphological identification (Oldroyd, 1952, Mullen and Durden, 2002). At the vertex, three distinct ocelli can be observed in both Pangoniinae and Chrysopinae, however, these ocelli are reduced or completely absent in Tabaninae (Mullen and Durden, 2002, Chainey, 2017).

The facial region, the area between the antennae and base of the proboscis, is divided into three segments: the facial lobe, parafacials and the buccae (Oldroyd, 1952). The facial lobe is the median area, consisting mainly of the clypeus (Chainey, 2017). The parafacials are the regions on the lateral areas which underlies the eyemargins. The buccae is divided from the parafacials by the foveal groove which anteriorly runs into the anterior tentorial pits (Oldroyd, 1952).

The antennae, which originates from the subcallus, plays a crucial role in identification at all levels (Oldroyd, 1952, Baldacchino *et al.*, 2014a). The antennae are divided into three sections: the scape, the pedicel, and the flagellum (Figure 1). The flagellum is the longest which can be further divided into three to eight flagellomeres or terminal segments (Chainey, 2017). In both Chrysopsinae and Heamatopini, the basal flagellum and scape is elongated, where the basal flagellum is also slender. In contrast, Tabanini has enlarged and flattened basal flagellum with a developed dorsal tooth, with the scape generally attenuated. The pedicel in both Tabanini and Haematopotini are attenuated, whereas the Chrysopsinae pedicel is elongated (Figure 1) (Oldroyd, 1952, Baldacchino *et al.*, 2014a).

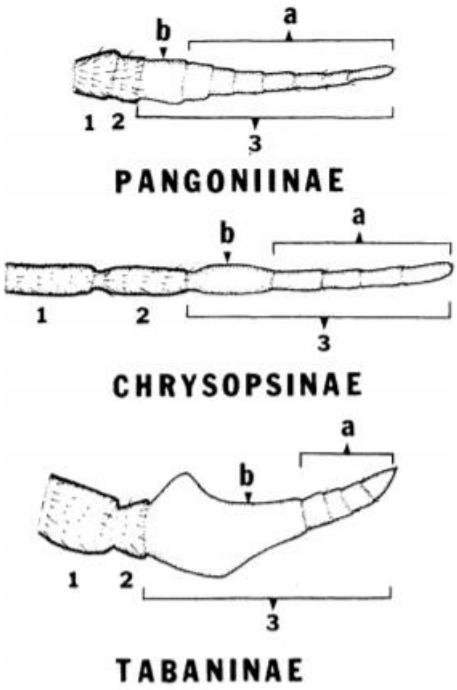

Figure 1: Basic structure of the antennae of the subfamilies of Tabanidae. 1= scape, 2= pedicel, 3= flagellum, a=flagellomeres b=base flagellum. Obtained from Cheng (1976).

The tabanid females have a proboscis that constitutes of six components: a labrum, a labium, a pair of toothed mandibles and a pair of maxillae. The females have a rigid hypopharynx and firm proboscis, where males lack mandibles, recurved teeth and have an inflexible hypopharynx (Cookson, 1967, McKeever and French, 1997, Müller *et al.*, 2011). The labium consists of a stalk with a pair of spongy lobes known as the labella. These labella have an abundance of open tubes known as pseudo-tracheae and, together, the labuim acts as a sheath for the other components (Oldroyd, 1952). The toothed mandibles and the pair of maxillae are adapted for piercing and are drawn out into stylet-like organs. During rest, the mandibles and maxillae lies in a dorsal groove within the labium, forming the walls of the food canal (Oldroyd, 1952).

Thorax:

Tabanid adults are stout-bodied flies (6–25 mm long). The thorax is unremarkable excluding the highly developed notopleural lobes. All Tabanidae have predominant notopleural lobes, which varies not only in the shape, but in the relation to the base of the wing as well (Oldroyd, 1952). These features, however, has not been incorporated into classification as of yet. Tabanids have large and prominent prothoracic and metathoracic spiracles, which displays structural differences within the family (Oldroyd, 1952). The thoracic pattern is useful in identification and consists of a variety of

components including: the median line, sublateral stripes, supra-alar stripes, median prescutellar patch and prescutellar crescents (Oldroyd, 1952). Some of these components may or may not be present in all species.

Wings:

Wings are useful in identification (Baldacchino *et al.*, 2014a). Colouration of the wings can vary from clear to a greyish or brownish tint to partially or completely infuscated (Oldroyd, 1952, Baldacchino *et al.*, 2014a). The presence or absence of the spur vein of R4 or the open-closed condition of the anal cell has always failed as an identification factor (Oldroyd, 1952). Wing shape and length, however, may be useful for identification alongside the colouration. In contrast to Tabaninae's broader and blunter wings; Pangoniinae has longer, pointier wings with costal margin undulation (Oldroyd, 1952). Genera such as *Chrysops* and *Ancala* have distinct darkened bands, while *Haematopota* has rosettes. The basicosta, bare or setulose, is another structure of the wing used for identification (Chainey, 2017).

Legs:

Legs differ little within Tabanidae, however, colouration, form, and patterning may be used for identification. The tarsi of all tabanids has pulvilliform empodium, hence each tarsi has three pulvilli (Chainey, 2017). All middle tibia has two apical spurs, however, the Pangoniinae and Chrysopsinae have apical spurs on the hind tibiae, which are absent in the Tabaninae (Oldroyd, 1952, Mullen and Durden, 2002, Baldacchino *et al.*, 2014a). On the surface of the fore tarsi some fine sensory hairs, known as touch hair, are located ventrally (Oldroyd, 1952). Apart from erected spicules on the fore tibia and long hair fringes on one or several legs, no other bristles can be found (Oldroyd, 1952, Chainey, 2017)

Abdomen:

The abdomen of tabanids is generally as broad as the thorax but is slightly compressed dorsoventrally or may be cylindrical (Oldroyd, 1952, Baldacchino *et al.*, 2014a). Usually a distinctive colour or pattern can be observed which is divided into two broad types: 1) a reddish or pale colour on the first two/three segments with the rest being darker; 2) all segments with a grey hind-margin, and with either one, two or three rows of grey triangles or spots that may or may not be fused into longitudinal stripes (Oldroyd, 1952). These colourations are used during identification (Baldacchino *et al.*, 2014a).

Biology and Lifecycle

Species of the family also exhibits sexual dimorphism in feeding habit; while both sexes of all adult tabanid species require carbohydrates to provide energy for general metabolic maintenance, mating, and flight, females require a blood meal to produce eggs (Herczeg *et al.*, 2014). Tabanids uses one suction organ, the proboscis, for both nectar intake and blood-feeding (Karolyi *et al.*, 2014).

The feeding style of the Pangoniinae, however, does differ from the remainder of the family. In general nectar feeding occurs during the sheathed position of the proboscis (mandibles and maxillae within the labium), however, during blood feeding the mandibles and maxillae are released from the dorsal groove. In most Tabanidae the mandibles and maxillae are as long as the labella, and piercing of the skin for blood meal intake occurs only when the labella is folded backwards (Oldroyd, 1952). The long-tongue horse flies (Pangoniinae) have the stalk of the labium that can be three to four times the length of the body, with labella comparably small (Oldroyd, 1952, Ren, 1998). These Pangoniinae have the unique ability where they adjust their proboscis for either nectar or blood feeding. During nectar feeding, the labium is inserted either straightforward or at a slight downward angle into the corolla (petals arranged circularly around the center of the plant) (Karolyi *et al.*, 2014). During blood feeding, only the mandibles and maxillary structures are used for skin piercing and blood uptake trough the epipharyngeal food canal. During this stage the labium has been folded backwards, diagonally to the main body axe (Karolyi *et al.*, 2014).

Sugars are mainly obtained from flowers or honeydew production by sap-sucking bugs (Hemiptera) (Kniepert, 1980). Tabanids are believed to have co-evolved with angiosperms and subsequent vertebrates since the Late Jurassic, roughly 180 million years ago, and are considered as amongst the first pollinators of flowering plants (McKeever and French, 1997, Ren, 1998). Within South Africa, tabanids are considered keystone species in pollination of floristically rich areas such as the Cape Floristic Region; where they are believed to be frequent visitors and pollinators of at least 170 plant species from various families (Karolyi *et al.*, 2014).

Since males do not produce eggs, they do not require blood meals and are solely reliant on carbohydrate feeding (McKeever and French, 1997). Most females require one large blood meal from a vertebrate host to produce a batch of eggs and are thus considered anautogenous (Mullen and Durden, 2002, Guimarães *et al.*, 2017). However, some females practice autogeny, where they are capable of laying one batch of eggs without a blood meal (Chainey, 2017). It is noted that in

some species, mainly of the Pangoniinae, both sexes require only carbohydrates for the completion of their lifecycle (Kniepert, 1980).

All tabanids have the same basic life history (Cheng, 1976, Baldacchino *et al.*, 2014a, Chainey, 2017). Eggs are laid in masses of 100 to 800 eggs (either in a single layer or in tiers) on vegetation close to niches in which the larvae develop (Baldacchino *et al.*, 2014a, Chainey, 2017). Development of the eggs occurs within two to 21 days; where the development and the amount of eggs laid is highly reliant on the species and the size of the blood meal that was taken (Mullen and Durden, 2002, Baldacchino *et al.*, 2014a). Eggs generally hatch within two to three days when temperatures are between 30-35℃ (McAlpine *et al.*, 1981, Mullen and Durden, 2002).

The larvae are cylindrical and vary in length, depending on the species (Chainey, 2017). The larvae can survive in several habitats; however, they are broadly divided into three morpho-ecological groups: (i) rheophilous and subrheophilous, (ii) hydrobionts and hemihydrobionts, (iii) and, edaphobionts (Baldacchino *et al.*, 2014a, Chainey, 2017). The former group develops within rivers and streams, the hydrobionts/hemihydrobionts inhabit stagnate or slow-moving water bodies, or even the banks to such water bodies. The latter group is generally found far from water bodies, underneath forest litter or within drier soil (Baldacchino *et al.*, 2014a). The first-instar larvae are armed with an “egg burster” or “egg tooth”, which is used to split the chorion (McAlpine *et al.*, 1981, Mullen and Durden, 2002).

Most tabanid larvae are predaceous where the mandibles are connected with a duct containing venom, used to subdue their prey (Chainey, 2017). They generally feed on other invertebrates and cannibalistic behaviours have been observed in captivity (Mullen and Durden, 2002, Chainey, 2017). Attacks on vertebrates have also been observed, ranging from frogs to humans (Kniepert, 1980). Tabanids generally undergo six to 13 larval moults. Most species are thought to be univoltine (have one generation per year), however, some are bi- or multivoltine (two or more generations per year) (Mullen and Durden, 2002). Large species of mainly Tabaninae, such as *Tabanus atratus,* can spend up to two or three years as larvae, all dependent on the environmental factors.

Pupation generally occurs underground within four to 21 days but are species and temperature-dependent (McAlpine *et al.*, 1981, Mullen and Durden, 2002, Chainey, 2017). The pupa is positioned vertically to enable movement to the surface for adult eclosion (Chainey, 2017). Males emerge

earlier compared to females, where the sex emergence ratio is 1:1 (Cheng, 1976, Baldacchino *et al.*, 2014a).

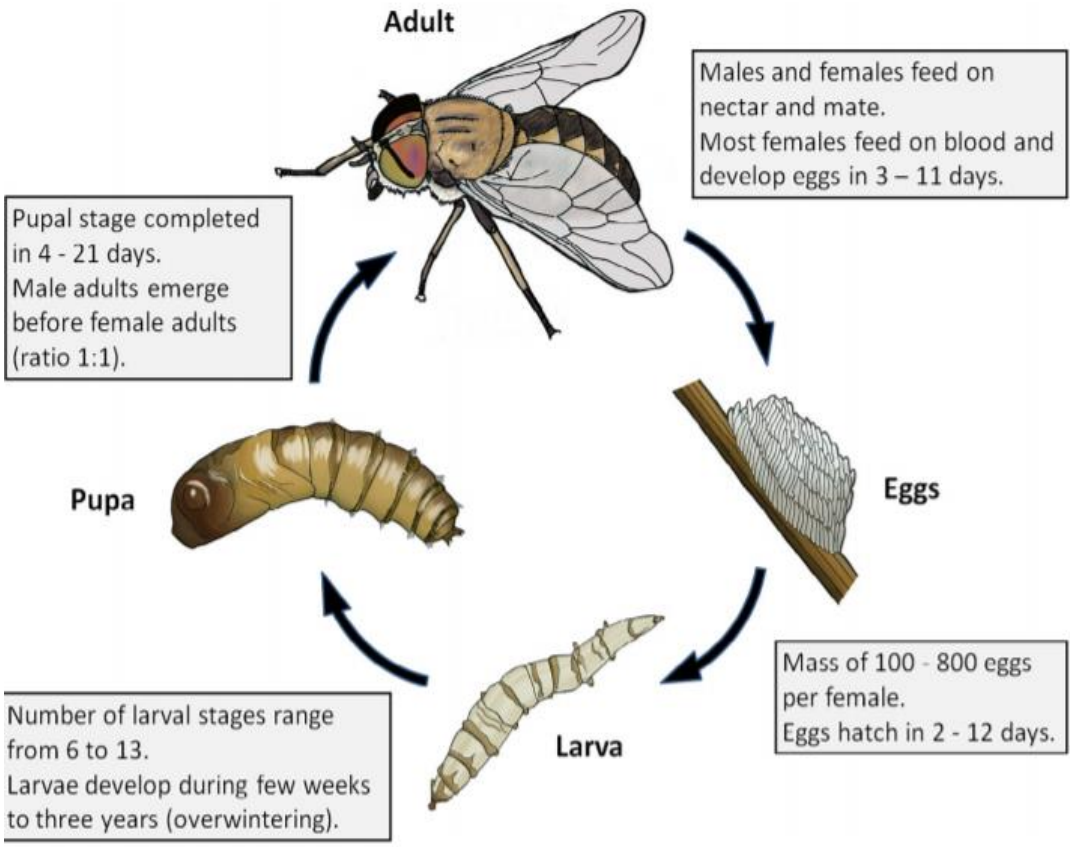

Figure 2*:* Summarised life cycle of Tabanidae obtained from Baldacchino *et al. (2014a)*.

Vectors of pathogens

Among the subfamilies, Chrysopsinae and Tabaninae are regarded as the most medically and veterinary important tabanids (Baldacchino *et al.*, 2014b). Tabanids are described as important vectors of pathogens, yet comparatively severely neglected in research (Baldacchino *et al.*, 2014a). Their bites are known to be painful and is capable of inducing allergic reactions (Herczeg *et al.*, 2015). Females are known to be persistent biters and can bite at rates that can exceed ten times per minute and results in roughly a 0.5mL blood lost per tabanid (Foil and Hogsette, 1994, Mullen and Durden, 2002).

In humans, the discomfort and annoyance of the bites have been shown to negatively affect outdoor recreational activities, field worker efficiency and even lower tourist attraction (Mullen and Durden,

2002, Al Dhafer *et al.*, 2009). Tabanids do however, transmit very few pathogens that are harmful to humans, of which the most important is the African eyeworm (*Loa loa*), causing loiasis and the bacterium *Francisella tularensis* which causes Tularemia (Krinsky, 1976, Chainey, 2017). Unfortunately, the population most affected with both these diseases are in developing countries in central to West Africa, where general access to health facilities are sometimes difficult.

Livestock suffers a greater deal under tabanid infestation. A dense tabanid population can be of a great economic burden to livestock farmers, especially farmers in developing countries. A direct attack on livestock may result in loss of weight, reduced milk production, hide damage, loss of appetite and reduced food consumption (Foil and Hogsette, 1994, Karolyi *et al.*, 2014). The punctures produced by tabanid feeding may lead to secondary hematophagy, provide sites for ovipositioning of myiasis causing dipterans as well as lead to secondary bacterial infections (Foil and Hogsette, 1994).

Tabanids are known mechanical and biological vectors of over 35 livestock pathogens including viruses, bacteria, protozoans, and helminths; all of which causes a range of diseases such as trypanosomosis, equine infectious anemia virus, anaplasmosis, anthrax, surra and, bovine besnoitiosis, in both wild and domesticated animals (Krinsky, 1976, Mullen and Durden, 2002, Hackenberger *et al.*, 2009, Baldacchino *et al.*, 2014b). Some of these pathogens has the potential of being zoonotic and include examples like *Trypanosoma* spp*., Bacillus anthracis* and *Dirofilaria roemeri* (Anthony, 1962, Anthony, 1977, Baldacchino *et al.*, 2014a). However, 17 of these pathogens have commercial vaccines available that provides at least some protection, while several other vaccines are currently in development. This, in turn, places limitations on small-scale farmers who do not have access to or cannot afford the vaccines and rely on their animals for their livelihood. Unfortunately, this results in incalculable economic losses due to tabanid activity with only unreliable or commercially biased data available. (Baldacchino *et al.*, 2014a).

Besnoitia besnoiti

The parasitic disease bovine besnoitiosis, commonly known as elephant skin disease, is one of the most problematic diseases believed to be transmitted by tabanids. Bovine besnoitiosis is caused by an obligate intracellular cyst-forming apicomplexan protozoan parasite which belongs to the *Besnoitia* genus (Bigalke, 1968, Ellis *et al.*, 2000, Ryan *et al.*, 2016). *Besnoitia* is classified as a member of the family Sarcocystidae and is included in the subfamily Toxoplasmatinae (Gutiérrez-Expósito *et al.*, 2016).

There are currently 10 *Besnoitia* species recorded of which most are found in marsupials and small mammals and cysts are presented in the viscerotropic form (internally) (Cortes *et al.*, 2014). The four *Besnoitia* species which infects ungulates and mainly present with external cysts are *B. besnoiti* (cattle), *B. tarandi* (deer), *B. bennetti* (equines) and *B. caprae* (goats) (Bigalke, 1968, Gutiérrez-Expósito *et al.*, 2016).

To date, no species of *Besnoitia* is believed to infect humans (Cortes *et al.*, 2014). For *B. besnoiti*, cattle are considered to be the intermediate host, however, the definitive host remains unknown (Diesing *et al.*, 1988, Ryan *et al.*, 2016). Due to the uncertainty of the definitive host, little of the life cycle and the routes of transmission of the pathogen are understood. Cattle-to-cattle transcutaneous contamination is believed to be the most common way of infection via hematophagous insects (Liénard *et al.*, 2011, Liénard *et al.*, 2013). In experimental trials, tachyzoites in blood or cutaneous bradyzoites have been successfully transmitted from an infected animal to a susceptible one. This was achieved, by transfusion of large volumes of infected bovine blood or through the mechanical transmission by biting flies such as tabanids and stable flies, where tabanids are found to be more efficient (Liénard *et al*., 2013).

Currently, little seasonal data is available for *B. besnoiti* however, studies by Liénard *et al.* (2013) suggests that the seasonality might be linked to that of the hematophagous insects. Bovine besnoitiosis is predominantly found in sub-Saharan Africa, the Arabian Peninsula and southern Europe (areas of Portugal, northern Spain and southern France), however *B. besnoiti* has spread geographically to countries such as Germany, Italy, Switzerland, Hungary, Belgium and Ireland, believed to be the cause of a geographical spread in the vectors (Ryan *et al.*, 2016, Gollnick *et al.*, 2018). Generally, mortality rates in endemic localities are less than 1% however, this may increase to 50% in animals which has mild clinical signs in endemically infected herds (Ryan *et al.*, 2016).

The course of the disease is well described. The disease has two different clinical stages and is considered a severe chronic infection. (Dubey *et al.*, 2013). The stages are known as the acute and chronic stage (Langenmayer *et al.*, 2015).

The acute stage occurs 11 to 13 days after infection and generally last only six to 10 days (Cortes *et al.*, 2014). This stage is characterized by the proliferation of tachyzoites within macrophages, fibroblasts and endothelial cells within the blood vessels (Dubey *et al.*, 2013) (Figure 2). The proliferation occurs via endodyogeny, where the replication rate of the tachyzoites varies between

0, 14 and 20 tachyzoites per hour depending on the type of cell lines in which it is present (Cortes *et al.*, 2014). This causes thrombosis and vasculitis, mainly in the capillaries and small veins within the dermis, subcutaneous tissue, testes and upper respiratory mucosae (Basson *et al.*, 1970).

The acute stage is rarely diagnosed due to animals not exhibiting all symptoms or in some instances, the animals appear to be asymptomatic (Liénard *et al.*, 2011). The reported clinical symptoms include fever (above 40℃), lethargy, pyrexia (accompanied by anorexia and photophobia), nasal and ocular discharge, increased heart and respiratory rates, subcutaneous oedema and necrosis of the epidermis (Basson *et al.*, 1970, Schares *et al.*, 2011a, Dubey *et al.*, 2013). During this acute stage, pregnant animals may abort, and cysts start to develop (Basson *et al.*, 1970, Cortes *et al.*, 2014).

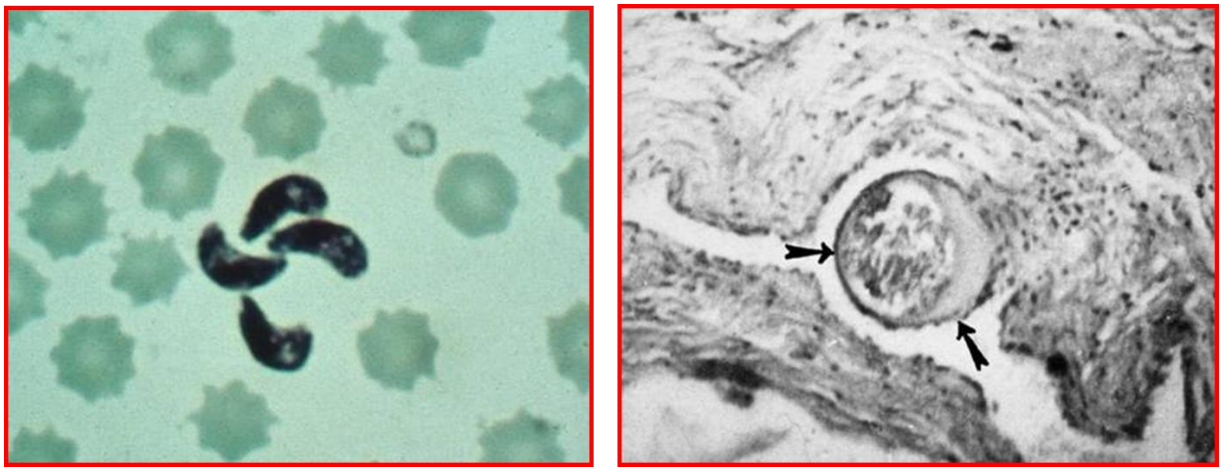

Figure 3: *Besnoitia besnoiti* acute stage indicating merozoites (left); merogony in the endothelium (right).

The chronic stages consist of only large (0.4 mm) tissue cysts containing bradyzoites, which persists for extended periods (Cortes *et al.*, 2014)(Figure 3 and 4). These cysts are characterized by two distinct cyst walls and generally contains hundreds of the parasite within (Cortes *et al.*, 2014). It has been found that besnoitiosis cannot reactivate from the chronic stage to the acute stage (Dubey *et al.*, 2013). Other signs observed include scleroderma in the hind limbs resulting in difficult and painful movement, severe anorexia and weight loss, dermal lesions and some bulls which develop orchitis resulting in temporary or permanent infertility (Schares *et al.*, 2011a, Dubey *et al.*, 2013). Skeletal muscles, tendons, the periosteum of the limbs, the testicular parenchyma and the upper respiratory mucosae is believed to be extensively involved during the chronic stage (Basson *et al.*, 1970). Scleroderma, alopecia and infertility are permanent disfigurements in surviving animals (Bigalke, 1968, Basson *et al.*, 1970, Bigalke, 1981, Diesing *et al.*, 1988).

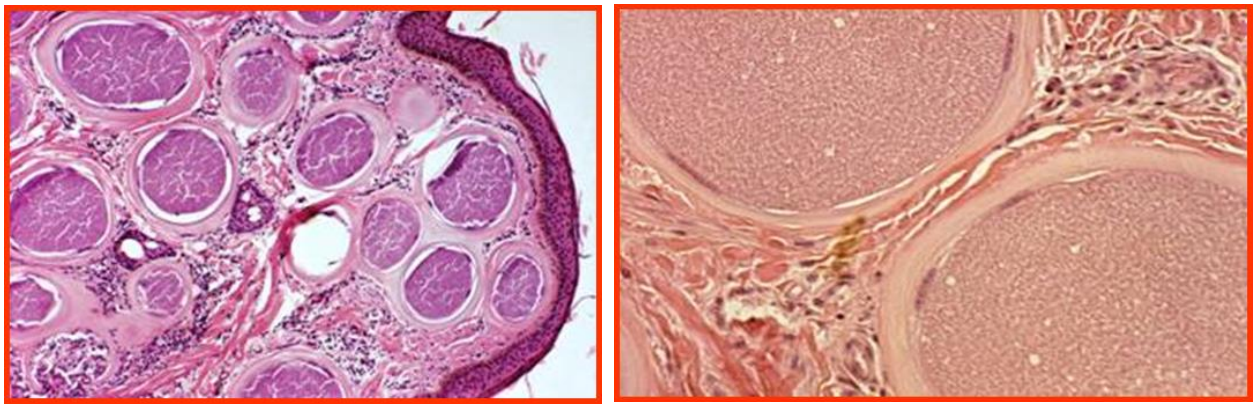

Figure 4: Cysts with bradyzoites in the endothelium (Left); Collagen deposited around the histiocyte (Right).

Both stages of bovine besnoitiosis infect cattle of all ages, gender and breeds, causing great economic loss. This is due to several factors such as weight loss in the herd, abortions, infertility of cows and sterility of bulls, decrease in milk production while the quality and hide value is substantially reduced (Ellis *et al.*, 2000, Ryan *et al.*, 2016).

Though several clinical signs are well described for *B. besnoita*, the symptoms are common for several different diseases leading to the miss diagnoses and confusion with other etiological agents, such as fungi or viruses (Cortes *et al.*, 2014).

Currently, there is no golden standard method for diagnosis of *B. besnoitia.* The mainstay methods for diagnosis can be divided into direct and indirect methods (García-Lunar *et al.*, 2013). Direct methods include histopathology, polymerase chain reaction (PCR) and the use of transmission electron microscopy (TEM). Histopathology is performed via hematoxylin and eosin (HE)–stained histological sections obtained from chronically infected cattle (Langenmayer *et al.*, 2015). In general, a "biopsy punch" of 8mm diameter is sufficient and should be obtained from cysts (Dubey *et al.*, 2013, Cortes *et al.*, 2014). When a densely infected skin biopsy is obtained, tissue cysts and bradyzoites can be visualized by TEM (Figure 4). However, histopathological diagnosis in animals that are still in the acute stage is difficult and no anti-*Besnoitia besnoiti* antibodies are present and thus cannot be detected by serology so more sensitive methods such as PCR is preferred (Cortes *et al.*, 2007, Cortes *et al.*, 2014). A positive result on PCR does not assure an active infection with an alive and infectious parasite, just merely that the DNA is present. False PCR negatives can also be observed when the infected animal is still very early in the acute stage and *B. besnoiti* DNA is present at sub-detection levels (Schares *et al.*, 2011b, Cortes *et al.*, 2014).

Indirect methods include serological methods. Serological methods are only applicable when anti-*Besnoitia besnoiti* antibodies are present in the subclinical and clinical disease. Indirect fluorescence antibody test (IFAT) and Western blot are both used in the diagnosis of *B. besnoiti*; however, IFAT is preferred since its more cost and time efficient. Enzyme-linked immunosorbent assay (ELISA) is used when a large number of samples are present and currently a commercialised ELISA kit is available (Cortes *et al.*, 2014). The surface antigens (39 and 42 kDa) used in ELISA has proven to have 100% sensitivity for sera of chronically infected animals and had a specificity of 99.8% in cattle from herds with *Neospora caninum*-associated abortions (Schares *et al.*, 2013). Schares *et al.* (2013), however, did find that the use of real-time PCR was still superior to serological tests in detecting positives at earlier stages of the infection.

Even though identification of the pathogen is possible, several treatments have been investigated with little positive results (Shkap *et al.*, 1987). Gerbils and rabbits are both effective *in vivo* models for *B. besnoiti* infection treatments. Shkap *et al.* (1987) testes several drugs including oxytetracycline, sulfonamides, trimethoprim, halofuginone, diminazene aceturate and pentamidine, of which only oxytetracycline prevented the death of gerbils and only when it was administered at the time of infection. New studies include studies about arylimidamides (pentamidine derivatives), which has been observed to have profound effects against *B. besnoiti* in Vero cells; the animal test is yet to be performed (Cortes *et al.*, 2011). Since no viable treatment is available, other methods to prevent the disease from spreading was investigated.

In South Africa, a live vaccine was used to prevent the spread of the disease (Cortes *et al.*, 2014). The live vaccine was produced from tachyzoites obtained an isolate from blue wildebeest that was grown in cell cultures (Bigalke *et al.*, 1974). These strains have been proven to be of low virulence in cattle and produce immunity when used to challenge cattle and rabbits (Bigalke *et al.*, 1974). The vaccine protected against clinical disease but was not as effective against sub-clinical infections (Cortes *et al.*, 2014). The vaccines had its limitations; not only was it geographically limited, but the live vaccine also posed a threat of introduction of the parasite to uninfected herds and areas and may result in carriers in vaccinated animals (Cortes *et al.*, 2014). Currently studies are conducted on the possibility of developing sub-unit vaccines; however, this attempt is hindered by the incomplete knowledge available on the molecular aspects of the parasite and by the absence of a good experimental animal model (Cortes *et al.*, 2014, Frey *et al.*, 2016).

DNA extraction methodologies

With the development of PCR and its use thereof in molecular studies; an important aspect is to extract the highest quality and quantity of DNA possible from the specimens available (Dillon *et al.*, 1996). The extraction method used and the preservation technique of the samples both play a vital role in the quality and quantity of the DNA extracted (Psifidi *et al.*, 2010). Several variables have been shown to reduce the quality of DNA extracted from specimens, including: the age of the specimen, the temperature in which the specimen is store and whether it was preserved in alcohol and the concentration thereof (Dillon *et al.*, 1996). Historically, DNA was extracted from a variety of biological samples using techniques which included separation and purification steps, with the utilization of phenol-chloroform extractions and/or ethanol precipitation (Walsh *et al.*, 2013). Inorganic extraction techniques commonly include the use of high concentrations of salt, a surplus of proteinase K digestion and the excessive use of glass powder (Walsh *et al.*, 2013).

The very first DNA isolation was performed by a Swiss physician, Friedrich Miescher in 1869 (Dahm, 2005). Miescher isolated leukocytes which he washed with fresh solutions of diluted (1:1000) hydrochloric acid, after which he shacked the nuclei in a mixture of water and ether, resulting in clean nuclei drifting in the water phase which he filtered out. The clean nuclei were subsequently extracted with an alkaline solution. Miescher added highly diluted (1:100,000) sodium carbonate to the nuclei, after which he noticed a "yellow solution of a substance" from these nuclei which he isolated. Miescher then added acetic acid or hydrochloric acid in excess to this isolated yellow solution which resulted in an insoluble, flocculent precipitate (DNA) (Dahm, 2005). He later refined this first protocol by including pepsin to remove proteins which were still present in the cleaned precipitate (Dahm, 2005). After Miescher's DNA extraction protocol was accepted, several different strategies were investigated and used in routine laboratories. Such strategies included density gradient centrifugation and differences in solubility of large chromosomal DNA, plasmids, and proteins in an alkaline buffer (Tan and Yiap, 2009).

Currently, three main categories of DNA extraction methods are known, including: Conventional extractions, Solid-phase Nucleic Acid Extractions and All-in-One Biomolecules Extractions Methods (Tan and Yiap, 2009). All these methods were discovered within the 1900's and will be listed as per group and not in chronological order. The Ethidium Bromide (EtBr)-Cesium Chloride (CsCl) Gradient Centrifugation method that was developed in 1970 (Kit *et al.*, 1970). This method is highly complicated and expensive and requires a large amount of sample for extraction. This was followed by the Alkaline Extraction Methods which are commonly used for plasmid DNA extraction. This

method was first described in 1979, which is reliant on Sodium Dodecyl Sulfate (SDS) which removes all contaminants (Bimboim and Doly, 1979). Subsequently, one of the most commonly used conventional extraction methods was developed in 1987 and is known as the Guanidinium Thiocyanate-Phenol-Chloroform extraction method. This method is reliant on a specific mixture of phenol, chloroform and isoamyl alcohol which removes all proteins, lipids and desalts the nucleic acid; after which DNA is precipitated with ethanol (Tan and Yiap, 2009).

Solid-phase nucleic acid extraction methods allow for quick and efficient purification compared to conventional methods (Tan and Yiap, 2009). Most of the commercial extraction kits use this form of extraction since it avoids the problem of incomplete phase separation which is found in conventional methods. Solid-phase nucleic acid extraction methods include one of the first deviations of Miescher protocol known as the Diatomaceous Earth method. Diatomaceous Earth was added to the filters to remove impurities and was developed in 1968 (Sutcliffe and Sharp, 1968). Several deviations followed, such as the use of glass particles developed in 1980 to extract DNA from gels (Smith, 1980), silica matrices that were developed in 1987 (Lorenz and Wackernagel, 1987), and Magnetic Bead Based Nucleic Acid Purifications which is the use of magnetic beads and a current to purify nucleic acid was developed in 1989 (Hultman *et al.*, 1989).

All-in-One Biomolecules Extraction, extract DNA, RNA and proteins from the same sample in a progressive manner. This group of extraction methods constitute the remaining automated kits that combine solid-phase extractions with protein extraction methods.

All these methods do produce high concentrations of extracted DNA, however, all of them requires several steps which allow for increased opportunities for cross-transfer of samples or introduction of contaminants (Walsh *et al.*, 2013). Manual methods for DNA extraction are simple and reliable; however, they are mostly time-consuming and more suitable for a lower number of samples. With the increase of samples and specimens that needs to be extracted, automated methods of DNA extractions have been developed and is available in the form of extraction kits (Smith *et al.*, 2003). These kits, however, are very expensive and generally requires expensive and specific equipment for the completion of the procedure (Tan and Yiap, 2009). With the increasing amounts of extraction methods being developed, a never-ending debate exists in which methods are comparable more efficient with regards to optimize DNA yield, minimize DNA degradation, while considering efficiency in terms of cost, time, labour, and consumables (Chen *et al.*, 2010).

Molecular markers for identification and phylogenetic inference

The first and most fundamental step required in vector-diseases association, is accurate identification of the vector (Banerjee *et al.*, 2015). Identification of Tabanidae is currently mostly reliant on morphological keys (Banerjee *et al.*, 2015). However, identification keys may lead to many misdiagnoses, even when used by experts. These mistakes are attributed to the differences in intraspecific and/or regional morphological clines, morphologically cryptic taxa which often is excluded or vaguely discussed, as well as the use of difficult to find life stages and/or gender-specific characteristics, including dissection of genitalia (Hebert *et al.*, 2003, Banerjee *et al.*, 2015).

The use of DNA barcoding is becoming increasingly common and has successfully been utilised in several taxa, including tabanids (Banerjee *et al.*, 2015). For accurate identification, genetic databases with comparative sequences are fundamental. According to Waugh (2007): "a DNA barcode is a short sequence of nucleotides taken from an appropriate part of an organism's genome that is used to identify it at species level". Increased studies on suitable genetic markers for several gene regions, including the mitochondrial genome, may yield in phylogenetic information suitable for identification at different taxonomic levels (Lunt *et al.*, 1996). Historically, two main sections of the genome were used for barcoding; the mitochondrial genome and the nuclear ribosomal DNA.

The mitochondrial genome is the target for analysis, mainly because this genome lacks introns, has limited exposure to recombination and its mode of inheritance is haploid, thereby simplifying analysis (Hebert *et al.*, 2003). The gene region generally accepted for species-level barcoding is the protein-coding mitochondrial gene, cytochrome oxidase I (COI). The COI gene is the largest of the mitochondrion encoded cytochrome oxidase subunits and acts as the terminal catalyst of the mitochondrion respiratory chain (Sone *et al.*, 1988, Lunt *et al.*, 1996). It aids in electron transport and associated proton translocation across the mitochondrial membrane. The COI gene contains several different types of functional domains (Ferguson-Miller *et al.*, 1978, Saraste, 1990).

Within the domain's reaction centres, there are several highly conserved amino acids, however, these regions do not constitute the whole domain (Ferguson-Miller *et al.*, 1978). There are several regions which are highly variable; the combination of highly variable and conserved regions in the same gene allows for it to be valuable in evolutionary distinction (Saraste, 1990, Lunt *et al.*, 1996). The amount of knowledge available on the COI gene is substantial due to many studies on this section. The consistency in the size and structure of the gene region in all life forms are the key

characteristics for the use of this region in the modern evolutionary characterization (Lunt *et al.*, 1996).

COI is most commonly used in DNA barcoding initiatives and is proficient in identifying and/or distinguishing between the known and unknown species (Li *et al.*, 2010). Several complications are, however, recognised with the use of mitochondrial genes including single linkage group, lineage sorting and insects which exhibit extreme A/T bias (Moulton and Wiegmann, 2004). Another complication with using the COI gene is that consensus barcodes are shared by several species, thus, complicating studies on molecular evolution (Meier *et al.*, 2006). COI has also been found to be inefficient when used alone, in cases where rapid speciation occurs in taxa, due to little interspecific COI divergence (Waugh, 2007).

Another region of interest for barcoding is the ribosomal DNA (rDNA). Nuclear rDNA occurs in multiple copies, organized in tandem repeats which are separated by non-transcribed spacers. Each nuclear rDNA repeat is composed of three genes (18S, 5.8S and 28S) and two internal (ITS1 and ITS2) and one external (ETS) spacer (Zardoya and Meyer, 1996). The two internal spacers are generally used for low-level identification such as for species and sub-species, whereas the 18S and 28S genes are used in assessing deeper evolutionary relationships (Schultz *et al.*, 2005). Each of the genes contains several divergent domains which are subjected to insertions and deletions accounting for variability. These divergent domains are, however, separated by conserved cores which are readily subjected to substitutions, rather than deletions or insertions, and are thus evolving at lower rates (Zardoya and Meyer, 1996), making sections of these gene regions suitable for barcoding.

When considering phylogenetic analyses on rDNA genes, the 18S rDNA gene (the small subunit) is generally used due to its slower evolving nature (Zardoya and Meyer, 1996). The 18S rDNA gene, however, is highly variable in length between genera and provides difficulty when aligning sequences. The 28S rDNA gene (large subunit) is nearly double the length of the 18S rDNA gene, however, struggles with the same form of size variation between different taxa (Wang *et al.*, 2013). The 28S rDNA gene contains more and longer variable regions as compared to the 18S gene, allowing it to divulge somewhat deeper relationship than that of the 18S gene (Wang *et al.*, 2013).

Uniquely the 28S rDNA gene in insects and other protostomes dissociates into two subunits (α and β) of equal size, due to the cleavage of a hydrogen bond when places under denaturing conditions (Fujiwara and Ishikawa, 1986). Some of the pitfalls in using the 28S rDNA gene for phylogenetic

analysis includes hindered attempts to amplify the gene by the hairpin structures that form due to the presence of tandem repeats and a direct correlation to a time scale cannot be suggested (Ludwig and Schleifer, 2005, Wang *et al.*, 2013).

Mitochondrial rDNA constitute of two gene ribosomal regions (12S and 16S), 13 essential oxidative phosphorylation subunit proteins/polypeptides and 22 tRNAs that are required for mitochondrial protein synthesis (Gillespie *et al.*, 2006, Yang *et al.*, 2014). The mitochondrial rDNA evolves at a faster rate than that of their nuclear homologs, thus it is more readily used for genus to population level identification (Medina and Walsh, 2000). These genes, however, share the same problem as that of the COI, where the genes may have a A/T bias and high rates of substitution (Gillespie *et al.*, 2006).

The complications for the aforementioned markers have led to the introduction of new makers; of which aminoacyl-tRNA synthetases (AARS) is widely used. Aminoacyl-tRNA synthetases are a family of enzymes which are responsible for the covalent bonding of amino acids to their respective tRNAs (Schimmel, 1993, Rajendran *et al.*, 2018). Aminoacyl-tRNA synthetases have several domains for several different functions, including: aminoacylation domain, tRNA binding, oligomerization, and amino acid proofreading. Aminoacylation occurs in three regions: the nucleus, cytoplasm and the mitochondria (Schimmel, 1993, Guiliani *et al.*, 1997). There are currently two classes of AARSs divided based on their aminoacylation domains: Class I, with five subclasses and Class II, with three subclasses.

Class I enzymes share the conserved HIGH and KMSKS motif regions in the active site of the Rossmann fold (Rajendran *et al.*, 2018). These Class I AARSs are generally monomeric excluding the rare few that are dimeric. With the use of their open pocket binding site, they are responsible for the charge of larger, bulkier amino acids (Guiliani *et al.*, 1997). Class II AARSs evolved early and are highly conserved, sharing three degenerate motifs in their active binding site. The first two subclasses of Class II (IIa and IIb) are dimeric, whereas subclass IIc is tetrameric (Guiliani *et al.*, 1997).

Alanyl-tRNA synthetase (AlaRS) resides within the IIc subclass, thus contains four domains. These domains include the N-terminal aminoacylation domain, the tRNA-recognition domain, the editing domain, and the C-terminal oligomerization domain (Schimmel, 1993). AlaRS is unique in that it has a G3: U70 wobble base pair in the acceptor stem, dictating the tRNA identity to AlaRS (Schimmel, 1993, Naganuma *et al.*, 2009). This wobble base pair allows AlaRS to aminoacylate small, isolated

portions of tRNA. With the proofreading ability of AlaRS, it is crucial in accurate translocation proses, and if not functional, leads to cell death (Rajendran *et al.*, 2018). AlaRS is used in barcoding since it is believed to have co-evolved with tRNA since the beginning of life, it has no misaminoacylation, it has high levels of conserved and variable regions and is present in all organisms (Schimmel, 1993, Rajendran *et al.*, 2018). AlaRS in different species are more similar in structure than to any other AARSs in the same organism, allowing it to differentiate between closely related species (Rajendran *et al.*, 2018).

Another marker, only recently introduced, is the CAD gene. CAD is a fusion protein which encodes the first three enzymatic activities of the de-novo pyrimidine biosynthetic pathway: carbamoylphosphate synthase (CPS), aspartate transcarbamylase (ATC), and dihydroorotase (DHO) (Boyd and Farnham, 1997, Moulton and Wiegmann, 2004). The CPS is the largest domain within the CAD gene and is present in the genome as a single copy. The synthase domain of CPS is similar in mostly all organisms; it is complex and contains areas which are variable to change and regions which are conserved. Thus, the phylogenetic signal produced when used is strong enough allowing use for phylogenetic inference (Lawson *et al.*, 1996, Moulton and Wiegmann, 2004). The CAD gene is found on the x-chromosome and thus is subjected to less heterozygosity than genes on the autosomes (Moulton and Wiegmann, 2004). It is a protein-coding gene which enables the gene to be aligned easier than that of ribosomal DNA. It provides phylogenetic information of the Mesozoic divergence of an organism, thus providing more comprehensive information for phylogenetic analysis (Moulton and Wiegmann, 2004, Meiklejohn *et al.*, 2013). Ultimately, resulting in the use of the CPS region of CAD to distinguish between cryptic species or species from different populations (Lawson *et al.*, 1996, Moulton and Wiegmann, 2004).

The use of only one marker for phylogenetic inference is generally discouraged and will likely not provide adequate signal for branch formation and support and thus may provide misleading information (Meiklejohn *et al.*, 2013). It has been shown the use of multiple markers with varying mutations rates will provide increased phylogenetic signal across evolutionary time, which will yield increased accuracy and support for phylogenetic inference (Wendel and Doyle, 1998, Beltrán *et al.*, 2002, Sang, 2002, Botero-Castro *et al.*, 2013, Wang and Wu, 2013).

Motivation

Kruger National Park (KNP) (Limpopo and Mpumalanga), South Africa (SA), has a high diversity of tabanids, representing 26 of the total indigenous species (n=213) present in SA (Usher, 1972). KNP

receives rain in summer months (December to February) where the park has an average of 47 days in which it rains. The average rainfall KNP receives ranges from 458mm to 746mm depending on the campsite (Venter and Gertenbach, 1986, Zambatis, 2003). The temperature within KNP ranges from 16.4°C to 32.4°C in the spring and summer months and from 9.5°C to 29.6°C in autumn and winter (Venter and Gertenbach, 1986). KNP has an estimated 2000 species of flora including trees, grasses, ferns, aloes, forbs and woody lianes divided into 16 macro ecozones (Venter and Gertenbach, 1986). KNP has an estimated 150 mammal species and over 500 recorded bird species.

Tabanidae species were sampled from several locations within KNP from 2018 to 2019 for abundance and distribution comparisons. Collection data from various museums throughout the country was collated for spatiotemporal analysis. The collection data from the museums are, however, incomplete. Some of the species are awaiting identification, while others have been identified incorrectly. These samples will be revised together with the newly collected samples with the use of identification keys from Oldroyd (1952, 1954, 1957) with amendments from Chainey (2017).

Currently little to no modern taxonomical work has been done on tabanids within South Africa, thus, there is no supporting evidence from a molecular side which either supports or rejects the current morphological identification of tabanids (Morita *et al.*, 2016). The aim of the study was to integrate molecular methods with the classical morphological and biogeographical methods. This was intended to test the current classification system, and if need be, hypothesize a new and robust system. Collected and identified tabanid samples were used for molecular identification to determine support or incongruence between the molecular and morphological identification systems. The COI and AATS gene regions were used to determine species relationships respectively via the use of molecular methods (Simon *et al.*, 1994). Sequences that were obtained was analysed using phylogenetic methods and the resulting phylogenetic hypotheses were compared to the existing classification system. After species identification, via both morphological and molecular methods, historic collection data and present tabanid data (this study) were used to compile an accurate distribution map of medically and veterinary important tabanid species.

In congruence, the tabanids were screened for *Besnoitia besnoiti,* which is of great concern to all African countries due to the great economic burden it causes. The disease has been detected in Limpopo, Mpumalanga and Kwazulu-Natal. Even though the pathogen is well understood, the mode of transmission is still under revision, and a definitive host is yet to be identified. It is believed that

Tabanidae can transmit the disease (Liénard *et al.*, 2013), but the extent of the tabanid involvement in the life cycle of *B. besnoiti* is unclear. The study aimed to determine which species, if any, are partaking in the life cycle of *B. besnoiti*.

Aim

To compare the traditional alpha-taxonomic approach to tabanid species delimitation, with molecular methods and investigated the role of tabanids in the transmission of *Besnoitia besnoiti*.

Objectives

- To determine an efficient, cost-effective DNA extraction method via comparative analysis
- To evaluate efficacy of molecular markers for Tabanidae species delimitation
- To evaluate the classification of Tabanidae using molecular methodologies
- To determine the presence of *Besnoitia besnoiti* in sampled tabanids

I would like to state that the dissertation will be sectioned into five chapters (Including this one), which may have some overlap in terms of the introductions, methods and discussions.

Comparison of four genomic DNA extraction methods on arthropods

Abstract

Molecular methods have become common place in almost all biological fields. Even though these methods have become more cost-effective, it is often still a burden to labs in developing countries. Generally, molecular studies require a high quality and quantity of nucleic acid from the available specimens. Standardized, commercially available DNA extraction methods, better known as extraction kits, are believed to be the golden standard for DNA extractions. In this study four DNA extraction methods are compared, to determine an effective, time and cost-efficient method that can be used on arthropods. We compared the Chelex 100 resin extraction, Qiagen DNeasy tissue kit, phenol chloroform isoamyl extraction (PCI) and phenol chloroform isoamyl sodium acetate extraction (PCI-SA). The extractions were compared based on quantity (ng/uL) and quality (260/280 ratio and 230/280) of the DNA as well as time and cost per extraction. These methods were conducted on three groups of arthropods: horseflies (Tabanidae), mosquitoes (Culicidae) and ticks (Ixodidae). Five individuals per group were selected. One leg per individual, per extraction method, was used to allow for comparison purposes. The Qiagen DNeasy tissue kit was the most expensive and the quality and quantity of DNA obtained was comparatively low. The Chelex 100 resin extraction was both the quickest and easiest, with no overnight incubation of samples. It produced the highest quantity of DNA, with a varying quality. Both PCI and PCI-SA extractions were significantly cheaper than the commercial kit, producing good quantity and quality of DNA, but both are time consuming and produce chemical waste that requires specialised disposal.

Keywords: DNA, Extraction, Arthropods

Introduction

DNA extraction is a fundamental step in several areas of biological studies, including molecular identification, phylogenetic inference, population genetics, and genomics (Chen *et al.*, 2010). The quality and quantity of DNA obtained from samples during the extraction process is of vital importance, thus, the selection of an appropriate extraction method is one of the main aspects in all studies (Psifidi *et al.*, 2010). Ideally, the criteria that should be considered for the selection of the extraction method entails: the optimization of DNA yield and purity, minimization of DNA degradation and hazardous waste production, as well as the cost efficiency, labor, time and the consumables used. The method must also be adaptable to a large number of samples (Chen *et al.*, 2010).

Currently, there are several highly specialized methods of DNA extraction. These methods can be divided into two broad groups, solution based and column based protocols, most of which has been industrialized into commercial kits (Tan and Yiap, 2009). These kits however are generally expensive and not readily available, to developing countries worldwide (Margam *et al.*, 2010).

In arthropods, extractions are generally conducted by macerating the whole insect or using only one or two legs. This results in the damage or loss of a sample, which museums or collections with TYPE specimens cannot afford (Castalanelli *et al.*, 2010, Chacon-Cortes *et al.*, 2012). On the other hand, using only a few legs has been shown to, at times, produce insufficient amount of DNA (Watts *et al.*, 2007). These low amounts of DNA obtained from legs can be associated to either the age of the sample or the way in which the sample was preserved (Nishiguchi *et al.*, 2002b, Watts *et al.*, 2007, Lienhard and Schäffer, 2019). Due to the varying suitability of different extraction methods, a laboratory that handles numerous arthropod taxa may be required to establish and validate several methods (Castalanelli *et al.*, 2010, Chen *et al.*, 2010).

In this study we compare the efficacy of four DNA extraction protocols, using three different arthropod families. We aimed at determining a protocol which will not only produce a high quality and quantity of genomic DNA per extraction, but one that is effective in all aspects and can be replicated on several different arthropod families.

Material and Methods

Samples

Three different arthropod families were used during this study: *Ixodidae*, *Culicidae* and *Tabanidae*. The Ixodidae samples, all *Amblyomma hebraeum*, were preserved in 70% ethanol. The *Culicidae* were preserved by storage with silica gel beads to remove moisture. All *Culicidae* samples were *Culex pipiens*. *Tabanidae* samples, included: *Tabanus par*, *Tabanus taeniola* and *Atylotus agrestis* species and were kept at -20℃. All samples used were adults and part of the University of Pretoria (Pretoria, South Africa), Department of Veterinary Tropical diseases entomological collection.

Five specimens per family was used. With sterile forceps, one leg per individual per extraction was removed and stored in separate sterile Eppendorf tubes (Hauppauge, New York, US).

Extraction methods

Four extraction methods were selected for comparison: Phenol Chloroform Isoamyl (PCI) protocol, Phenol Chloroform Isoamyl protocol with Sodium Acetate (PCI-SA), Chelex 100 resin protocol and lastly the QIAamp DNA mini kit. These protocols were followed precisely as stated. Extraction methods were compared with regards to the quantity (ng/uL) and quality (260/280 and 260/230 ratio) of the DNA extracted. We also compared the time per extraction and cost per extraction.

Phenol Chloroform Isoamyl (PCI)

This protocol was modified form protocols by Barker (1998). Tissue lysis was conducted by adding 400uL of extraction buffer (100mM Tris-HCl, 5mM EDTA and 0.2% SDS) to each sample. After the addition of 10uL proteinase K (600 mAU/mL), the legs were manually crushed using an Eppendorf pestal fitting tightly in the tubes. The samples were incubated overnight at 56℃. Extraction commenced with the addition of an equal volume of phenol-chloroform-isoamyl alcohol. Samples were placed on a rocking platform and gently mixed for 5 minutes followed by centrifugation for 10 minutes at 9968 x g at room temperature. The aqueous phase was transferred to a new Eppendorf tube and the abovementioned extraction steps were repeated twice. Subsequently, an equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed for 1 minute followed by a two-minute centrifugation at 9968 x g. After the removal of the aqueous phase, 2.5 times the obtained volume of chilled 100% ethanol was added. The samples were incubated at -70℃ for one hour, centrifuged at maximum speed (9968 x g or more) for 20 minutes and the supernatant was discarded. Afterward, 1 mL of 70% ethanol was added and centrifuged at maximum speed (9968 x g or more) for 20 minutes. The supernatant was discarded, and the pellets were air dried for 5 minutes at room

temperature before resuspending the pellets in 20 uL of TE buffer, pH 8. The samples were then stored at -20°C for downstream uses.

Phenol Chloroform Isoamyl Sodium Acetate (PCI-SA)

This protocol was modified form Ausubel *et al.* (1995). The tissue lysis step used was as for the PCI extraction. For the extraction, an equal volume of phenol-chloroform-isoamyl alcohol was added, followed by vigorously vortexing the samples for 10 seconds and micro-centrifuging the samples for 15 seconds. Subsequently, the aqueous phase was transferred to a new Eppendorf tube and the aforementioned extraction steps were repeated twice. A tenth of the volume of 3M sodium acetate, pH 5.2, was added and samples were vortexed for 2 seconds. Afterward, 2.5 volumes of chilled 100% ethanol was added, samples were vortexed for 2 seconds and incubated in ice for 5 minutes. Samples were centrifuged at maximum speed (10 000 x g or more) for 5 minutes. After discarding the supernatant, 1 mL 70% ethanol was added and centrifuged at maximum speed (10 000 x g or more) for 5 minutes. The pellets were air dried for 15 minutes after the supernatant was discarded and resuspended in 100 uL of double distilled water. The samples were then stored at -20°C for downstream uses.

Chelex 100 Resin

This protocol is modified from Nishiguchi *et al.* (2002b). Under stirring, 200 uL of a 5% Chelex 100 resin mixture was added to each sample. Samples were manually crushed using an Eppendorf pestal tightly fitting the tube, followed by an hour incubation at 56°C. Another incubation followed at 95°C for 30 minutes. The samples were then centrifuged at 12 000 x g for 3 minutes and stored at -20°C for downstream uses.

QIAamp DNA mini kit

The extraction was conducted as per the manual with some modifications. In short: 180 uL Buffer ATL and 20 uL proteinase K (600 mAU/mL) were added to each sample. Samples were manually crushed with an Eppendorf pestal tightly fitting the tube and incubated at 56°C for 3 hours on a rocking platform. Subsequently, 200 uL of Buffer AL was added, samples were vortexed for 15 seconds and incubated at 70°C for 10 minutes. After brief centrification, 200 uL of chilled 100% ethanol was added, and samples were mixed by vortexing for 15 seconds. The aqueous phase was transferred to the QIAamp Mini spin columns and centrifuged at 6 000 x g for 1 minute. The flow trough was discarded and 500 uL of buffer AW1 was added. Samples were centrifuged at 6 000 x g for 1 minute, the flow through was discarded and the collection column was replaced. Before the dry

spin, 500 uL of buffer AW2 was added and the samples were centrifuged at 20 000 x g for 3 minutes. The DNA was eluted by performing two elution spins of 200 uL buffer AE at 6 000 x g for 1 minute. The samples rested for 5 minutes after the addition of the elution buffer before each spin. The samples were then stored at -20℃ for downstream uses.

Quantity and Quality

The quantity (ng/uL) of extracted DNA and quality (260/280 and 260/230) thereof was determined with the use of two NanoDropH ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) from different departments to ensure comparable readings. Pure DNA is considered if the 260/280 ratio is 1.8, however not all proteins are always removed during the extraction process and a ratio of 1.7-2.0 is considered adequate. The 260/230 ratio is an additional measurement of purity and values exceeding that of the 260/280 ratios are considered adequate.

Time per extraction

A stopwatch was used during the extraction process. We included time spent on buffer preparation, sample preparation and the general extraction process. When overnight incubations were required, we did not measure the time of that period, we merely indicated that an overnight incubation was required. Time, excluding the base protocols, were divided by the number of samples then added to the base protocol time to account for the time per extraction.

Cost per extraction

In order to determine cost, we excluded our equipment and the number of pipette tips used, since both are dependent on the individual researcher as well as the lab in which the extractions are conducted. The prices were obtained from Inqaba Biotechnical Industries (Pty) Ltd. (Hatfield, Pretoria, South Africa) and Whitehead Scientific (Pty) Ltd (Brackenfell, Cape Town, South Africa). The prices were obtained during the time the study was conducted and thus may be subjected to change (at the time of writing, the exchange rate between ZAR and US$ were 13.72 and between ZAR and Euro were 16.81). Prices of the reagents and tubes that were used was added and then divided by the number of samples.

Gel Electrophoresis

All extracted DNA was assessed by gel electrophoresis to determine the DNA integrity. Precisely, 5 uL of each sample was analyzed in a 1.5% agarose gel containing 2% ethidium bromide and

visualized using the Gel Documentation system (Bio-Rad, Hercules, California, USA). A 1kb ladder (Thermo Fisher scientific, Waltham, Massachusetts, United States) was included in all runs.

Statistical analysis

One-way ANOVA with Post-hoc analyses, based on Least Squares Differences and a Bonferroni adjustment for multiple testing, were used to compare different DNA extraction methods for both the quality and quantity of the DNA obtained. Statistical significance level was set at 0.05. All analyses were performed using the statistical package IBM SPSS (Rogness *et al.*, 2002).

Results

Time and Cost analysis

The comparison of the four DNA extraction protocols for time and cost efficiency is presented in Table 1. The most rapid extraction method was the Chelex 100 resin protocol which took less than two hours to complete. The most time-consuming method was the Phenol Chloroform Isoamyl (PCI) extraction protocol, which required four hours excluding the overnight incubation. Both the PCI and Phenol Chloroform Isoamyl Sodium Acetate (PCI-SA) methods were relatively cost effective, whereas the QIAamp DNA Mini kit was the most expensive of the methods.

Table 1: Comparison of four genomic DNA extraction methods based on the time per extraction and cost per extraction.

Method used	Time (Hours:minutes) Average Lab time	Overnight	Price per extraction (R)
QIAamp DNA mini kit	03:56	x	83.54
Chelex 100 Resin	01:41	x	43.61
Phenol Chloroform Isoamyl	03:51	✓	18.03
Phenol Chloroform Isoamyl Sodium Acetate	01:34	✓	16.17

Spectrophotometer measurements

Averages of spectrophotometer and average quantity DNA yield ratios for each extraction method, as well as the comparisons between methods derived from the statistical analysis are shown in Table 2. Using the 260/280 ratio, Chelex 100 Resin produce the purest DNA followed by PCI-SA. However, no significant difference was observed between any of the extraction methods ($P>0.05$). For the 260/230 ratio, PCI-SA had on average the highest values indicating that the DNA was relatively free of guanidine contamination. The PCI method had the lowest scores; however, no statistically significant differences were observed between any of the methods ($P>0.05$). Statistical significance was observed between groups in relations to the quantity of DNA obtained (ng/uL). Chelex obtained

statistically higher quantity of DNA than all other methods (Kit(P<<0.01), PCI(P=0.01) and PCI-SA(P<0.01)), whereas no significant differences were observed between any of the other groups.

Gel Electrophoreses Analysis

Integrity of the extracted DNA was assessed with the use of gel electrophoreses (Figure 5). Gel electrophoreses indicated that the Chelex 100 resin protocol had the highest integrity of DNA, where bands remained within the wells, or produced smears. The QIAamp DNA mini kit and PCI produced DNA samples of different levels of integrity, of which most produced neither bands nor smears. PCI-SA, however, produced one faint band where all other samples produced no bands or smears.

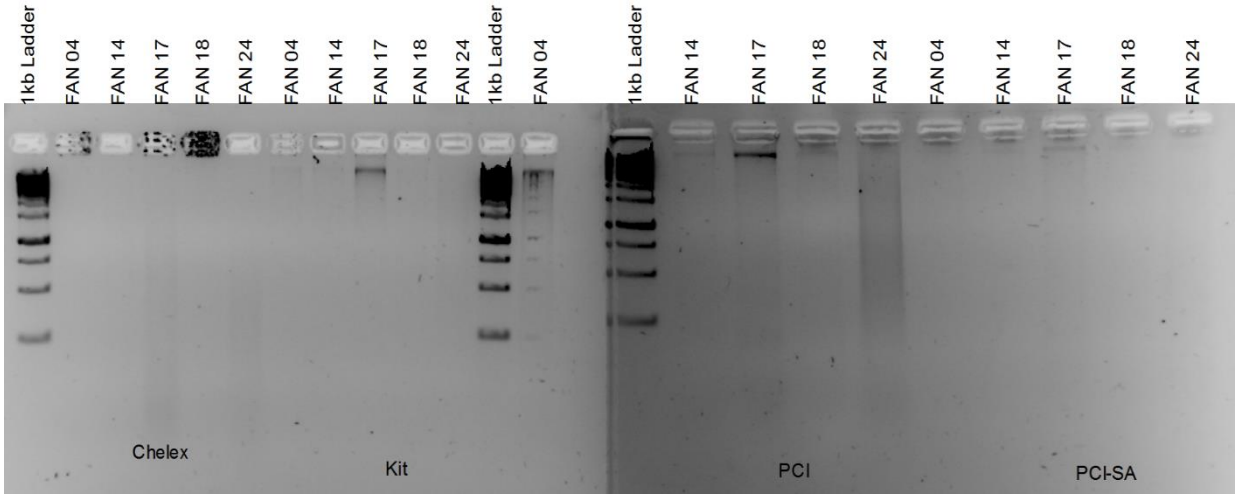

Figure 5: Representative results from gel electrophoresis analysis of four different genomic DNA extraction methods from five different tabanid samples. Electrophoresis was conducted on a 1.5% agrose gel with 2% ethidium bromide for 2 hours at 50V and a 1kb base pair ladder was used.

Table 2: Comparison of four genomic DNA extraction methods using three spectrophotometer measurements (ng/uL, 260/280 nm and 260/230 nm); standard errors of marginal means are indicated in parentheses.

Family	Extraction method	Average Quantity (ng/uL)	Average Quality (260/280)	Average Quality (260/230)
Tabanidae	QIAamp DNA mini kit	2.420 (1.29)	1.608 (0.23)	0.384 (0.14)
Culicidae	QIAamp DNA mini kit	-1.892 (1.29)	1.563 (0.23)	0.093 (0.14)
Ixodidae	QIAamp DNA mini kit	-5.153 (1.29)	2.210 (0.23)	0.831 (0.14)
Tabanidae	Chelex 100 resin	24.359 (7.02)	1.617 (0.25)	0.499 (2.96)
Culicidae	Chelex 100 resin	6.532 (7.02)	1.806 (0.25)	0.412 (2.96)
Ixodidae	Chelex 100 resin	50.996 (7.02)	1.124 (0.25)	4.345 (2.96)
Tabanidae	Phenol Chloroform Isoamyl	22.964 (2.99)	1.540 (0.37)	-1.60 (0.55)
Culicidae	Phenol Chloroform Isoamyl	0.676 (2.99)	2.310 (0.37)	0.601 (0.55)
Ixodidae	Phenol Chloroform Isoamyl	2.064 (2.99)	2.414 (0.37)	1.953 (0.55)
Tabanidae	Phenol Chloroform Isoamyl Sodium Acetate	9.909 (2.64)	1.705 (0.19)	1.702 (0.36)
Culicidae	Phenol Chloroform Isoamyl Sodium Acetate	1.455 (2.64)	2.287 (0.19)	3.595 (0.36)
Ixodidae	Phenol Chloroform Isoamyl Sodium Acetate	6.700 (2.64)	1.143 (0.19)	2.205 (0.36)

Discussion

Genomic DNA of high quality is of fundamental importance within all forms of molecular or genomic studies (Chacon-Cortes *et al.*, 2012). There is a number of methods available, ranging from classic protocols to commercially available kits, each with their own advantages and disadvantages to consider (Nishiguchi *et al.*, 2002b). Currently, very little comparative work has been done on DNA extraction methods using arthropods (Lienhard and Schäffer, 2019). Those, however, that have done comparative analyses have excluded salting out methods, disregarding their potential advantages in terms of cost (Chacon-Cortes *et al.*, 2012). These studies generally include methods that constitutes multiple steps, toxic and/or corrosive chemicals or expensive components (Castalanelli *et al.*, 2010).

There are, however, several conditions, unrelated to the extraction protocol itself, which may decrease the quality and the quantity of the extracted DNA. These conditions includes: 1) the preservation method, 2) the time that the sample has been preserved for and 3) the amount of such samples used for the extraction (Nishiguchi *et al.*, 2002b, Smith *et al.*, 2003, Watts *et al.*, 2007). Gilbert *et al.* (2007) struggled to amplify DNA that was extracted from old museum samples as well

as from younger samples of which the storage and preservation conditions was not known. This could relate to our variation of the quality and quantity of DNA obtained since the samples varied in both age and storage conditions. The Ixodidae was stored at room temperature in 70% ethanol, which, according to Vink *et al.* (2005), is not ideal and may decrease the amount of DNA extracted. I would also like to note that the Ixodidae samples used was not washed beforehand and thus residue of ethanol may have skewed the results. The use of silica gel beads for Culicidae and freezing for Tabanidae, is believed to not negatively affect the DNA obtained (Nsubuga *et al.*, 2004, Vink *et al.*, 2005). The amount of the sample used for the extraction, which in this study was merely a leg per individual, has been shown to effect the resulting quantity and quality of the DNA (Lienhard and Schäffer, 2019).

According to the 260/280 values (Table 2), Chelex 100 resin produced the purest DNA, corresponding with the results of Lienhard and Schäffer (2019). However, gel electrophoresis results (Figure 5) and the low 260/230 ratio (Table 2) indicates possible contamination by RNA and protein. The PCI and PCI-SA had several values lower than 1.7 for the 260/280 ratio, possibly due to phenol residue that remained during the extraction; however, there was also several values higher than 2 indicating possible RNA contamination (Glasel, 1995). Since no RNAase step is present in the above-mentioned protocols, RNA contamination is likely. The PCI-SA method had high values (>2) for the 260/230 ratio; a result of lower contamination, mostly due to the addition of the sodium acetate which aids in contaminants removal. The QIAamp DNA mini kit experienced the same problems with the contamination of RNA and protein. The values obtained for the quality of the DNA, however, does not correspond to other findings of silica-based spin columns by Chen *et al.* (2010), Dittrich-Schroder *et al.* (2012) and Athanasio *et al.* (2016). These authors not only found the silica-based spin columns to be most effective, they also found less contaminants. At this time, the lower quality and higher contamination obtained during this study is of unknown origin.

Other factors which may influence the 260/280 and 260/230 ratios includes the buffer used and the pH thereof. Many buffers and detergents absorb light themselves, thus, the buffers in which the DNA/RNA is suspended may influence the absorbency of the waves resulting in increased or decreased of ratio values (Wilfinger and Chomczynski, 1997, Wilfinger *et al.*, 1997). The pH of these buffer may also skew absorption ratios; Acidic solutions results in under representation by 0.2-0.3, where basic solution will over-represent by the same amount (Wilfinger and Chomczynski, 1997).

Gel electrophoresis showed a few bands with a smearing appearance indicating intact DNA (Figure 5) however, most of the samples produced no bands or smears. This could be the result of the low quantity of DNA that was obtained during the extractions and is not to be considered an indication that no DNA was obtained. In general, the Phusion Flash PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, United States), generally used in the DVTD laboratories, requires a minimum of 10ng/uL for a successful PCR result, leaving most of the sample unusable; Lienhard and Schäffer (2019) however, stated that the low quantity of DNA obtained should not be a major complication since most PCR procedures requires very little DNA to function well.

When considering the use of extraction methods in developing counties, the cost and access to specialized and/or expensive equipment becomes crucially important. Even though the PCI and PCI-SA methods are the most cost effective, the protocols are both labor intensive and time consuming, making these methods unsuitable for large sample processing. In this study, the pellets obtained were too small to be visualized with the naked eye, thus increasing the possibility of accidently discarding the DNA. This may be a major concern if the whole sample was used. Both these methods make use of phenol, a biohazardous chemical that requires specialized disposal which, in most cases, are expensive and not readily available in all areas. One should also note that in the PCI-SA method, the DNA pellet is dissolved in water, whereas PCI is dissolved in TE buffer. DNA dissolved in water is not stable cannot be stored as long as DNA dissolved in TE buffer (Kim *et al.*, 2012).

The QIAamp DNA mini kit requires limited labor, allowing for increased sample throughput compared to the previous methods (Nishiguchi *et al.*, 2002b). However, this method is expensive, nearly four times that of the PCI and PCI-SA protocols and, according to these results, produce less DNA (ng/uL) as well as lower quality DNA. The DNA extracted with the QIAamp kit can however be stored for 16 years at 2-8℃ or at -20℃ according to the QIAamp website.

The Chelex 100 resin method is quick and easy to perform, with limited labor that allows for an increased sample throughput. Chelex is more expensive than both the phenol-based methods, however, is nearly half that of the kit making it more affordable. A statistically significant difference was observed, where Chelex produced more DNA per microliter than all the other methods, which was also found by Lienhard and Schäffer (2019). The Chelex resin is also ecologically friendly, with no hazardous chemical waste (Lienhard and Schäffer, 2019). There are, however, drawbacks with Chelex. Firstly, the resin beads remains in the extracted DNA and may inhibit PCR procedures (Casquet *et al.*, 2012). This drawback can be overcome by centrifuging the samples before each use

to ensure that the resin and the aqueous phase is separated properly. Another major drawback is that the extracts are not stable, since the DNA is single stranded, and not suitable for long term storage (Lienhard and Schäffer, 2019). Casquet *et al.* (2012) found that Chelex extracted DNA could roughly be stored for two years.

To conclude, a significant difference was observed in the quantity of DNA extracted, where Chelex produced more nanograms of DNA per microliter. No statistical significance was observed for the quality of the extracted DNA between any of the methods. All methods, however, has their own advantages and disadvantages that will suit different requirements of different research laboratories.

Contribution to the systematics of Tabanidae of South Africa, including species pages

Abstract

Tabanids are true hematophagous flies, important in pollination and pathogen transmission. The family, however, is in need of systematic treatment. Little research has been conducted on this family in South Africa, and most studies were limited to one gene or small geographical locations within the region over comparatively short collection periods. In the present study, tabanids were collected at three major locations in Kruger National park with the use of octanol and three different traps. DNA from the flies was extracted and amplified using both cytochrome oxidase I (COI) and alanyl-tRNA-synthetase (AATS) gene regions and phylogenetic analyses were conducted using the COI sequences. The resulting topologies were compared to the current morphological classification system. In total 856 flies were captured belonging to 16 morphologically unique species within five genera. Several incongruences were observed in the topologies. The COI barcode revealed that some species are very uniform such as *Tabanus pullulus* while others such as *Tabanus taeniola* and *Tabanus gratus* forms co-occurring haplotypes. COI was not adequate in distinguishing between all species from South Africa but still had some value in other species and/or species groups. Further studies on Tabanidae are required, not only in South Africa but worldwide. A revision of the family is highly suggested, this will be aided by more studies producing barcodes and other molecular markers of available species for phylogenetic analyses.

Keywords: COI, Afrotropical, horse flies, Kruger National Park, Barcoding

Introduction

Tabanids are free-living, robust, hematophagous flies more commonly referred to as horse flies or deer flies (Cheng, 1976). The family Tabanidae belongs to the infraorder Tabanomorpha and comprise approximately 4450 described species within 114 genera; of which 800 species of 35 genera occurs in the Afrotropical region (Baldacchino *et al.*, 2014a, Morita *et al.*, 2016, Votýpka *et al.*, 2019). The family was previously divided into five subfamilies: Adersiinae, Chrysopsinae, Pangoniinae, Scepsidinae and Tabaninae; however the status of the subfamilies requires revision (Mackerras, 1954, Mackerras, 1955, Morita *et al.*, 2016). Currently the accepted classification system groups Adersiinae within the Scepsidinae (Mackerras, 1954, Mackerras, 1955, Morita *et al.*, 2016).

The subfamily, Scepsidinae, are thought to be supported as a monophyletic group due to their unique genitalia and reduced mouthparts and are of no medical concern according to Service and Service (1980). Pangoniinae, more commonly known as the long-tongue horse flies are regarded as the most primitive of the subfamilies and contains the most specialized pollinator species (Morita, 2008, Morita *et al.*, 2016). The deer flies, formally known as the Chrysopinae, are tenacious feeders with intricate eyes (Turcatel, 2014). Last of all, Tabaninae, the most diverse of the subfamilies with nearly 3200 species, with the majority of them residing in one genus, *Tabanus*, is in dire need of revision (Morita *et al.*, 2016).

Tabanids are important pollinators in the Drakensberg region where they are known to be one of few pollinators of the orchid, *Disa pulchra,* as well as in the Cape floral region where they are known to pollinate over 170 of the plant species (Jersáková et al., 2012, Karolyi et al., 2014). Tabanids, predominantly of the subfamilies Chrysopinae and Tabaninae, are also important vectors of several human and livestock pathogens including *Loa loa*, *Trypanosoma* spp., *Bacillus anthracis*, *Anaplasma* spp. and *Francisella tularensis*, to mention a few (Foil and Hogsette, 1994, Baldacchino et al., 2014). Their bites are painful and may cause allergic reactions as well as damage to skin or hide which may result in blood loss (Foil and Hogsette, 1994). In animals this may reduce appetite, milk production and/or weight, resulting in economic damage (Baldacchino et al., 2014).

Tabanids have a cosmopolitan distribution which range from the tropics through temperate areas to oases in deserts. While the temperate fauna has been documented extensively, the tropical fauna has been studied less, with most studies taxonomic in nature (Mullen and Durden, 2002, Baldacchino et al., 2014, Herczeg et al., 2015, Taioe et al., 2017). The identification of tabanids has thus far been mainly reliant on morphological methods and literature that was published over half a

century ago (Oldroyd, 1952, 1954, 1957, Banerjee *et al.*, 2015, Mugasa *et al.*, 2018). There are some complications with identifications with the use of identification keys including: 1) differences in intraspecific and/or regional morphological clines, 2) morphologically cryptic taxa, frequently excluded or vaguely discussed, and 3) the use of difficult to find life stages and/or gender-specific characteristics which include dissection of genitalia (Hebert et al., 2003, Banerjee et al., 2015). Identification keys are available for the Afrotropics (as the Ethiopian region) (Oldroyd, 1952, 1954, 1957, Chainey, 2017), however in the attempt to reduce misidentifications, barcoding is being utilized more frequently (Banerjee *et al.*, 2015).

Several studies have been conducted using barcodes to investigate the species level classification of tabanids such as Cywinska *et al.* (2010), Morita *et al.* (2016), Mugasa *et al.* (2018), Mazibuko (2018) and Votýpka *et al.* (2019). These studies however, primarily use cytochrome oxidase I (COI), a gene commonly used for Tabanidae and other arthropod families phylogenetic work, but may not provide enough signal to make accurate conclusions/inferences (Meier *et al.*, 2006). Other barcoding markers such as alanyl-tRNA-synthetase (AATS) and the carbamoyl-phosphate synthase (CPS) domain of carbamoyl-phosphate synthetase 2, aspartate transcarbamylase and dihydroorotase (CAD) has only recently became more regular in phylogenetic studies of arthropods (Morita, 2008, Bayless, 2012). Morita *et al.* (2016) used a wide variety of genes including: COI, CAD, AATS and 28S, providing a framework for tabanids, however, little to no attention was placed on South African species. All aforementioned studies, excluding Mazibuko (2018), were conducted in other regions, leaving a gap in South African tabanid systematics. Thus, this study aimed at building on the work of Mazibuko (2018) by comparing the morphological approach to tabanid species delimitation, with molecular methods of identification, in species collected in the Kruger Park region of South Africa.

Material and Methods

Study site, sampling and identification

The study was conducted from 2018 to 2019, in the central and southern areas of Kruger National Park at three main locations: Lower Sabie, Skukuza and Orpen. Overall, three different traps were used, including: Manitoba trap (either with a black bucket or black ball), Ngu trap and a Horizontal trap (H-trap). All traps were baited with 2 mL of octenol to increase tabanid capture. Tabanids caught outside of the sampling sites were collected as opportunistic samples.

Lower Sabie

Sampling at Lower Sabie constituted a total of three sampling periods, each four days long. In total, seven sites were utilized during sampling in this area (Table 3). Each site was roughly 1 km apart from one another. At each of these sites, excluding the Sabie bucket and Shishangeni watering hole sites, one of each of the traps were placed an estimated 100 m apart of each other in a clustered format (Figure 6). Traps were rotated clockwise the following trip to avoid position favourability (Figure 6). Samples were collected daily and frozen, after which they were transported to the Department of Veterinary Tropical Diseases at Onderstepoort, University of Pretoria, South Africa (DVTD) for identification and processing.

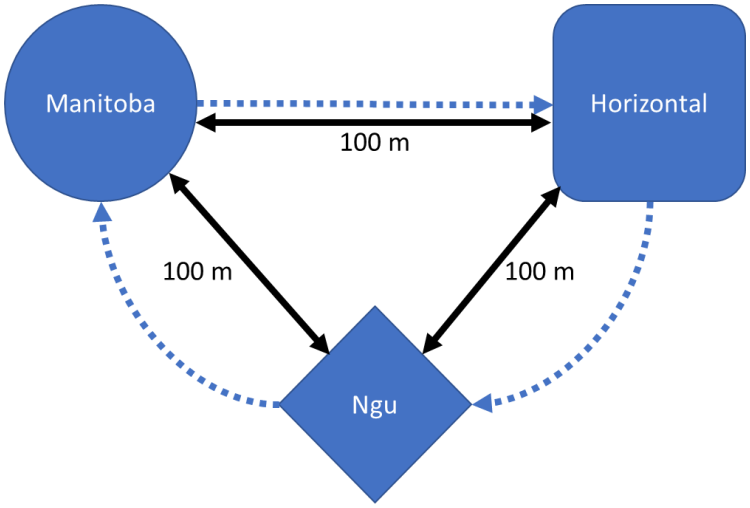

Figure 6: Diagram illustrating the cluster format of the three traps at each of the sites (excluding Sabie bucket and Shishangeni watering hole sites). The dotted blue line indicates the directing of the rotation of the traps; solid black line indicates the approximate distance between the traps.

Skukuza

Sampling at Skukuza constituted only one sampling period, which extended over a nine-day period. In total 10 sites were utilized (Table 3). Each site only had one trap which was randomly selected with use of a dice, and placed 200 m apart. The sites were along the upper Sabie river, in shaded areas. Samples were collected every second day and frozen, after which they were transported to DVTD for identification and processing.

Orpen

Sampling at Orpen constituted of only one sampling period, extending over a four-day period. Two separate locations within the Orpen area were selected for sampling, including: the Hans Hoheisen Wildlife Research Station (HHWRS) and the South African Wildlife Collage (SAWC). At HHWRS only three sites were incorporated which were 200 m apart and traps were selected at random (Table 3).

At SAWC, 10 sites were used that were 200 m apart. Traps were placed at random in open areas (Table 3). Samples were collected daily and frozen, after which they were transported to DVTD for identification and processing.

Tabanids were identified to species level using a stereomicroscope and identification keys from Oldroyd (1952,1954,1957) with amendments from Chainey (2017). Reference flies were pinned and preserved in entomological drawers as part of a reference collection, each with a leg removed that was placed in 100% EtOH to preserve genetic material. One representative fly of each species in the reference collection was photographed to document morphological characteristics. The reference collection is currently housed in the ecto-parasitology laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, Pretoria, South Africa. This chapter aims to build and contribute to the Tabanidae website, http://tabanidae.myspecies.info/, a digital initiative complied and maintained by the PEET project and the Smithsonian institution, USA.

Distribution maps were compiled using GPS locations taken during each sampling period at each trap location as well as GPS locations from museum records. Seasonality of the species was determined with the use of the current collection records and museum records with some assistance of Oldroyd (1952, 1954, 1957). Museums, from which records were obtained, included: Iziko National Museum (Cape town), Ditsong Museum of Natural History (Gauteng), National Museum of Natural History (Bloemfontein), South African National Collection (Roodeplaat), Rikomitjie Research Station (Zimbabwe), British Museum of Natural History (London) and Kruger collection (Skukuza). For the distribution map construction, QGIS version 3.10 was used, enabling the OpenStreetMap function (Baghdadi *et al.*, 2018).

Table 3: Locations and site names of sampling area within Kruger National Park, including the dates and type of traps used for the collections.

Lower Sabie			
Site Name	**Location**	**Trap Used**	**Dates of Collection**
Moamba river	S25,250005° E31,592331°	Ngu, Manitoba and Horizontal Rotational base (Figure 6)	06/03/2018 -10/03/2018
Sabie drainage	S25,177872° E31,975624°	Ngu, Manitoba and Horizontal Rotational base (Figure 6)	06/03/2018 – 10/03/2018 26/03/2018 - 29/03/2018 17/04/2018 - 20/04/2018
Sabie matrix	S25,181522° E32,002162°	Ngu, Manitoba and Horizontal Rotational base (Figure 6)	06/03/2018 – 10/03/2018 26/03/2018 - 29/03/2018 17/04/2018 - 20/04/2018
Sabie reeds	S25,179097° E31,963002°	Ngu, Manitoba and Horizontal Rotational base (Figure 6)	26/03/2018 - 29/03/2018 17/04/2018 - 20/04/2018
Sabie bucket	S25,184580° E32,008112°	Manitoba-bucket Manitoba-ball	26/03/2018 – 29/03/2018 17/04/2018 - 20/04/2018
Sabie river	S25,186237° E32,012945°	Ngu, Manitoba and Horizontal Rotational base (Figure 6)	06/03/2018 – 10/03/2018 26/03/2018 - 29/03/2018 17/04/2018 - 20/04/2018
Shishangeni watering hole	S25,230926° E31,586215°	Manitoba-bucket Manitoba-ball	06/03/2018 – 10/03/2018

Skukuza			
Site Name	**Location**	**Trap Used**	**Dates of Collection**
Upper Sabie River 1	S24.97416° E31.65417°	H-Trap	24/01/2019 - 01/02/2019
Upper Sabie River 2	S24.97517° E31.65204°	Manitoba-bucket	24/01/2019 - 01/02/2019
Upper Sabie River 3	S24.97594° E31.64984°	Ngu-Trap	24/01/2019 - 01/02/2019
Upper Sabie River 4	S24.97635° E31.64741°	Manitoba-bucket	24/01/2019 - 01/02/2019
Upper Sabie River 5	S24.97697° E31.64481°	H-Trap	24/01/2019 - 01/02/2019
Upper Sabie River 6	S24.97731° E31.64212°	Ngu-Trap	24/01/2019 - 01/02/2019
Upper Sabie River 7	S24.97836° E31.63955°	Manitoba-bucket	24/01/2019 - 01/02/2019
Upper Sabie River 8	S24.97928° E31.63753°	H-Trap	24/01/2019 - 01/02/2019
Upper Sabie River 9	S24.98039° E31.63510°	Ngu-Trap	24/01/2019 - 01/02/2019
Upper Sabie River 10	S24.98160° E31.63224° E31.38778°	Manitoba-bucket	24/01/2019 - 01/02/2019

Table 3 (Continue): Locations and site names of sampling area within Kruger National Park, including the dates and type of traps used for the collections.

Orpen			
Site Name	**Location**	**Trap Used**	**Dates of Collection**
SAWC 1	S24.543555° E31.333258°	Ngu-Trap	07/01/2019 - 11/01/2019
SAWC 2	S24.543351° E31.332502°	Manitoba-ball	07/01/2019 - 11/01/2019
SAWC 3	S24.543293° E31.331832°	H-Trap	07/01/2019 - 11/01/2019
SAWC 4	S24.542136° E31.329568°	Ngu-Trap	07/01/2019 - 11/01/2019
SAWC 5	S24.540726° E31.329218°	H-Trap	07/01/2019 - 11/01/2019
SAWC 6	S24.539484° E31.328889°	Manitoba-ball	07/01/2019 - 11/01/2019
SAWC 7	S24.538443° E31.328580°	H-Trap	07/01/2019 - 11/01/2019
SAWC 8	S24.537350° E31.328329°	Ngu-Trap	07/01/2019 - 11/01/2019
SAWC 9	S24.536142° E31.328224°	Manitoba-ball	07/01/2019 - 11/01/2019
SAWC 10	S24.534602° E31.327707°	Manitoba-bucket	07/01/2019 - 11/01/2019
HHWRS 1	S24.478368° E31.388563°	Ngu-Trap	07/01/2019 - 08/01/2019
HHWRS 2	S24.479613° E31.388023°	Manitoba-ball	07/01/2019 - 08/01/2019
HHWRS 3	S24.480325°	H-Trap	07/01/2019 - 08/01/2019

DNA extraction

From the reference collection, only one leg per tabanid was used for DNA extraction, whereas for the remainder of the flies, the whole fly was used. DNA was extracted using the Chelex 100 resin method obtained from Nishiguchi *et al.* (2002a) with slight modification. In short: 200 uL of a 5% Chelex 100 resin mixture was added, under stirring, to each sample. Subsequently, three sterilized glass beads were added to each sample. Samples were homogenised using the Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) using the 1st setting (5200 rpm, for 18 sec per direction). The glass beads were removed from each sample, after which samples were incubation at 56℃ for an hour. Another incubation followed at 95℃ for 30 minutes. The samples were then centrifuged at 12 000 g for 3 minutes and stored at -20℃ for downstream uses.

Amplification

Cytochrome oxidase I (COI)

Reference collection samples were amplified with primers obtained from Folmer *et al.* (1994) (Table 4). A 20 uL total volume reactions was prepared per sample. A master mix was made which constituted of 6.5 uL of ddH2O, 10 uL of Phusion Flash PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, United States) (final concentration of x1) and 0.5 uL of both the forward and reverse primers (final concentration of 0.5uM each) per sample. This master mix was aliquoted, 17.5 uL per sample, to which 2.5 uL of extracted DNA was added. The PCR was performed on the 2720 Thermal Cycler (Applied Biosystems™), with the following cycling conditions: 98˚C for 10 sec, (98˚C for 1 sec, 55˚C for 5 sec, 72˚C for 15 sec) x 30 cycles, 70˚C for 1 min and 4˚C on hold.

Alanyl-tRNA synthetase (AATS)

All reference samples were amplified using primers obtained from Morita *et al.* (2016) (Table 4). A 20 uL total reaction volume was prepared. A master-mix was made for all samples and constituted of 4 uL ddH2O, 10 uL of Phusion Flash PCR Master Mix (final concentration of x1) and 0.5 uL of both the forward and reverse primers (final concentration of 0.5uM each) per sample. The master mix was then aliquoted, 15 uL per reaction, to which 5 uL of extracted DNA was added. The PCR was performed on the 2720 Thermal Cycler, with the following cycling conditions: 98˚C for 10 sec, (98˚C for 1 sec, 49˚C for 5 sec, 72˚C for 15 sec) x 30 cycles, 70˚C for 1 min and 4˚C on hold. Amplified products were subjected to a reamplification with the same primers. The master mix was as follows: 7 uL ddH2O, 10 uL of Phusion Flash PCR Master Mix (final concentration of x1) and 0.5 uL of both the forward and reverse primers (final concentration of 0.5uM each) per sample. This master mix was then aliquoted, 18 uL per reaction, to which 2 uL of amplified DNA was added. The PCR cycle was altered as follows: 98˚C for 10 sec, (98˚C for 1 sec, 51˚C for 5 sec, 72˚C for 15 sec) x 30 cycles, 70˚C for 1 min and 4˚C on hold.

Table 4: Primer information for this study, including: target region, primer name, length, sequence, expected product size and reference.

Target Region	Primer	Length	Sequence (5'-3')	Expected product size	References
COI	LCO1490	25-mer	GGT CAA CAA ATC ATA AAG ATA TTG G	708bp	Folmer *et al.* (1994)
	HC02198	26-mer	TAA ACT TCA GGG TGA CCA AAA AAT CA		
AATS1	M13A1x40F	40-mer	TGT AAA ACG ACG GCC AGT GNA TGA AYC ART TYA ARC CAN T	587bp	Morita *et al*. (2016)
	M13rA1x244R	38-mer	CAG GAA ACA GCT ATG ACC ATN CCR CAR TCN ATR TGY TT		

Gel electrophoresis

Gels were used to confirm amplification. To prepare the amplified products for visualization, 2 uL of amplified DNA was added to 2 µL of 6x TriTrack DNA Loading Dye (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The amplified products were then visualized by means of agarose gel electrophoresis (1.5 % (w/v) agarose, 120V for 60 minutes) in 1 x Tris-acetate EDTA (TAE) electrophoresis buffer. The gel was analysed using the ChemiDoc XRS+ System with corresponding image lab software (Bio-Rad, Hercules, California, United States). A 100 bp molecular ladder was used as reference (Thermo Fisher Scientific, Waltham, Massachusetts, United States). If bands were observed, the remaining amplified products were sent to Inqaba Biotec™ (Pretoria, South Africa) for Sanger sequencing.

Phylogenetic analysis

Sequences for AATS and COI were viewed and edited, and contigs were constructed using CLC main workbench version 8.1.2 (developed by CLC Bio, http://www.clcbio.com). Assembled COI sequences were aligned alongside reference sequences that were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Annexure A, Table 1) with the use of the online version of MAFFT version 7 (developed by http://mafft.cbrc.jp/alignment/server/index.html) with default parameters. The aligned matrix for COI was manually viewed, edited and truncated using MEGA 7 (Kumar et al., 2015).

The format of the aligned matrices was changed with the use of the HIV sequence database (https://www.hiv.lanl.gov/content/sequence/FORMAT_CONVERSION/form.html) to accommodate several programs (Nexus, Fasta and Phylip). The matrix was translated and aligned to display no stop codons with the use of Mesquite version 3.04 using the minimise stop codons function. Data-display

networks (neighbour-networks) were constructed with SplitsTree version 4 (Huson and Bryant, 2005), using all characters and uncorrected p-distance. Bootstrap support values were calculated using 1000 replicates.

Evolutionary model estimations were conducted using the jModelTest2 via the Cipres science gateway (www.phylo.org). Maximum Likelihood (ML) analysis was conducted in RAxML (Stamatakis, 2014). A random starting tree (12345), under the GTR + Gamma model with the autoMRE function invoked to calculate bootstrap support values (Felsenstein, 1985). Separate datasets for Pangoniinae + Chrysopsinae, Haematopotini, Tabanini and *Atylotus* were assembled as an attempt to increase resolution and support.

Ethic consideration

This project obtained both animal ethics (v059-18) and faculty ethics (REC057-19) clearance, provided by the University of Pretoria, South Africa. All samples were collected with the aid of the State Veterinarians and support from South African National Parks (SANparks permit number: NEVL1497), according to the guidelines provided by the ethical committees. Permission was obtained to do research in terms of section 20 of the ANIMAL DISEASE ACT, 1984 (ACT NO. 35 of 1984) (Ref number: 12/11/1/1/6). All permits can be located in Annexure C.

Results

Tabanid abundance and composition

A total of 856 tabanids were sampled, morphologically identified and stratified into 16 morphologically unique species in five genera (Figure 7). The most predominate genus was *Tabanus*, making up a total of 87.5% of the specimens, with *Tabanus minuscularius* as the most prevailing species, constituting 68.3% of the catches. The minority of the catch belonged to the *Haematopota* genus (1.1%), whilst *Philoliche medialis* (0.1%), *Tabanus par* (0.1%) and *Tabanus taeniola* (0.1%) were the least caught species. Out all the samples collected, five specimens (0.6%) remain unidentified and forms part of the reference collection.

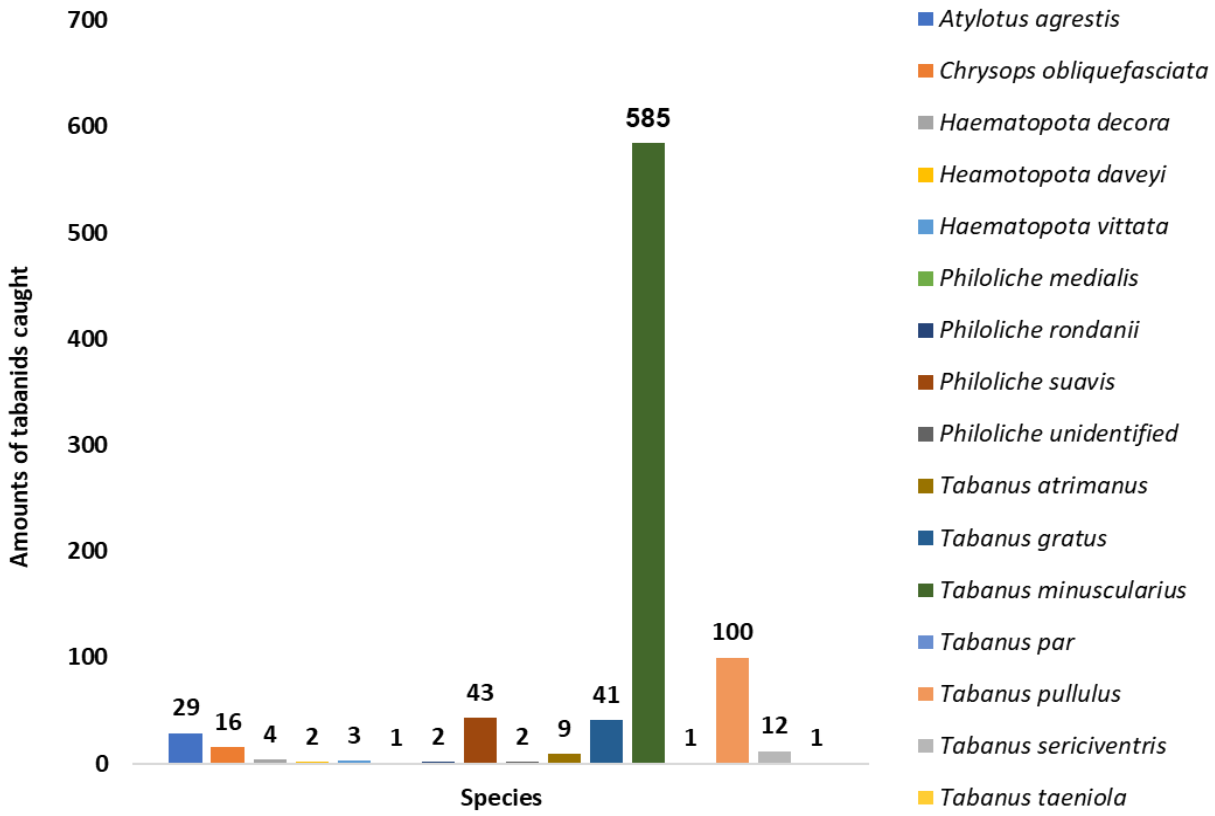

Figure 7: The composition and abundance of tabanids caught in Kruger National Park from 2018 to 2019.

Species pages of tabanids collected

Tribe: Chrysopsini

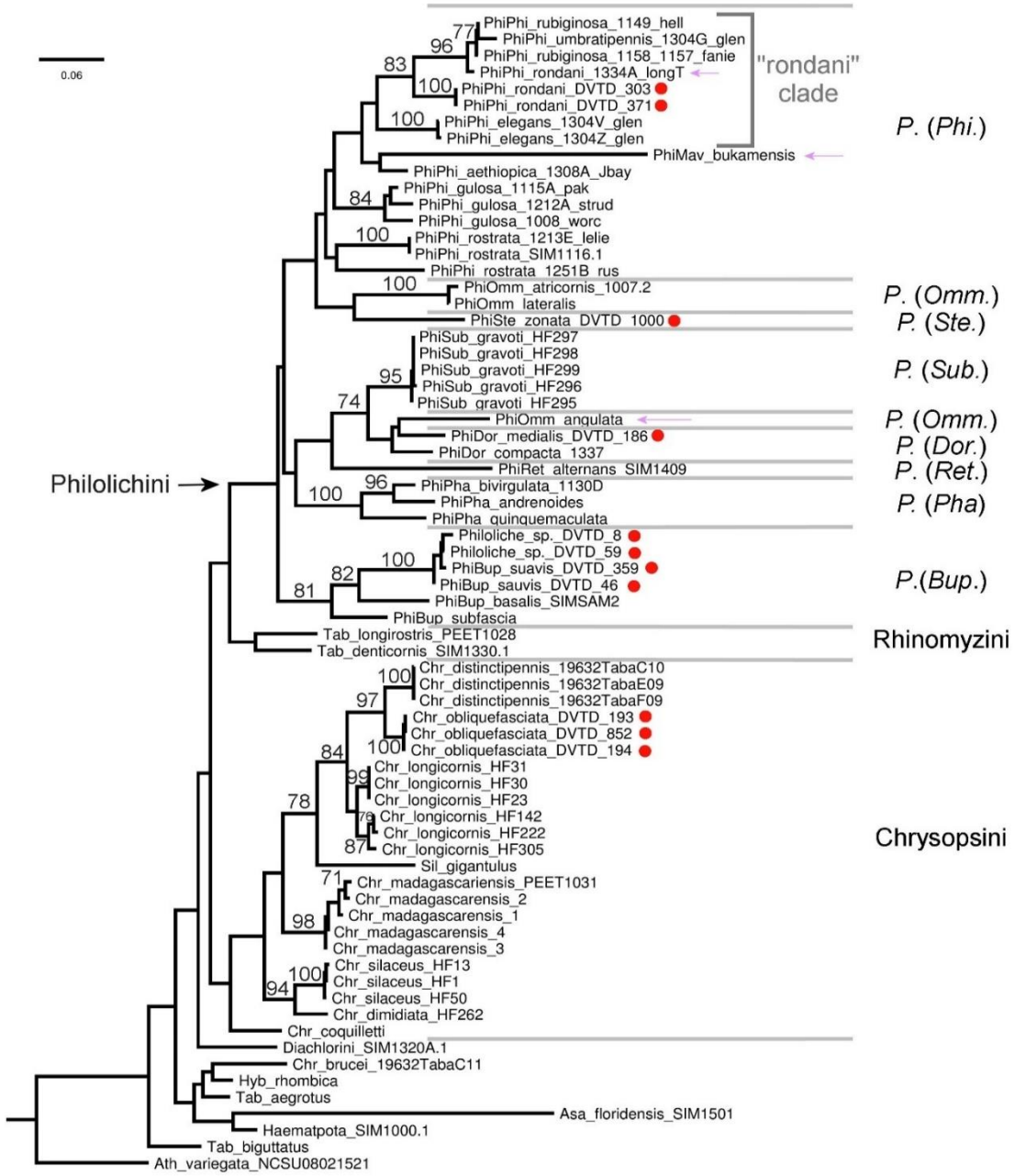

Figure 8: COI phylogenetic analysis using RaXML of the Chrysopini, Rhinomyzini and Philolichini tribes by Maximum likelihood (ML) alongside reference sequences obtained from GenBank. A total of 70 taxa were evaluated with a matrix length of 676 characters. The grey lines indicate the separation between the three tribes as well as between the subgenera within the Philolichini (Subgenera abbreviations: *Phi*.: *Philoliche*; *Omm*.: *Ommatiosteres*; *Ste*.: *Stenophara*; *Sub*.: *Subpangonia*; *Dor*.: *Dorcaloemus*; *Ret*.: *Returneria*; *Pha*.: *Phara*; *Bup*.: *Buplex*). Red dots indicate new sequences obtained during this study; purple arrows are discussed in text.

Chrysops obliquefasciata Macquart, 1838

http://tabanidae.myspecies.info/taxonomy/term/648 (as *C. obliquefasciatus*)

Chrysops obliquefasciata Macquart, 1838:161 by original designation. South Africa; Angola, Mozambique, Rhodesia, Zaire, Zambia.

Chrysops natalis Macquart, 1846:171 by synonymy. South Africa.

Chrysops stigmaticalis Loew, 1858:338 [1860: 101] by synonymy. South Africa.

Chrysops confluens Loew, 18586:338 [1860: 102] by synonymy. South Africa.

Chrysops fuscus Ricardo, 1902: 368 by synonymy. Rhodesia.

Figure 9: Sequential images of *Chrysops obliquefasciata* from top left going clockwise: dorsal, facial, enlarged lateral and complete lateral views.

Morphological diagnosis: As seen by Figure 9, Chrysops obliquefasciata is a small fly, approximately 10mm in length, with a 9mm wing length. Predominantly a black species with golden tuffs of hairs visible on the sides of the pronotum as well as on the meso- and metapleural. The legs are predominantly yellow excluding certain sections such as the fore tarsi extending to the apical half of the fore tibia, which are black. Wings are brown at the base extending along the fore margin to the tip. A middle brown band is visible which extends to the fork of the R_{4+5} vein. The lower tip of the wings is smoky, and a clear patch is visible. A yellow stigma is visible on the wings.

Similar species: *Chrysops distinctipennis* and *Chrysops streptobalia*, both found in overlapping areas to that of *Chrysops obliquefasciata*. The distinguishing characteristics between *C. streptobalia* and *C. obliquefasciata* includes a broad median strip on the mesonotum of *C. streptobalia* as compared to the sublateral stripes but no median stripe in *C. obliquefasciata*. The distinction between *C. distinctipennis* and *C. obliquefasciata* lies in the middle band on the wing; *C. distinctipennis*'s band does not reached the fork of the R_{4+5} vein whereas *C. obliquefasciata*'s does.

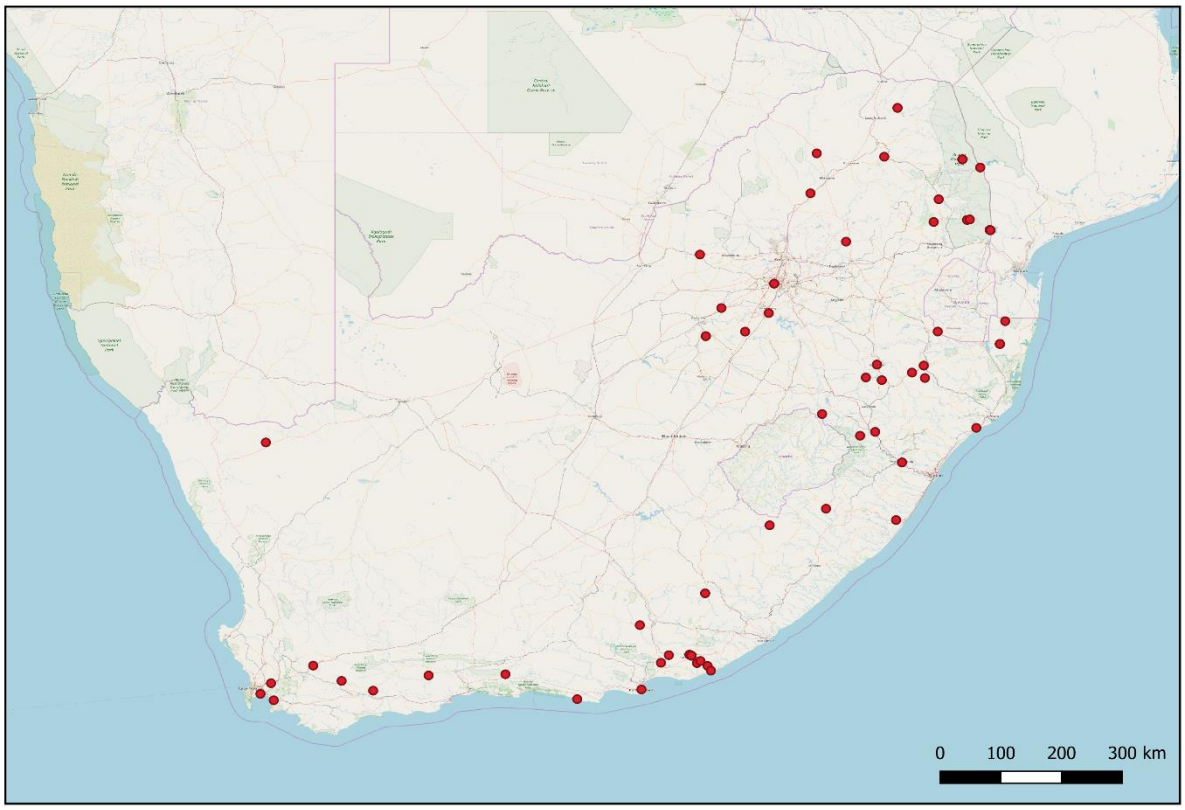

Figure 10: Distribution map representing locations at which *Chrysops obliquefasciata* (red dots) has been collected in South Africa (n= 101).

Distribution: Occurs along the southern coastal region and is widely distributed in the north-eastern parts of South Africa (Figure 10). This corresponds with records from Usher (1972). A suspect record in the north-western part of South Africa.

Biology: Currently no information regarding the life history of *C. obliquefasciata* is known. Sexual dimorphism is pronounced; the small plain black male is not holoptic, and the greenish-grey female is patterned. The species is often numerous around "vleis" and in marshy areas; Mr Van Rensburg (Usher, 1972) reports "All *Chrysops obliquimaclilatus* were caught in marshy places on edges of dams by means of a net". Usher (1972) found that this species did not attempt to bite humans and was commonly found in water-meadows which were occupied by cows.

Seasonality: Occurs during September to April (n=93).

Vector status: No studies to date have investigated the vector potential of *C. obliquefasciata*. Other *Chrysops* species have been implicated as vector of the microfilariae causing loiasis (*Loa loa*) via vector competency studies (Connal and Connal, 1922). While *Francisella tularensis, Bacillus anthracis, Trypanosoma gambiense*, *Trypanosoma rhodesiense*, *Trypanosoma evansi, Trypanosoma equinum, Trypanosoma simiae, Trypanosoma vivax* and *Trypanosoma brucei* all were confirmed by pathogen screening (Goddard, 2008, Kouam and Kamgno, 2017).

Molecular data: No sequence data is available from GenBank. *Chrysops obliquefasciata* forms a monophyletic group with *Chrysops distinctipennis* (Figure 8). This monophyly is sister to *Chrysops longicornis*. All relationships are supported with high bootstrap values. Both AATS and COI sequences obtained during this study will be submitted to GenBank.

Depositories: A holotype of *C. obliquefasciata* and *C. natalensis* is available in the Muséum National d'Histoire Naturelle, Paris; *C. stigmaticalis* and *C. confluens* are available at the Zoologisches Museum der Universität, Berlin; the holotype of *C. fusca* can be located in the British Museum.

References: Connal and Connal (1922), Usher (1972), Oldroyd (1957), Goddard (2008) and Kouam and Kamgno (2017).

Tribe: Philolichini

Philoliche (Buplex) suavis (Loew, 1858)

http://tabanidae.myspecies.info/taxonomy/term/2686

Pangonia sauvis Loew, 1858:337 [1860:89] by original designation. South Africa.

Ommatiosteres caffrica Enderlein, 1925:267 by synonymy. South Africa.

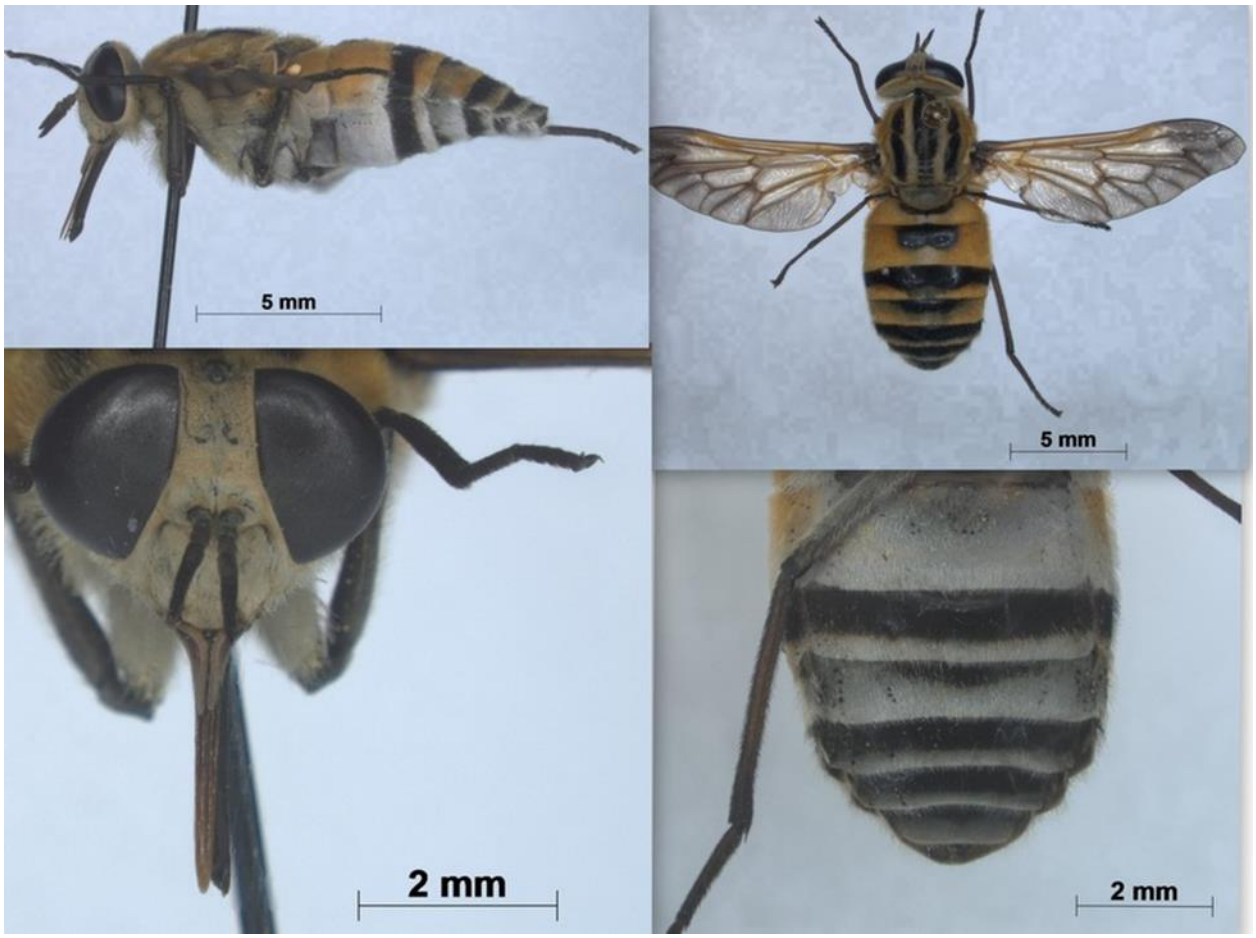

Figure 11: Sequential images of *Philoliche suavis* from top left going clockwise: lateral, dorsal, ventral and facial views.

Morphological diagnosis: As can be seen from Figure 11, *Philoliche suavis* is predominantly a yellow and black species resembling a bee, approximately 15-16mm in body length and 15mm in wing length. The wings are yellow with a distinct grey tip. The mesonotum is black with yellow median, sublateral and lateral complete stripes. Each segment of the abdomen is black basely and yellow apically. A unique bi-lobed black patch is visible on the second abdomen segment. Third segment onwards is mainly black with a faint yellow band.

Similar species: *P. sauvis* cannot easily be confused with other *P.* (*Buplex*) species due to the unique coloration. During this study, however, two “dark” specimens, lacking the characteristic longitudinal

lines on the thoracic dorsum were collected. The entire thoracic dorsum is dark and can therefore not be identified using the keys of Oldroyd (1954). This coloration difference requires further investigation.

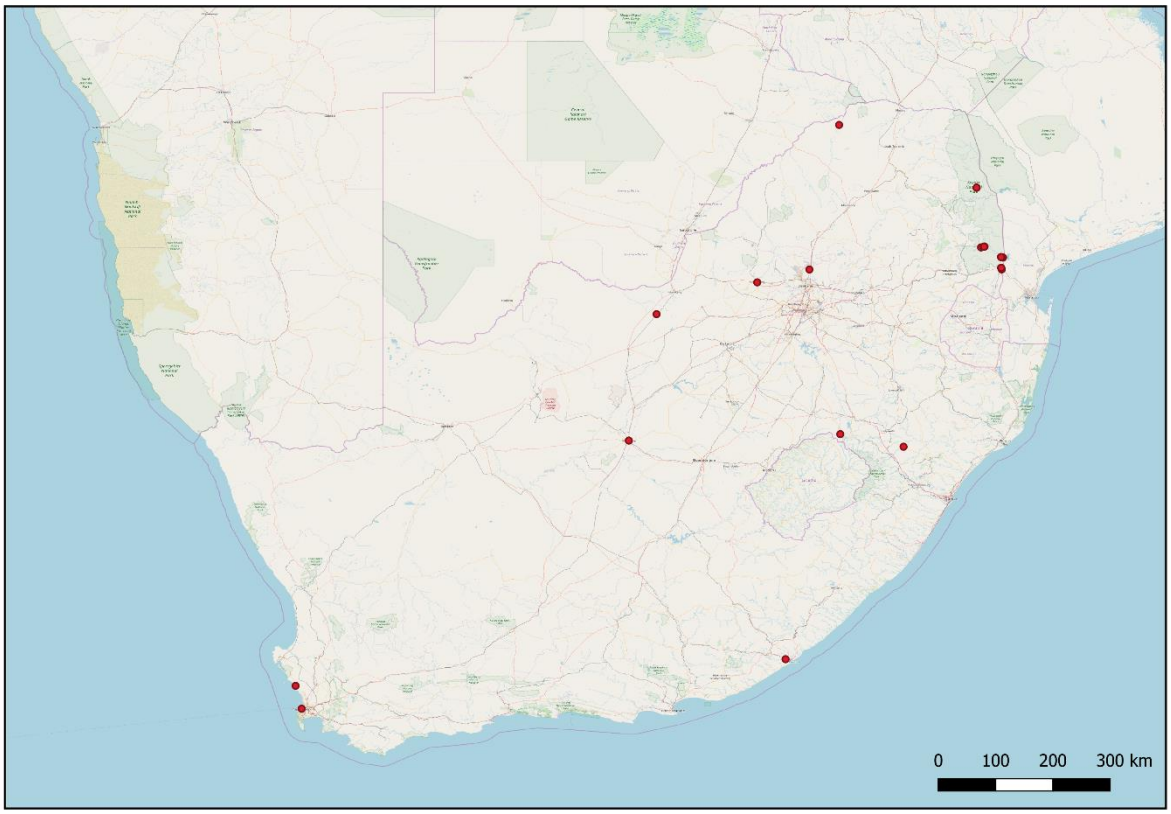

Figure 12: Distribution map representing locations at which *Philoliche suavis* (red dots) has been collected in South Africa (n= 60).

Distribution: Occurs widely across South Africa, found at Kruger National Park, Pretoria, Cape Town, Kimberly, East London and Ladysmith (Figure 12). Our records agrees with that of Usher (1972), excluding the specimens collected from the Cape.

Biology: Currently no information regarding the life history of *P. suavis* is known. Adults readily bite humans, a co-worker on this project was bitten by a *P. sauvis* while doing fieldwork in Kruger National Park.

Seasonality: Occurs during November to May (n= 58).

Vector status: No studies to date have investigated the vector potential of *P. suavis.*

Molecular data: No sequence data is available from GenBank. *Philoliche suavis* forms a well-supported monophyletic group sister to *Philoliche basalis* in support of the status of the subgenus

Buplex (Figure 8), (Oldroyd, 1957). The two dark specimens (Figure 8, DVTD 8; DVTD 59) (see Similar Species section) grouped within *P. sauvis* and with only two specimens available, placement in relation to the typical from of *P. sauvis* remains unclear and require further investigation. No AATS sequences were obtained during this study, however, all COI sequences will be submitted to GenBank.

Depositories: A holotype of *P. suavis* and a lectotype, *O. caffrica*, is available in the Zoologishes Museum der Universität, Berlin.

References: Mackerras (1955), Usher (1972) and Oldroyd (1957).

Philoliche (Dorcaloemus) medialis Oldroyd, 1957

http://tabanidae.myspecies.info/taxonomy/term/2647

Philoliche medialis Oldroyd, 1957:439 by original designation. Rhodesia; Mozambique,

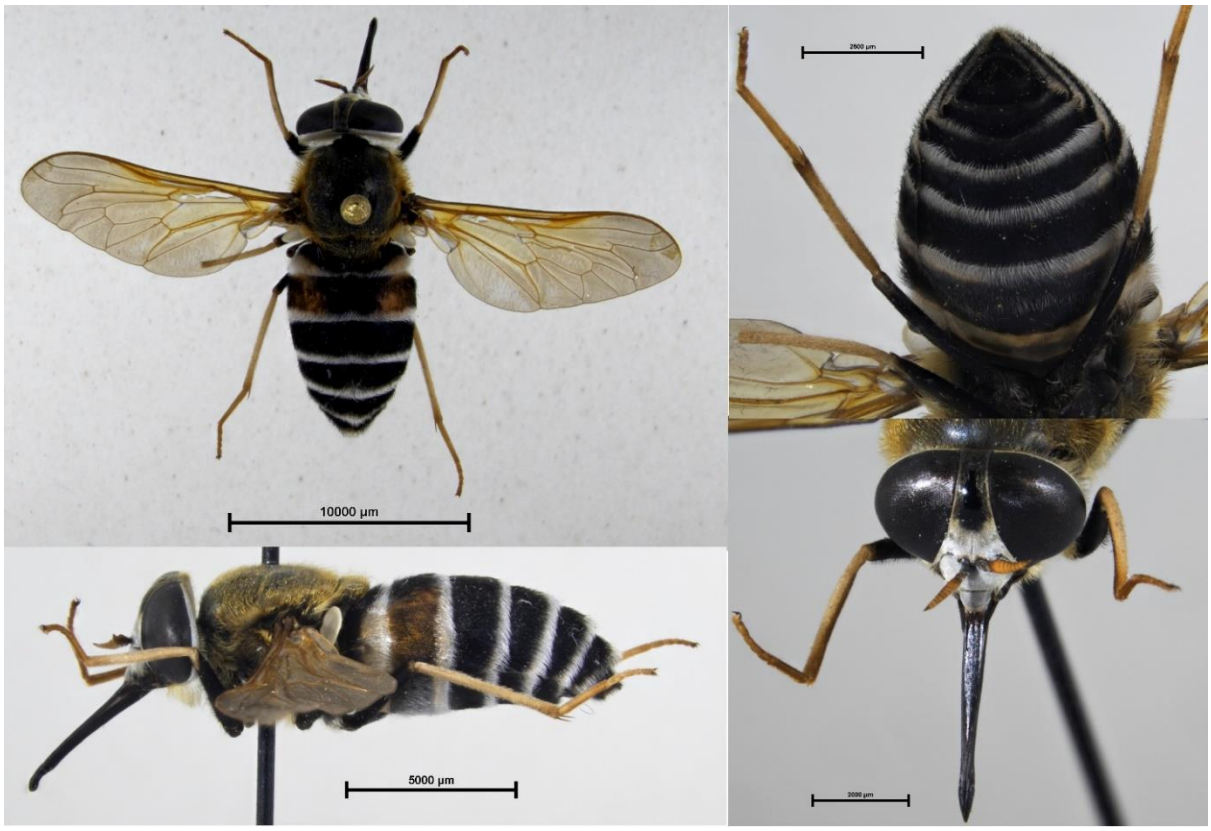

Figure 13: Sequential images of *Philoliche medialis* from top left going clockwise: dorsal, ventral, facial and lateral views.

Morphological diagnosis: A black, yellow and white species as seen on Figure 13, roughly 14mm in body length and 13mm in wing length. Both the proboscis and palpi are black, whist the palpi is reduced. The callus is shining black and well defined. The abdomen is black with white hind margins with yellow-red sub lateral spots on the first two segments. The wings are tinted a slight brown yellow but is clear.

Similar species: *Philoliche silverlocki* and *Philoliche auricomus*. The abdomen of *P. silverlocki* has no yellow hairs/spots. The abdomen of *P. auricomus* has yellow hind margins and the frons are broader with a dull and poorly defined callus.

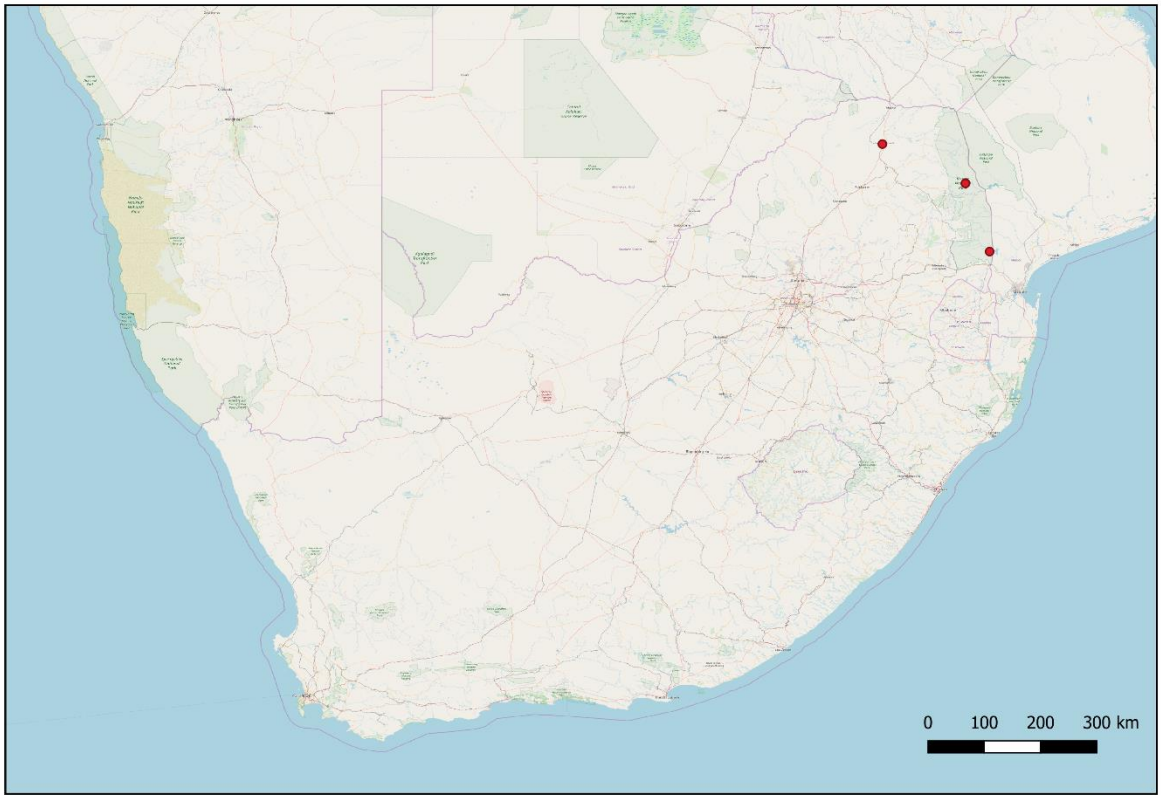

Figure 14: Distribution map representing locations at which *Philoliche medialis* (red dots) has been collected in South Africa (n= 5).

Distribution: Only five specimens have been collected in South Africa. According to available records, *P. medialis* occur in northeastern parts of South Africa, including Kruger National Park (Figure 14). This species was not recorded by Usher (1972) nor Oldroyd (1957) as occurring in South Africa and is here confirmed as present in South Africa.

Biology: Currently no information regarding the life history of *P. medialis* is known, however Usher (1972) has noted the species preference for forests.

Seasonality: Occurs during January to March (n=5).

Vector status: No studies to date have investigated the vector potential of *P. medialis.*

Molecular data: No sequence data is available on GenBank. *Philoliche medialis* clusters alongside *Philoliche angulate* and *Philoliche compacta* (Figure 8). While *P. medialis* and *P. compacta* are both species within the *Dorcaloemus* subgenus, the subgenus *Ommatiosteres* was recovered as paraphyletic, with *P.* (*Ommatiosteres*) *angulate* falling within *P.* (*Dorcaloemus*). No AATS sequences were obtained during this study. All COI sequences will be submitted to GenBank.

Depositories: A holotype is available in the British Museum.

References: Oldroyd (1957) and Usher (1972).

Philoliche (Philoliche) rondani (Bertoloni, 1861)

http://tabanidae.myspecies.info/taxonomy/term/2671

Pangonia rondani Bertoloni, 1861: 29 [1862: 56] by original designation. Mozambique

Diatomineura (Corizoneura) pallidipennis Ricardo, 1900: 110 by synonymy. Mozambique.

Corizoneura pallidipennis (Ricardo): Austen 1920: 140.

Pangonius rondanii (Bertoloni): Surcouf 1921: 128. [incorrect subsequent spelling].

Corizoneura rondanii (Bertoloni): Enderlein 1925: 266. [incorrect subsequent spelling].

Siridorhina pallidipennis (Ricardo): Enderlein 1925: 266.

Nuceria pallidipennis (Ricardo): Dias 1955: 176.

Philoliche (Philoliche) aethiopica ssp. *rondanii* (Bertoloni): Oldroyd 1957: 331. [incorrect subsequent spelling].

Philoliche (Philoliche) rondanii (Bertoloni): Dias 1966: 1182. [incorrect subsequent spelling].

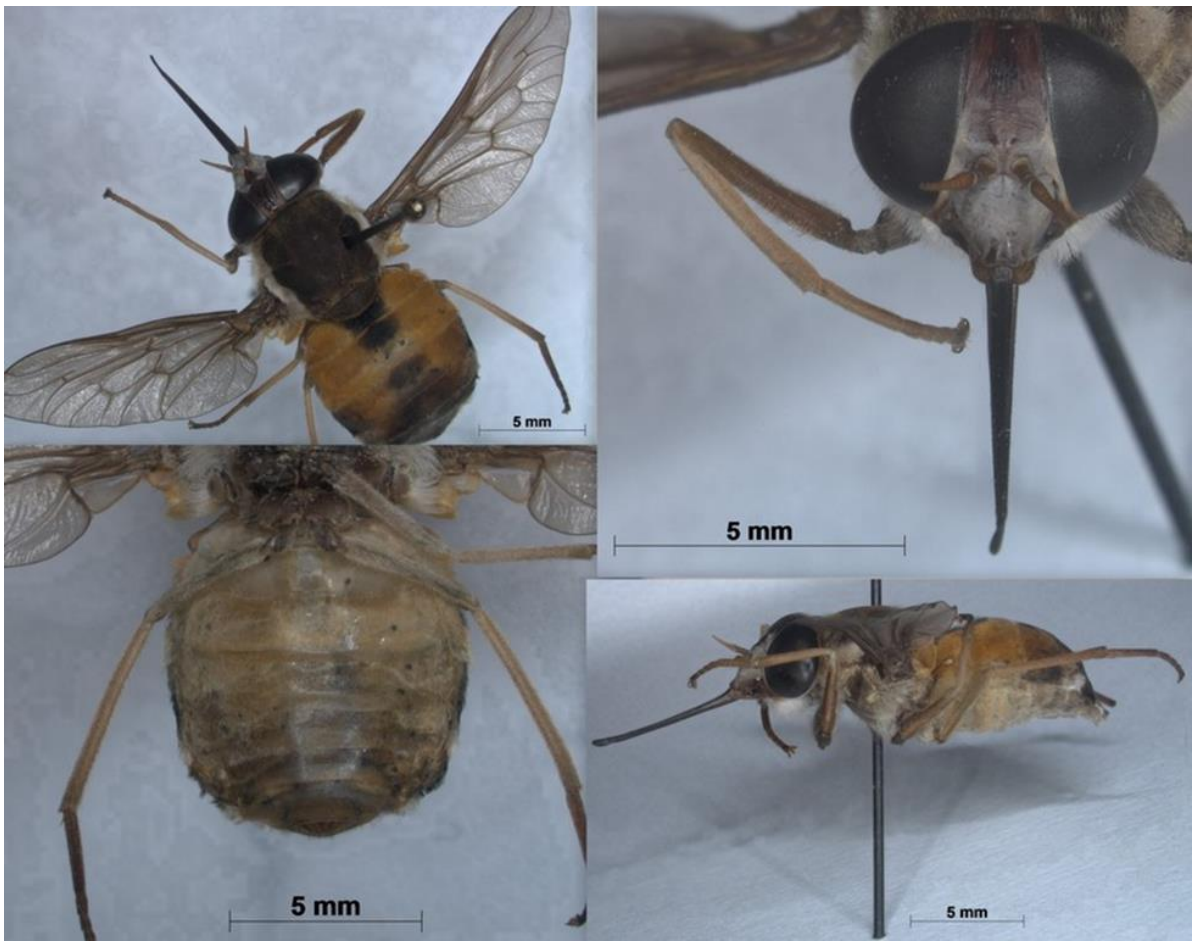

Figure 15: Sequential images of *Philoliche rondani* from top left going clockwise: dorsal, facial, lateral and ventral views.

Morphological diagnosis: A brown-yellow and black species, with an average body length of 16-17mm with a wing length of 16mm (Figure 15). The facial structure resembles a short cone. The

abdomen is an orange yellow in base colour with a black median spot expanding over the first two segments. A faint white median patch can be observed on the second or third segment. The hind abdomen has a dark triangular shape. The wings are clear with a slight smoky appearance.

Similar species: *Philoliche aethiopica*, which has more prominent abdominal markings, an extended face and dark spots on the wings. *Philoliche pallidipennis* is believed to be synonymous to *Philoliche rondani* but can be distinguished base on length of mouthparts, where *P. pallidpennis* is longer. This complex is discussed in detail by Morita (2008)

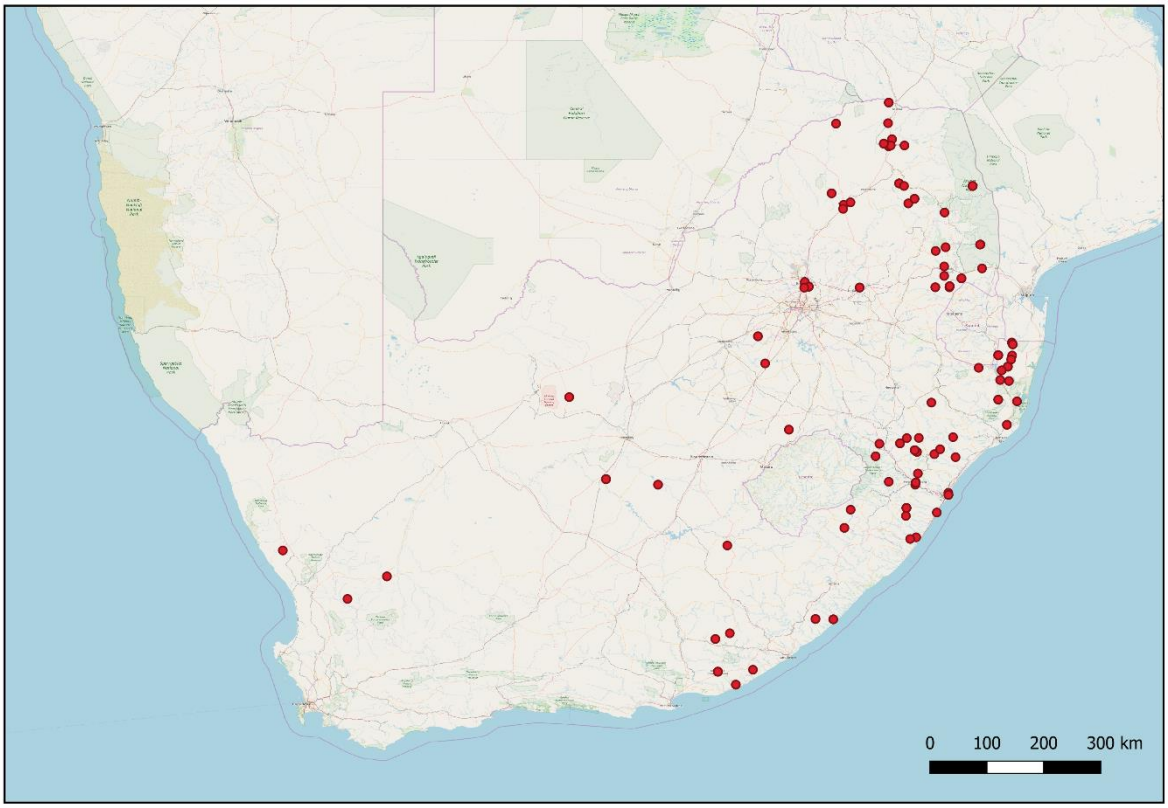

Figure 16: Distribution map representing locations at which *Philoliche rondani* (red dots) has been collected in South Africa (n= 218).

Distribution: Occurs mostly on the Eastern side from Kimberly of South Africa, with peak numbers along the costal and lowveld areas (Figure 16). Records agrees with that provided by Usher (1972) and detailed discussion of the distribution of the species complex is discussed in Morita (2008).

Biology: *P. rondani* is known to pollinate an orchid species (*Disa pulchra*) as well as flowers of *Agapanthus campanulatus* (Johnson, 2006). Jersáková *et al.* (2012) found that *P. rondani* preferred pink and blue flowers. Currently no other information regarding the life history of *P. rondani* is known.

Seasonality: Occurs during October to May (n= 209).

Vector status: No studies to date have investigated the vector potential of *P. rondani.*

Molecular data: On GenBank, four sequences are available, two CAD and two COI sequences. The sequences obtained during this study clustered within the "rondani" group, however, did not cluster with a *P. rondani* reference sequence from GenBank (DQ983528) (Figure 8). Interestingly, the *P. aethiopica* sample clustered with *P.* (*Maverica*) *bukamensis*, however, no support for the grouping was recovered and placement may be erroneous due to a small sample size. No AATS sequences were obtained during the study and al COI sequences will be submitted to GenBank.

Depositories: A holotype of *P. rondani* is available from the Natural History Museum in London. A holotype of *P. pallidipennis* is available from the British Museum.

References: Usher (1972), Oldroyd (1957), Johnson (2006), Morita (2008) and Jersáková *et al.* (2012).

Tribe Haematopotini

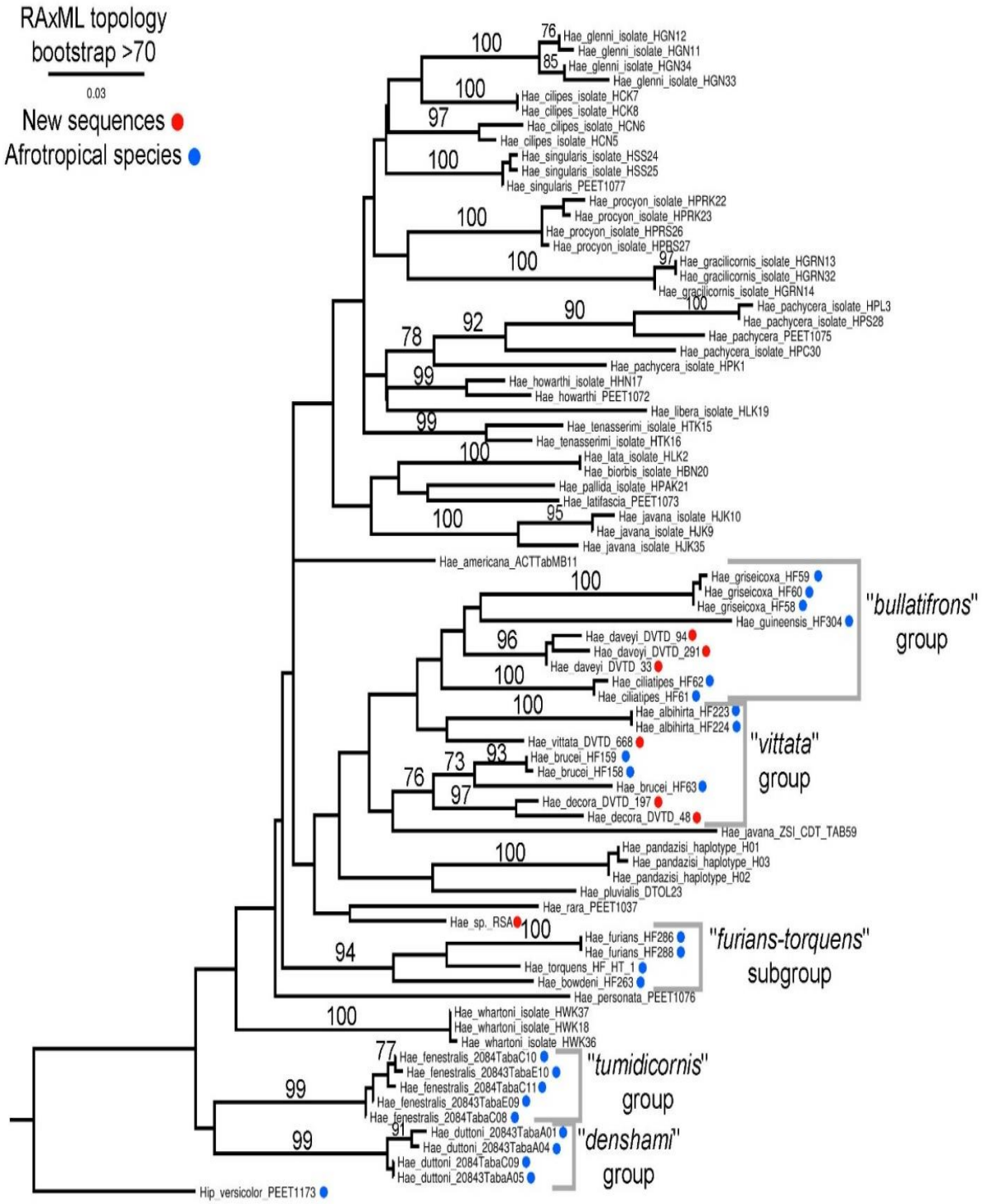

Figure 17: COI phylogenetic analysis using RaXML of the Haematopini tribe by Maximum likelihood (ML) alongside reference sequences obtained from GenBank. In total 78 taxa, with a matrix length of 658 characters, was analysed. Red dots indicate new sequences obtained during this study; blue dots are reference sequences of species that occur in the Afrotropics.

Haematopota daveyi Austen, 1912

http://tabanidae.myspecies.info/taxonomy/term/1624

Haematopota daveyi Austen, 1912:408 by original designation. Malawi; Mozambique, Rhodesia, South Africa, Tanzania, Zaire.

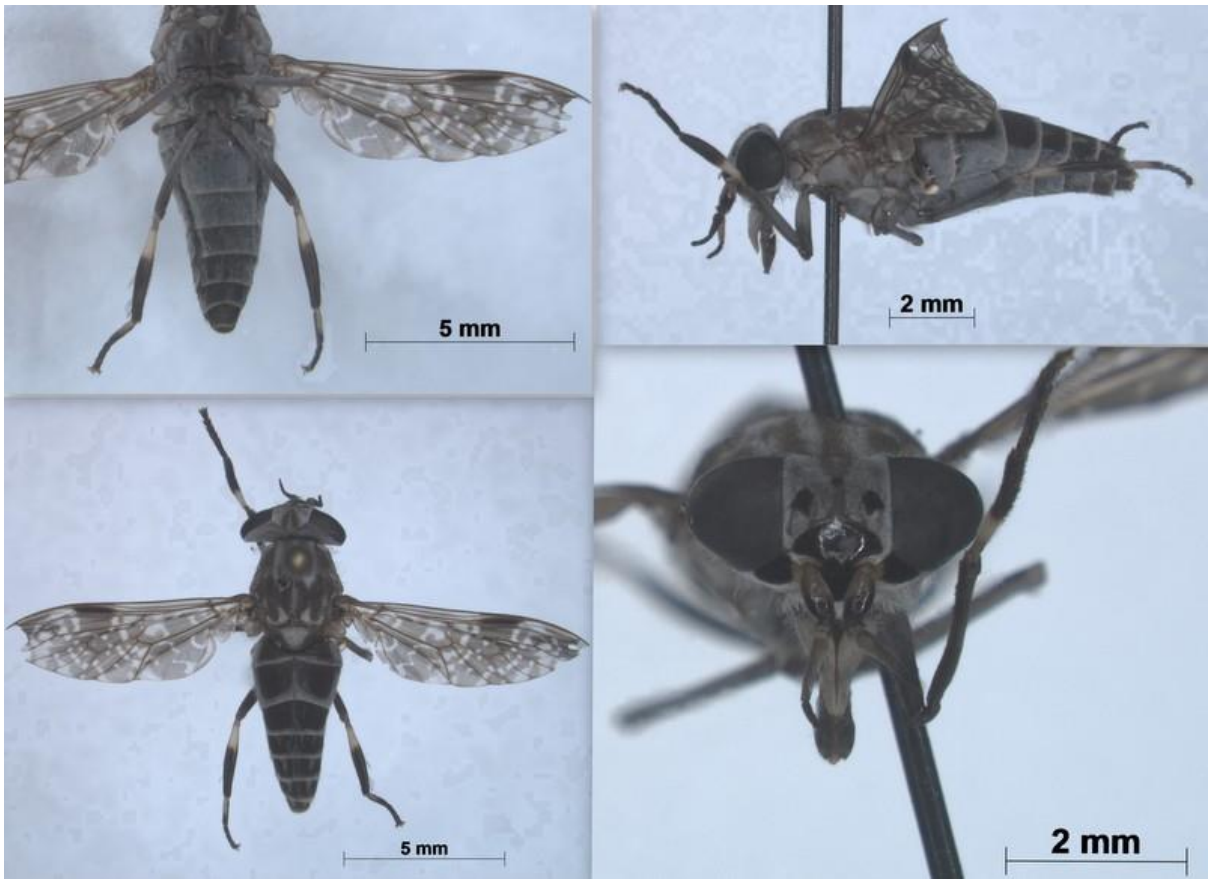

Figure 18: Sequential images of *Haematopota daveyi* from top left going clockwise: ventral, lateral, facial and dorsal views.

Morphological diagnosis: A brown-black and grey species, with an estimated 11mm in body length and 10mm in wing length (Figure 18). The callus is prominent but does not reach the eye margins, with large velvet spots under the eyes. The abdomen is a dark brown base colour with a narrow grey boarder around each segment. A narrow grey median strip is present with a pair of elongated narrow spots on the last four posterior segments. A single broad white basal ring is clearly visible on the flattened fore and hind tibia. The middle tibia has two pale rings. Wings have white marking in rosettes shapes evenly distributed along the wing.

Similar species: *Haematopota bullatifrons* and *Haematopota grahami. H. daveyi* is morphologically a combination of the aforementioned species; where the head structure resembles *H. bullatifons* with the abdominal and leg pattern of *H. grahami*.

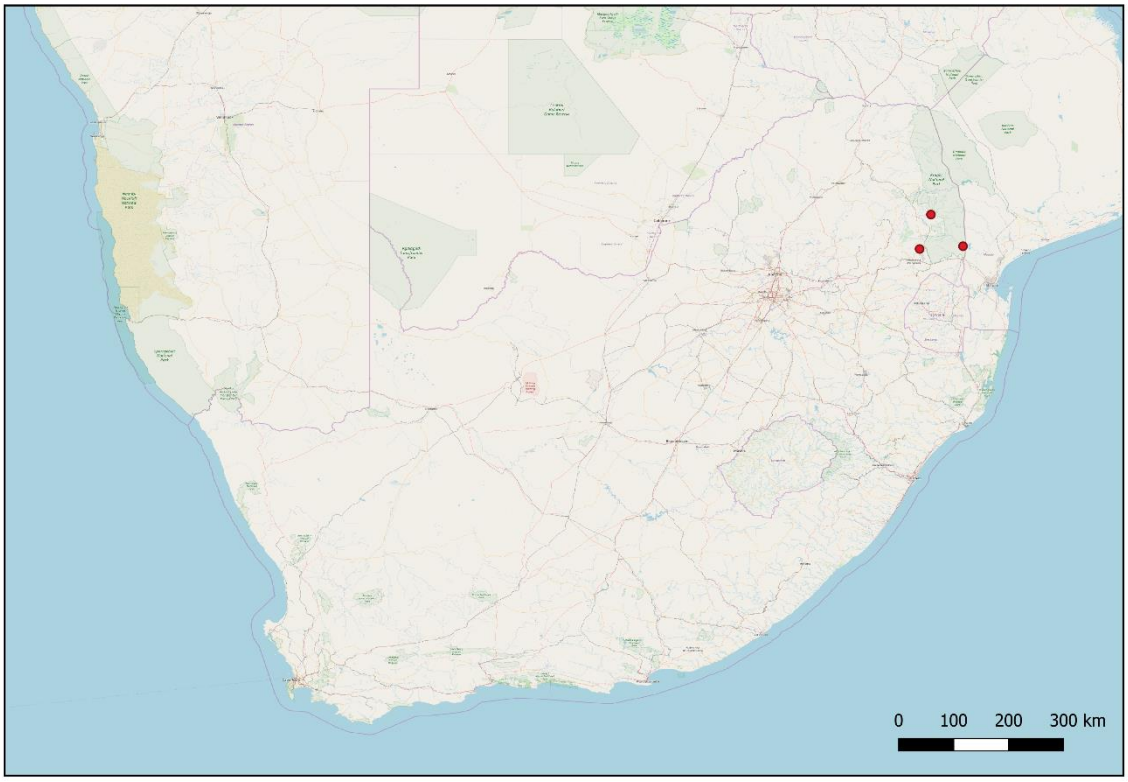

Figure 19: Distribution map representing locations at which *Haematopota daveyi* (red dots) has been collected in South Africa (n= 3).

Distribution: Only three records are available from South Africa, all restricted to the southern parts of Kruger National Park (Figure 19). Usher (1972) has not reported of any sample within South Africa, but some were obtained along the South African border on the Zimbabwe and Botswana side.

Biology: Currently no other information regarding the life history of *H. daveyi* is known.

Seasonality: Occurs during January to May (n=3).

Vector status: No studies to date have investigated the vector potential of *H. daveyi.*

Molecular data: No sequence data is available on GenBank. During this study*, H. daveyi* clustered with three other species in an unsupported monophyletic "*bullatifrons*" cluster with all species forming well supported monophyletic groups within the "*bullatifrons*" group (Figure 17). AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: At the time of writing, the deposition of the type specimen(s) could not be confirmed but are thought to be deposited in the British Museum.

References: Marchand (1920), Usher (1972) and Oldroyd (1952).

Haematopota decora Walker, 1856

http://tabanidae.myspecies.info/taxonomy/term/1625

Haematopota decora Walker, 1856:454 by original designation. South Africa; widespread through western Africa to eastern Africa, Congo basin & southern Africa.

Haematopota dorsalis Loew, 1858: 342 [1860: 124] by synonymy. South Africa.

Tylopelma niveipalpis Enderlein, 1925 :40 by synonymy. Togo.

Haematopota decora ssp. constricta Travassos Dias & de Sousa, 1955:49 by synonymy. Mozambique.

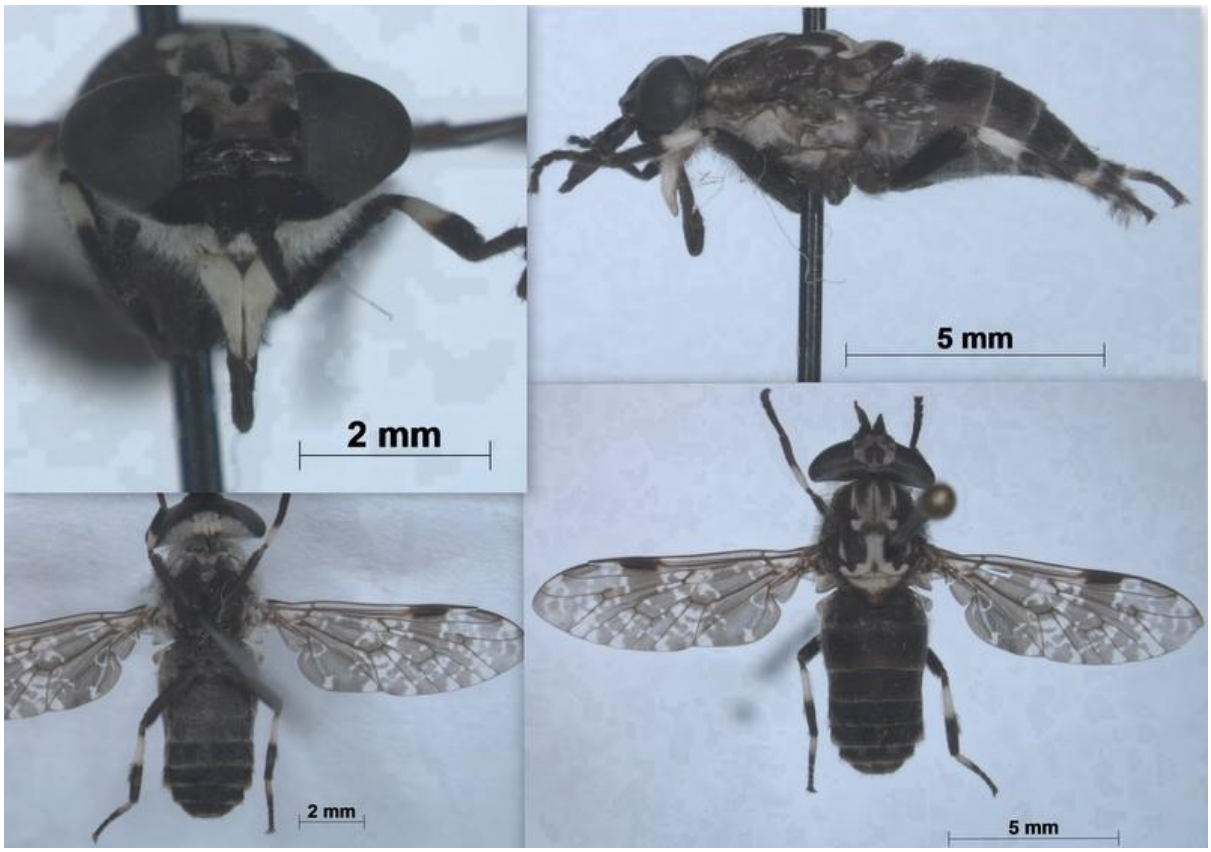

Figure 20: Sequential images of *Haematopota decora* from top left going clockwise: facial, lateral, dorsal and ventral views.

Morphological diagnosis: A brown and grey species, about 9mm in length and 9mm in wing length (Figure 20). A transverse callus, dark brown, touches the eye margins. The abdomen is mostly brown in base colour with grey areas alongside the hind- and lateral margins, with an indistinct narrow median line. Small paired spots are visible on the last few posterior segments. Distinct white rings are visible on all tibia, whilst a second white spot may be present on the hind tibia. Wings have white marking in rosettes shapes evenly distributed along the wing.

Similar species: *Haematopota brucei* has similar bold black-brown and white patterns but is clearly a bigger species with more of a blacker undertone than brown.

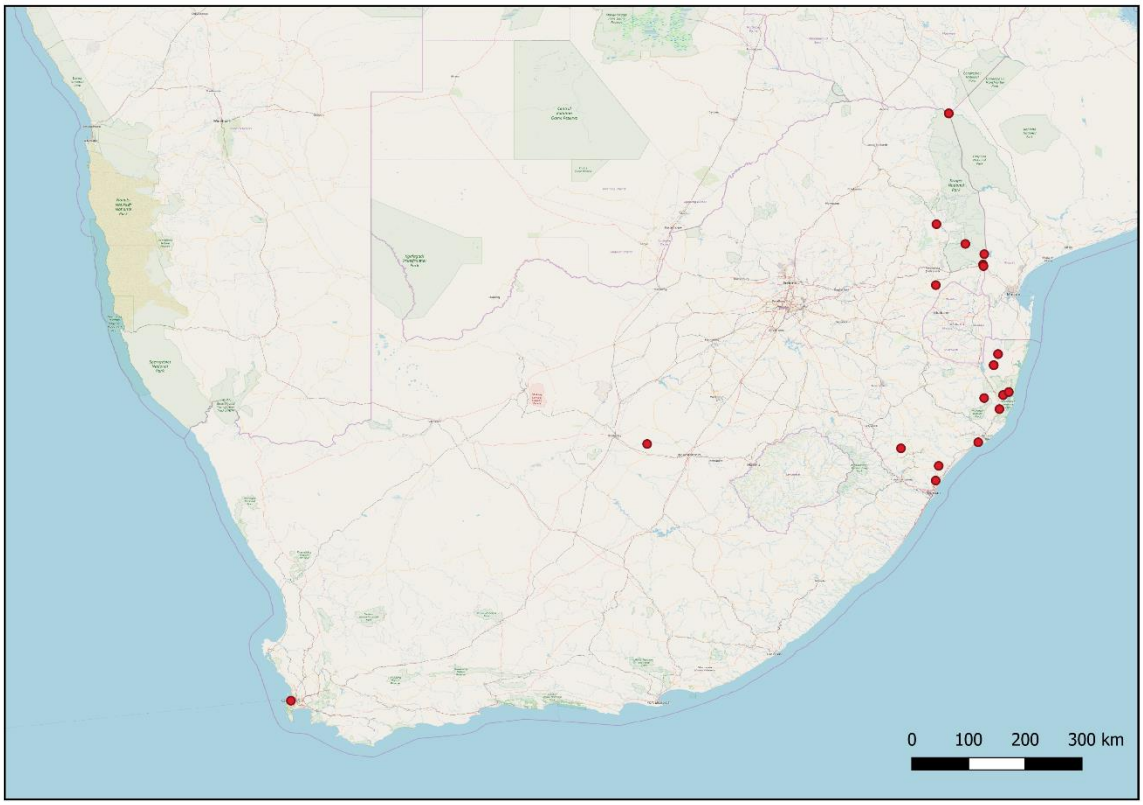

Figure 21: Distribution map representing locations at which *Haematopota decora* (red dots) has been collected in South Africa (n=27).

Distribution: Occurs in the KwaZulu-Natal area, as well as within Kruger National Park, one sample was obtained between Kimberley and Bloemfontein and one from Cape Town (Figure 21). Usher (1972) mention one sample from Pretoria as well.

Biology: *H. decora* prefers low-lying and dry areas. The larval stage resembles that of all *Haematopota* with the exception of orange pigmentation on the anal segments. The pupa has a regular shape with a dorso-lateral comb consisting of three spines. Usher (1972) noted that adults of the species tend to rest above the tail base of cows, avoiding the swishing.

Seasonality: Occurs during September to April (n=26).

Vector status: Partial development of *Loa loa* has been observed in *H. decora* samples collected from Nigeria during a vector competence study (Woodman and Bokhari, 1941).

Molecular data: No Genbank sequence data is available. During this study, *H. decora* clustered together within a paraphyletic "*vittata*" group forming a well-supported monophyletic group (bs.=97) (Figure 17). *H. decora* was recovered as sister to *Haematopota brucei*, an Afrotropical

species. AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: A holotype is available at the British Museum. A type sample of *Tylopelma niveipalpis* Enderlein is available in the Zoologisches Museum der Universität, Berlin.

References: Woodman and Bokhari (1941), Oldroyd (1952) and Usher (1972).

Haematopota vittata Loew, 1858

http://tabanidae.myspecies.info/taxonomy/term/2029

Haematopota vittata Loew, 1858:336 [1860:122] by original designation. South Africa; Angola, Botswana, Kenya, Malawi, Mozambique, Rhodesia, Tanzania, Uganda, Zaire, Zambia.

Haematopota pulchrithorax Austen, 1906: 54 by synonymy. Rhodesia.

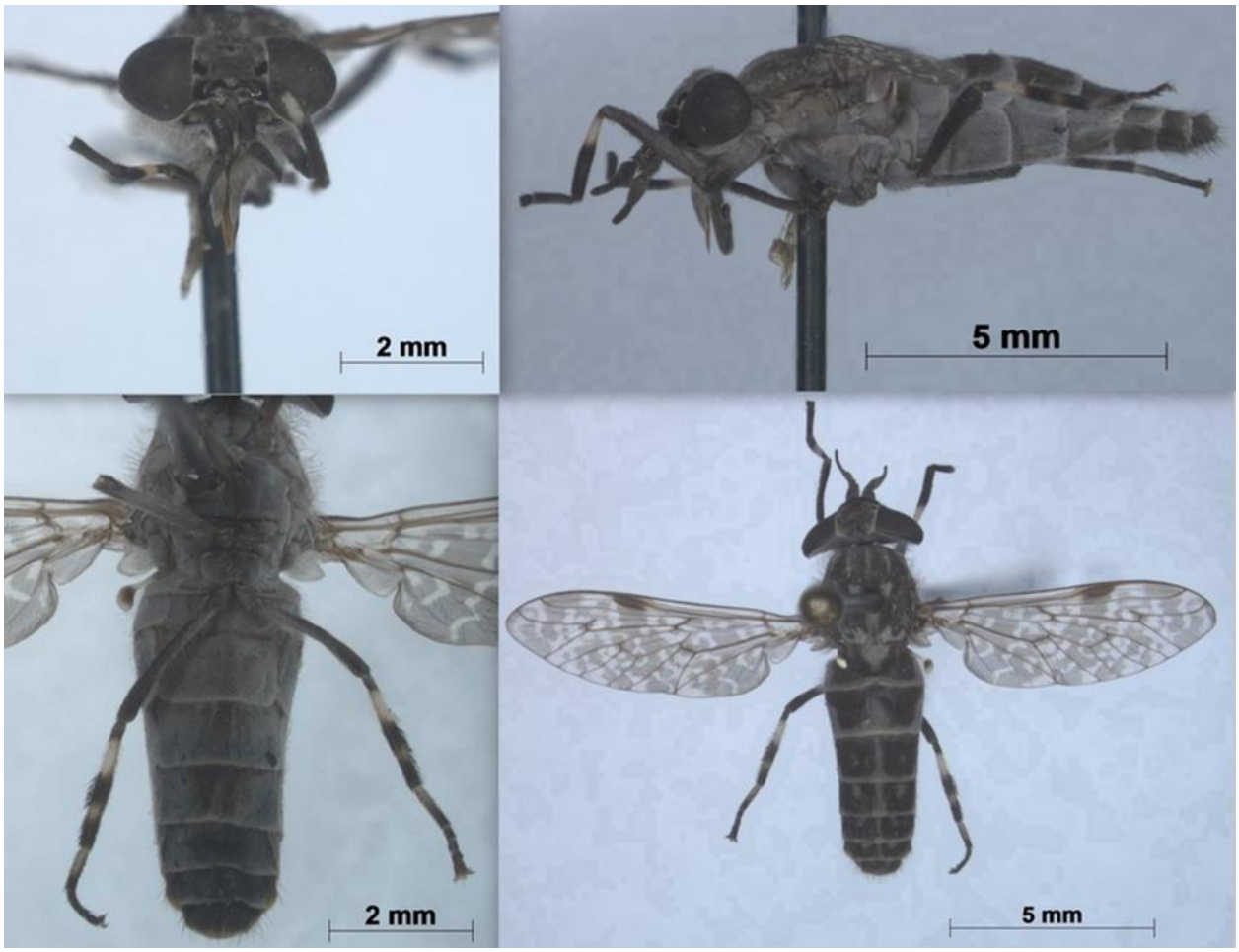

Figure 22: Sequential images of *Haematopota vittata* from top left going clockwise: facial, lateral, dorsal and ventral views.

Morphological diagnosis: A brown and grey species that's roughly 11mm in body length and has a 10mm wing length (Figure 22). The callus is a deep brown, low laying bare patch that reaches the eye margin and has a deep central notch. The abdomen is a red brown with grey boarders around each abdominal segment and a prominent medial grey line is visible. From the third segment onwards, pairs of distinct grey spots are present. Both the tibia and tarsi are black with a white band (second band may be pale). Wings have blurred rosettes that tend to merge together.

Similar species: None

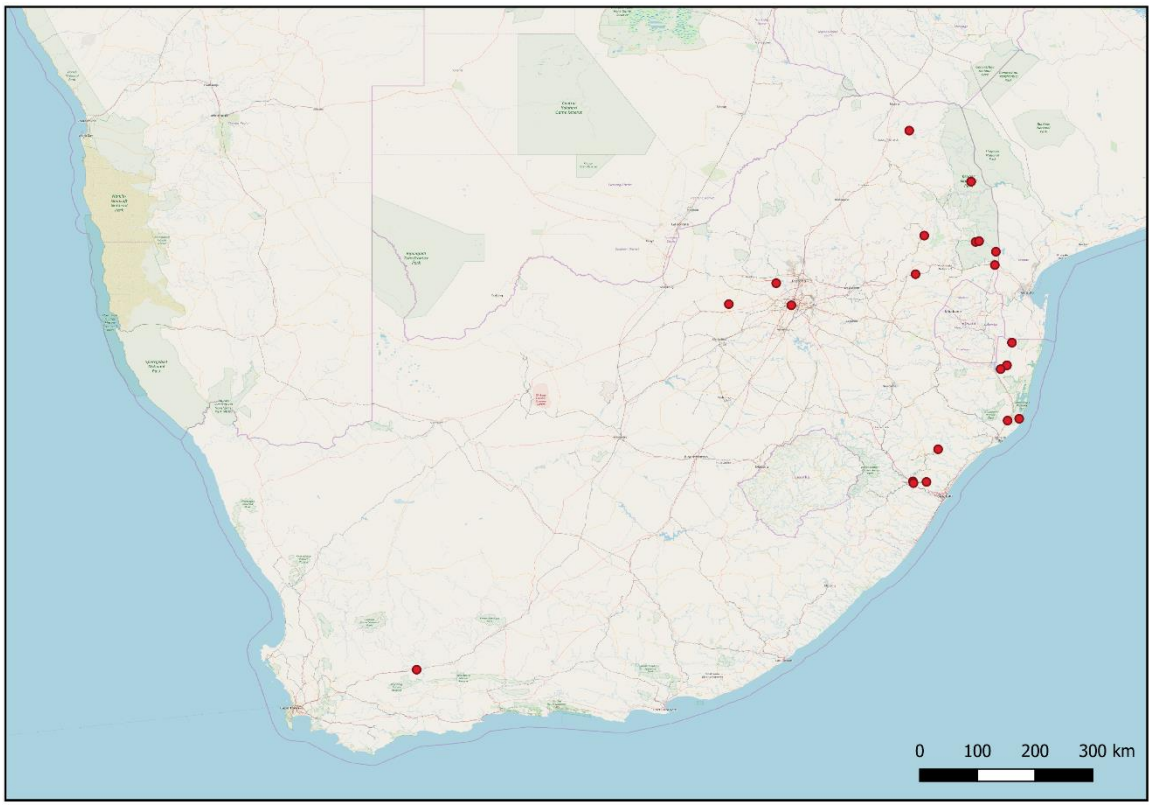

Figure 23: Distribution map representing locations at which *Haematopota vitatta* (red dots) has been collected in South Africa (n=26).

Distribution: Occurs in the KwaZulu-Natal and Limpopo (Kruger National Park) areas, with a few samples collected around Pretoria and one suspect locality from the Western Cape Province (Figure 23). Usher (1972) reported a similar pattern of distribution.

Biology: Currently no information regarding the life history of *H. vitatta* is known. Usher (1972) noted the species preference for bushveld regions.

Seasonality: Occurs during September to April (n=25).

Vector status: Partial development of *Loa loa* was detected in *H. vittata* during a competency study conducted in Nigeria (Woodman and Bokhari, 1941).

Molecular data: No sequence data is available on GenBank. During this study, *H. vittata* was recovered as sister to *Haematopota albihita*, an Afrotropic species, within a paraphyletic "*vittata"* group (Figure 17). AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: Holotype can be located at the British Museum.

References: Woodman and Bokhari (1941), Oldroyd (1952) and Usher (1972).

Tribe Tabanini

Figure 24: COI phylogenetic analysis using RaXML of the Tabanini tribe by Maximum likelihood (ML) alongside reference sequences obtained from GenBank. In total 116 taxa, with a matrix length of 662 characters were analysed. The GTR + G model was used, with a random starting tree and using the autoMRE bootstopping function for bootstrap support calculations. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Red dots indicate new sequences obtained during this study; purple arrows indicate incongruencies observed.

Atylotus agrestis (Wiedeman, 1828)

http://tabanidae.myspecies.info/taxonomy/term/250

Tabanus agrestis Wiedemann, 1828:557 by original designation. Egypt

Tabanus albicans Macquart, 1834-2:204 by synonymy. Senegal.

Tabanus ditoeniatus Macquart, 1838:130 by synonymy. Mauritius & Reunion.

Tabanus bipunctatus Wulp, 1885:75 by synonymy. Ghana & South Africa.

Tabanus soudanensis Cazalbou in Laveran, 1904: 352 a *nomen nudum*.

Tabanus ghidini Pechuman, 1949:84 by synonymy. Originally a replacement name for *Tabanus lacustris* Ghidini, 1938:343 as junior homonym of *Tabanus lacustris* Stone, 1935.

ditaeniatus. Incorrect subsequent spelling of *ditoeniatus*.

sudanicus. Incorrect subsequent spelling of *soudanensis*.

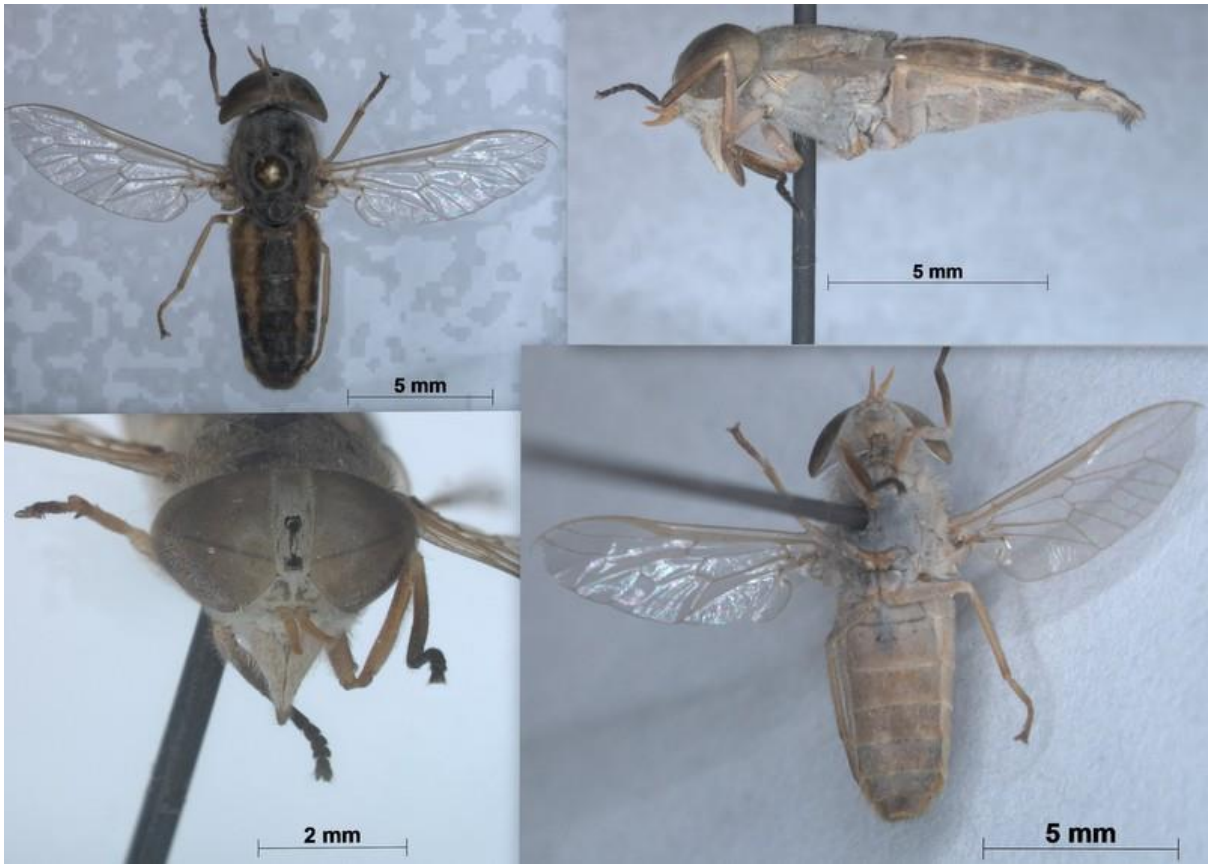

Figure 25: Sequential images of *Atylotus agrestis* from top left going clockwise: dorsal, lateral, ventral and facial views.

Morphological diagnosis: A grey-brown species, roughly 14mm in length, with a wing length of 11mm (Figure 25). The eyes have a central, very narrow single purple band, while the fronts have

two small, round, well separated calli. Two distinct golden yellow sub-lateral stripes on the abdomen. Wings are clear.

Similar species: *Atylotus fuscipes*, distinguishable by variation in leg colour as well as a longer post-occipital fringe and a more prominent subcallus. *Atylotus afghanistanicus* separated in distance where *A. afghanistanicus* occurs only in Arabia, India and China. *Atylotus diurnus*, distinguished by the larger size, more grey-like appearance and three well defined longitudinal stripes on the abdomen. *Atylotus nigromaculatus*, identified by a longer post-occipital fringe and three well defined longitudinal abdominal stripes.

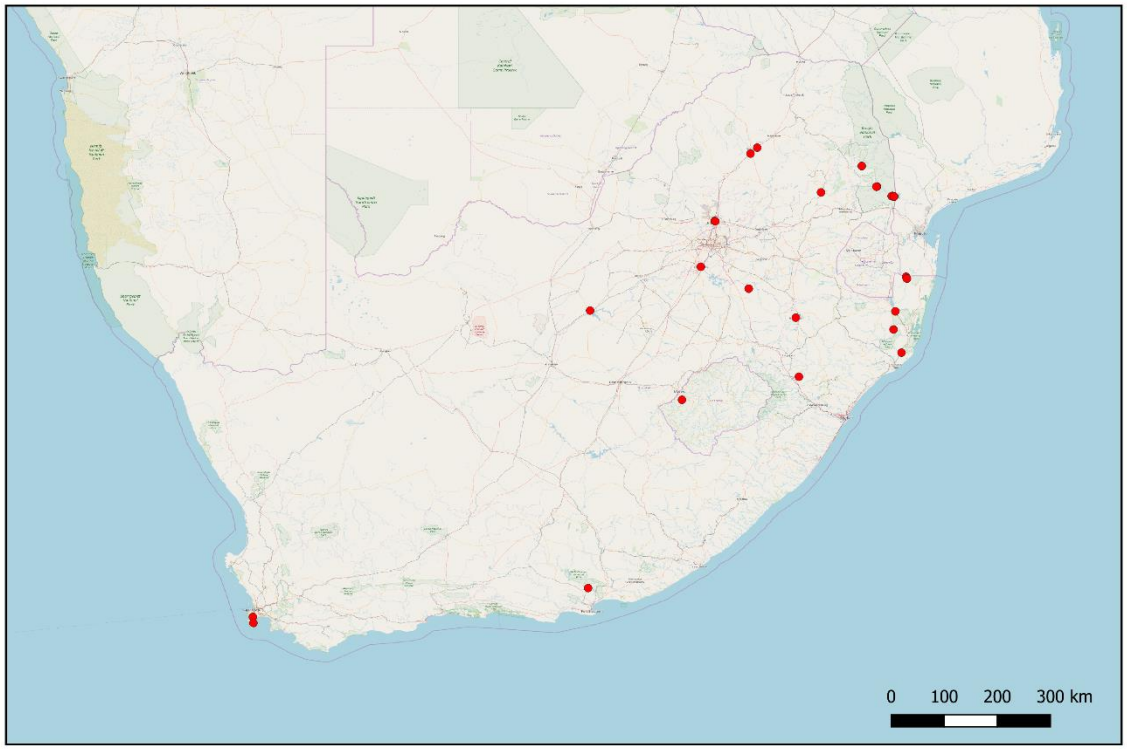

Figure 26: Distribution map representing locations at which *Atylotus agrestis* (red dots) has been collected in South Africa (n=54).

Distribution: A widespread species, occurring in Savanna and coastal regions (Figure 26).

Biology: On average, three generations occur per year (trivoltine). According to Veer (1999), *A. agrestis* lays eggs (1.2mm long and 0.2 mm broad) on grass blades bordering shallow streams or ponds. The larvae reside at the bottom of the stream/pool and occasionally rises via lashing motion to the surface to breath. The time between egg hatching and emergences as adults may vary and ranges from 26 to 100 days. The pupal period is an estimated six days. Larvae has been found to feed on plant matter surrounding the waterbody. The species is not known to bite humans and are silent flyers (Usher, 1972).

Seasonality: Occurs during September to April (n=49).

Vector status: Known as mechanical vectors of *Trypanosoma vivax* and *Trypanosoma congolense* confirmed by vector competency study conducted in Bobo-Dioulasso (West-Africa)(Desquesnes and Dia, 2003a, b), influenza virus detected by pathogen screening in Kazakhastan and *Babesia bigemina* detected by pathogen screening in Zambia (Taioe, 2017).

Molecular data: Five COI sequences are available on GenBank. During the present study, COI was able to distinguish between all Atylotus species, excluding those that occur in Africa (*A. agrestis*, *A. diurnus* and *A. nigromaculatus*) (Figure 24 and 27). The Afrotrpical *Atylotus* species cluster together, forming a monophyletic group, with a bootstrap value of 99 (Figure 27). Since COI readily distinguishes between *Atylotus* species elsewhere, it will be interesting if it does not have the same ability in the Afrotropics. Since the species in the Afrotropics are all similar morphologically, synonymy should be considered in subsequent studies including another molecular marker, ideally a nuclear region for alternative signal. AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank

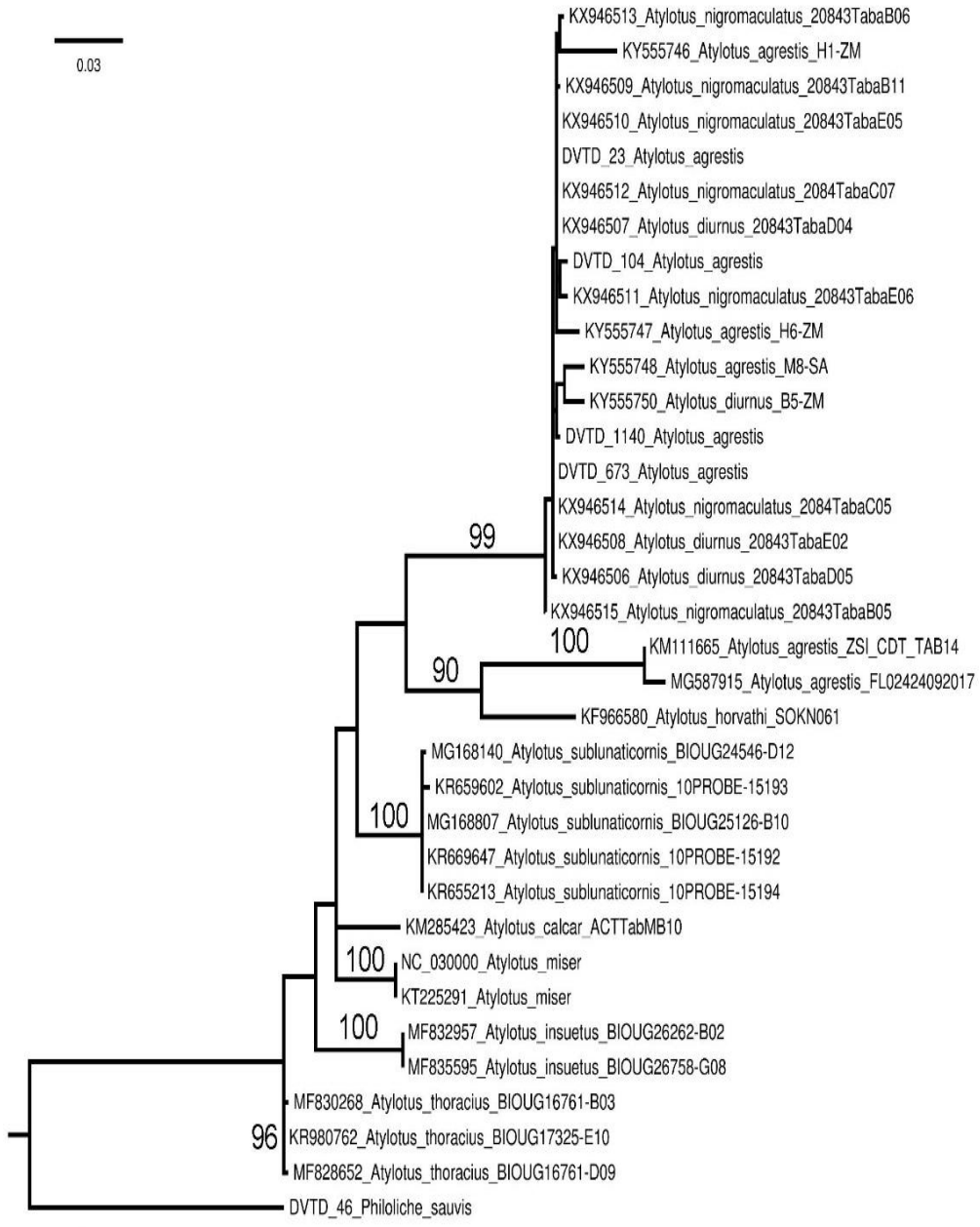

Figure 27: COI Phylogenetic analysis of the *Atylotus* genus by Maximum Likelihood using RaXML alongside reference sequences obtained from GenBank. In total 35 taxa, each with 658 characters were evaluated. The GTR + Gamma model was used with a random starting tree and using the autoMRE bootstopping function for bootstrap support calculations. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Depositories: Holotype is located in the British Museum.

References: Oldroyd (1954), Usher (1972), Ouhelli and Dakkak (1987), Veer (1999), Desquesnes and Dia (2003a), Desquesnes and Dia (2003b) and Taioe (2017).

Tabanus atrimanus Loew, 1858

http://tabanidae.myspecies.info/taxonomy/term/3293

Tabanus atrimanus Loew, 1858:340 [1860c: 112] by original designation. South Africa; Angola, Kenya, Malawi, Mozambique, Rhodesia, Tanzania.

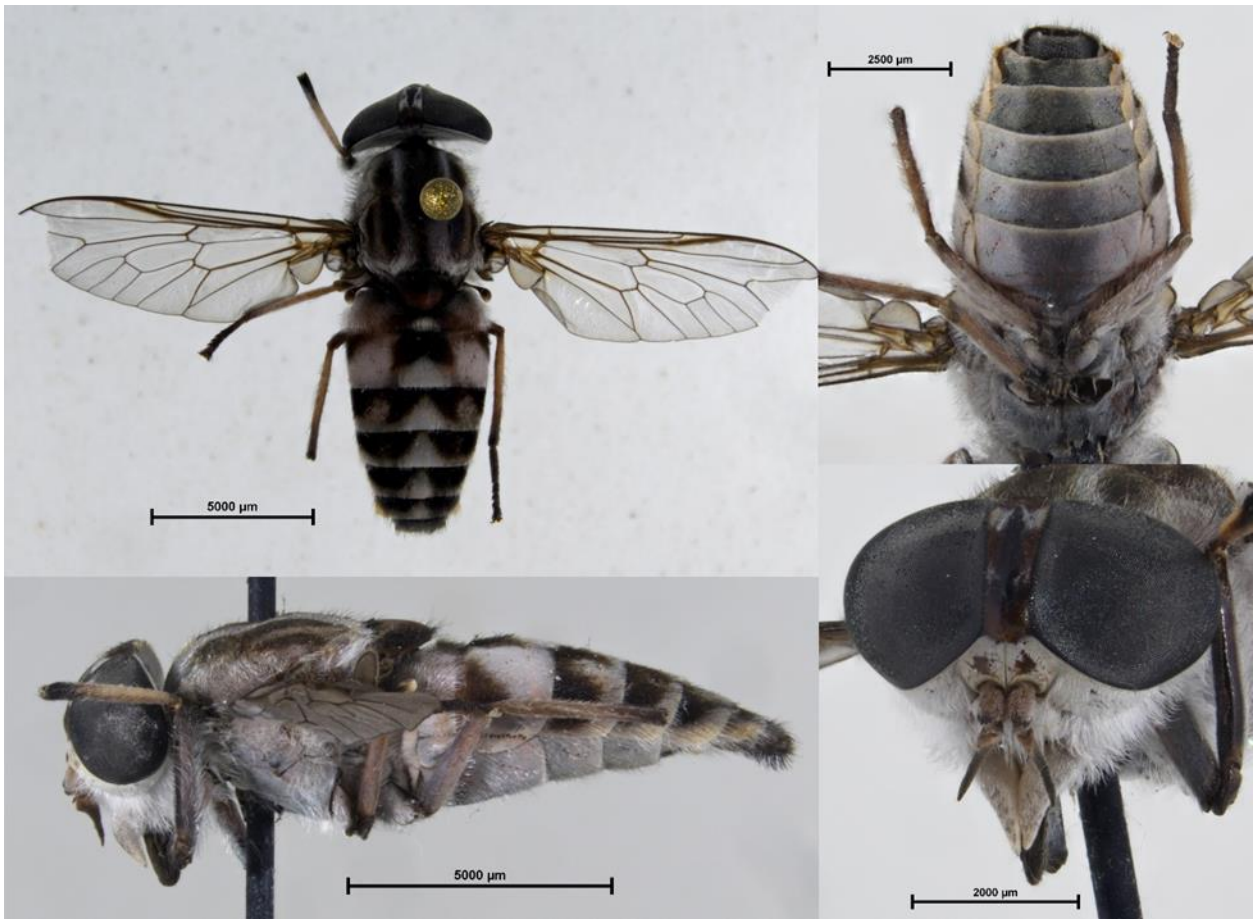

Figure 28: Sequential images of *Tabanus atrimanus* from top left going clockwise: dorsal, ventral, facial and lateral views.

Morphological diagnosis: A black and white species, estimated 13mm in body length and 11mm in wing length (Figure 28). A reddish tint can be seen along the sublateral stripes on the mesonotum. These sublateral stripes are white in colour and prominent on the hind margin of the mesonotum. The abdomen is black, adorned with a white median triangular pattern, with white paired lateral spots on the first three segments. All posterior segments have a whiteish narrow hind margin. The wings are clear with a yellowish stigma.

Similar species: *Tabanus variabilis* which could be distinguished from *T. atrimanus* by the absence of the prescutellar crescent on the mesonotum, the medial spots from the second segment onwards on the abdomen is smaller and no bands are visible in the eyes.

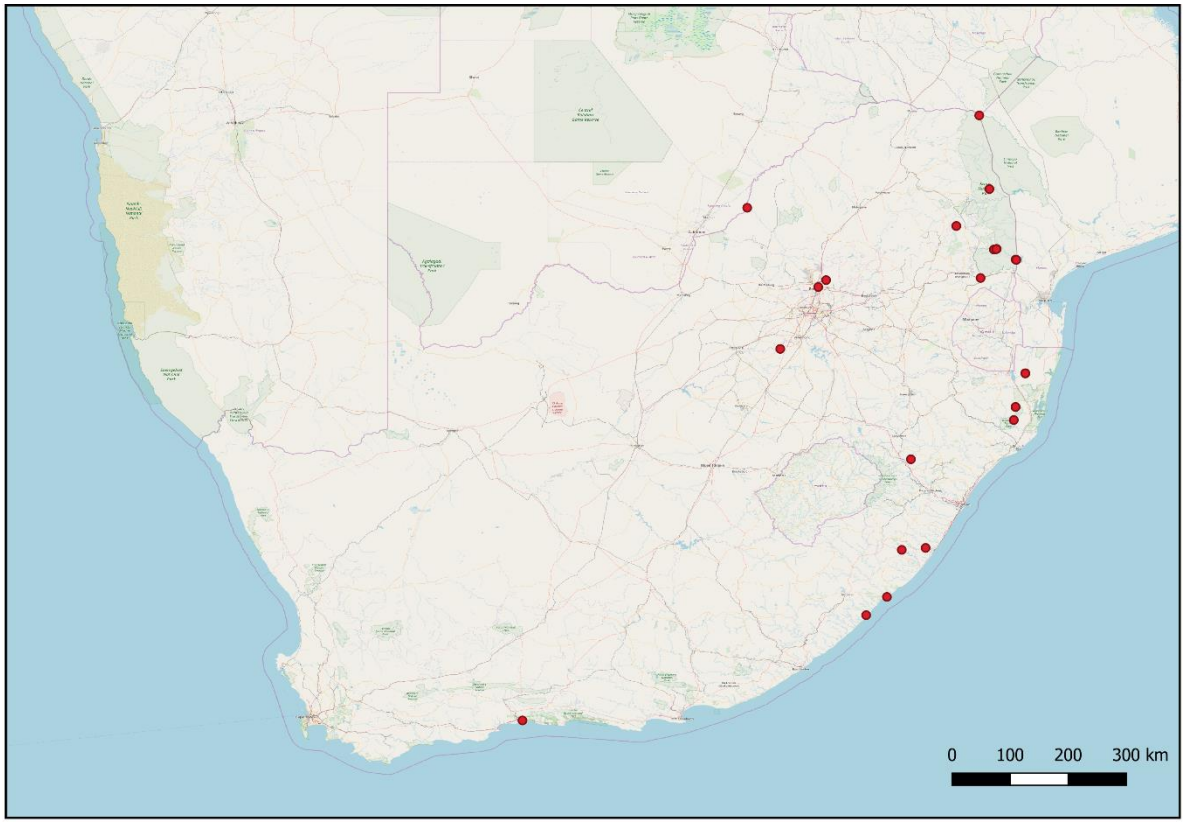

Figure 29: Distribution map representing locations at which *Tabanus atrimanus* (red dots) has been collected in South Africa (n=34).

Distribution: Occurs along the south-eastern costal area up into the Kruger National Park (Figure 29). Some samples obtained around the Pretoria area. Usher (1972) records a similar distribution.

Biology: Currently no information regarding the life history of *T. atrimanus* is known, however, this species has been documented several times as biting humans (Usher, 1972). The species is associated with running water and cool areas (Oldroyd, 1954).

Seasonality: Occurs during September to July (n=32).

Vector status: No studies to date have investigated the vector potential of *T. atrimanus.*

Molecular data: No sequence data is available on GenBank. Here, *T. atrimanus* forms monophyletic group, sister to *Tabanus leucostomus,* each clade with high bootstrap values (Figure 24). The sister grouping however, was not supported. AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: Holotypes for both *T. atrimanus* and *T. variabilis* can be located at the Zoologisches Museum der Universität, Berlin.

References: Oldroyd (1954), Usher (1972).

Tabanus gratus Loew, 1858

http://tabanidae.myspecies.info/taxonomy/term/3677

Tabanus gratus Loew, 1858: 340 [1860c: 114] by original designation. South Africa; widespread in Afrotropical region, outside forests, Egypt.

Tabanus tritaeniatus Ricardo, 1908:311 by synonymy. Angola; Zambia.

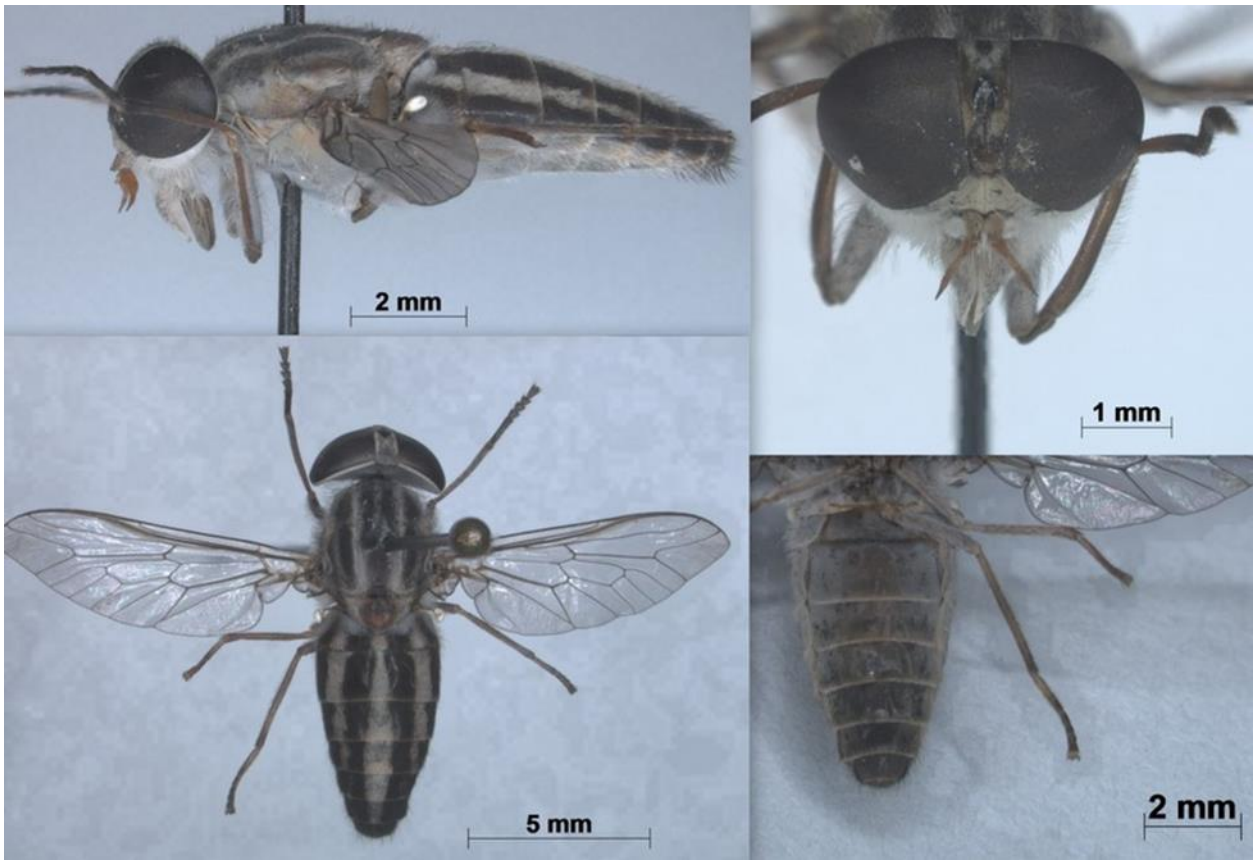

Figure 30: Sequential images of *Tabanus gratus* from top left going clockwise: lateral, facial, ventral and dorsal views.

Morphological diagnosis: A black and white species, roughly 12mm in body length and 10mm in wing length (Figure 30). The mesonotum is brown with a bold white patter. The pattern constitutes of medial, sublateral and supra-alar stripes as well as white side-margins. The abdomen has three characteristically white stripes, the medial one being the broadest. The medial stripe broadens towards the fourth abdominal segment, tapering to the posterior end afterwards. The sublateral stripes are broad anteriorly but also tapper towards the posterior side.

Similar species: *Tabanus pertinens* which can be distinguished by a more cylindrical abdomen, broader stripes on the abdomen and structural difference in the frons.

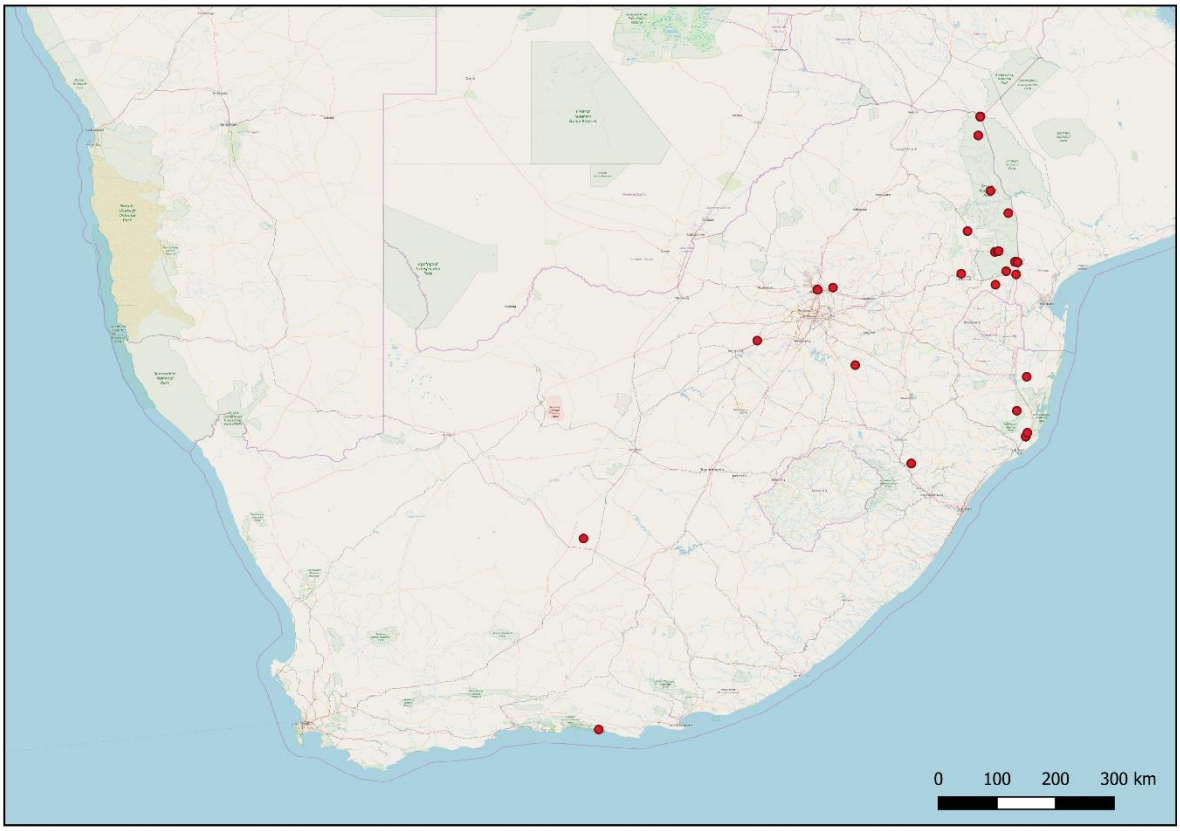

Figure 31: Distribution map representing locations at which *Tabanus gratus* (red dots) has been collected in South Africa (n=109).

Distribution: Occurs widely in Kruger National Park, Richards bay area, Pretoria and one from the Port Elizabeth area (Figure 31). This corresponds with Usher (1972), whom stated the species prefers open grasslands/savanna outside of indigenous forests.

Biology: The larva is moderately pigmented, mostly on the edges of the segments. A long syphon is present, and the species appears to prefer stream beds. The pupal aster is of the normal type, being regular in outline. The spines of the dorsolateral comb are reduced, especially in the male.

Seasonality: Occurs during June to August (n=108).

Vector status: *Trypanosoma theileri* was detected in South Africa via pathogen screening (Mazibuko, 2018). *Trypanosoma vivax* was detected on the mouth parts via pathogen screening in Nigeria (Odeniran *et al.*, 2019).

Molecular data: Five COI sequences and one combined 5.8S rRNA gene, ITS 2 and large subunit rRNA gene sequences are available from GenBank. In this study, *Tabanus gratus* formed one monophyletic

group subdivided into two *T. gratus* sister clades, both with high bootstrap values (Figure 24). One *T. gratus* sample (CB2-SA purple arrow Figure 24) did not group with the remainder of the clade. Since the groupings are not geographically separated, a possibility of haplotypic variability is likely. AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: A Holotype is available at the Zoologisches Museum der Universität, Berlin.

References: Neave (1915), Oldroyd (1954) , Taioe *et al.* (2017), Mugasa *et al.* (2018), Mazibuko (2018) and Odeniran *et al.* (2019).

Tabanus minuscularius Austen, 1912

http://tabanidae.myspecies.info/taxonomy/term/3948

Tabanus minuscularius Austen, 1912:31 by original designation. Mozambique; Lesotho, Rhodesia, South Africa.

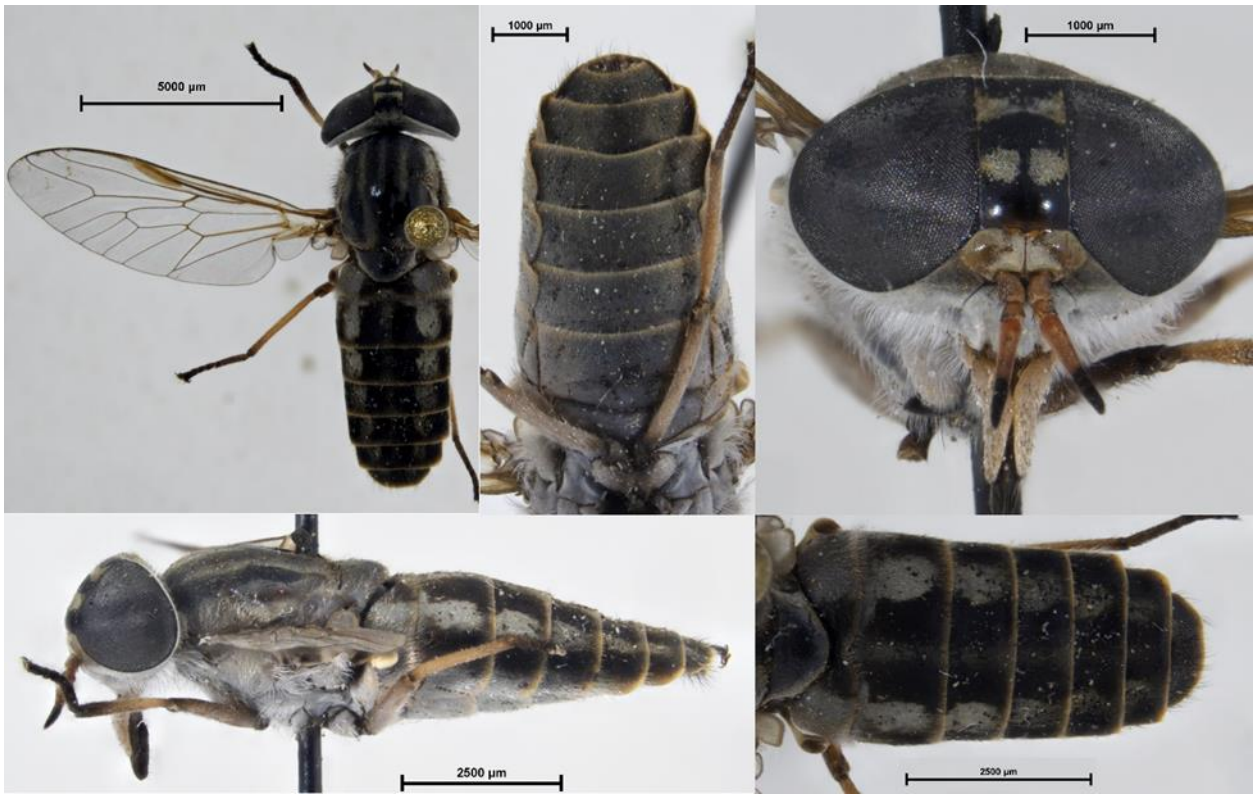

Figure 32: Sequential images of *Tabanus minuscularius* from top left going clockwise: dorsal, ventral, facial, abdominal and lateral views.

Morphological diagnosis: A black and greyish species with an average body length of 9-10mm and wing length of 8mm (Figure 32). The eyes have three bands which may be faint in older specimens. Both the upper and lower calli are rectangular in size, expanding to the eye margins. The mesonotum is black with a narrow median line and sublateral and supra-alar strips. The abdomen is black with an inconspicuous medial stripe and two broad prominent sublateral grey stripes. The wings are clear with yellowish veins and stigma.

Similar species: *Tabanus taeniatus* can be readily distinguished from *T. minuscularius* by its it larger body size and densely hairy eyes.

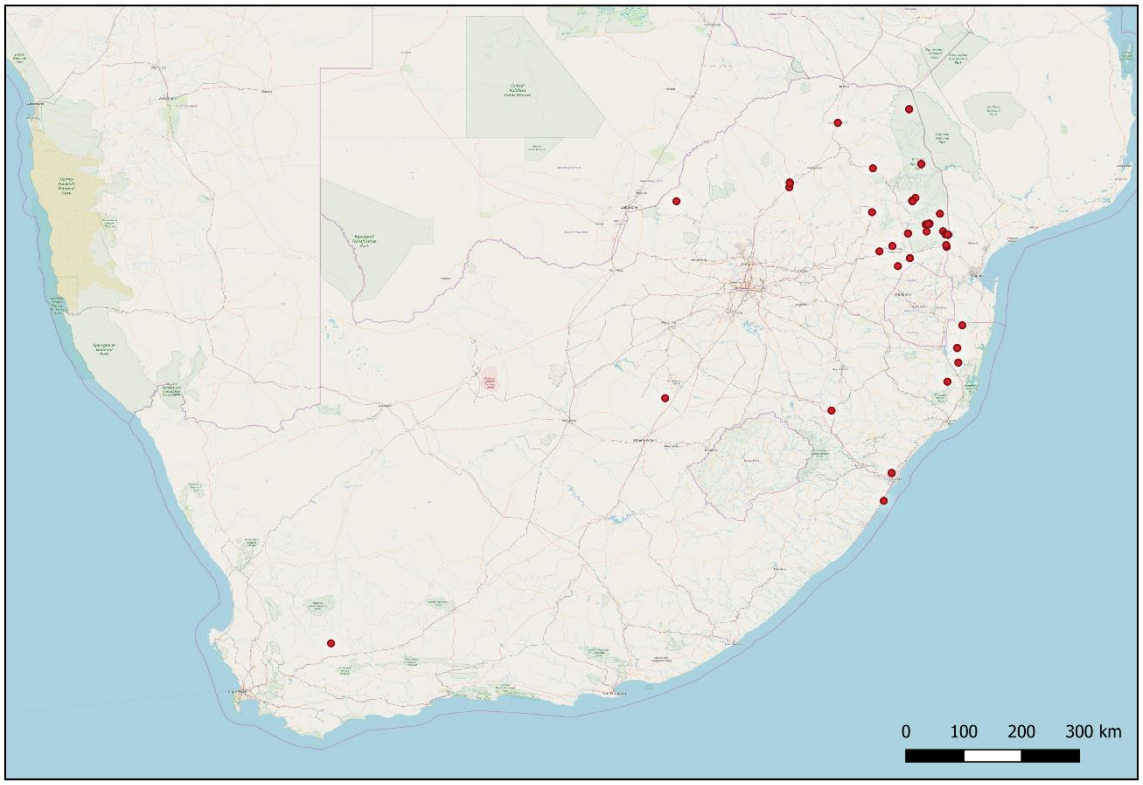

Figure 33: Distribution map representing locations at which *Tabanus minuscularius* (red dots) has been collected in South Africa (n=638).

Distribution: Occurs in the North-eastern parks of South Africa, with the exception of a sample collected from the Cape, which remains suspect (Figure 33). Usher (1970) discussed the possibility of a subpopulation south of the Tugela in KZN.

Biology: Usher (1972) found *T. minuscularius* flies inhabiting areas extending from the Drakensberg region to the eastern Mozambique plains. The flies shown preference for open grassland areas with clusters of trees and bushes. These flies have been found on numerous occasions to bite humans freely in the Pateni area in KwaZulu-Natal (Usher, 1970, Usher, 1972). Usher (1970) noted that the species avoided densely shaded areas of forests but were flying along the stream bed. No other information regarding the life history of *T. minuscularius* is known.

Seasonality: Occurs during November to March (n=634).

Vector status: No studies to date have investigated the vector potential of *T. minuscularius.*

Molecular data: No sequence data available from GenBank. In this study, T. minuscularius did not group together. One sample DVTD00003 formed a non-supported monophyletic group with

Euancala irrorata while the other, DVTD00006, was polyphyletic to DVTD00003 (Figure 24). AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: A holotype can be located at the British Museum.

References: Oldroyd (1954), Usher (1970) and Usher (1972).

Tabanus par Walker, 1854

http://tabanidae.myspecies.info/taxonomy/term/4105

Tabanus par Walker, 1854:235 by original designation. South Africa; throughout Afrotropical region, mainly outside Congo basin.

Tabanus rufipes Macquart, 18380:128 (124) by synonymy, a junior homonym of *Tabanus rufipes* Palisot de Beauvois, 1806. South Africa.

Tabanus luteolus Loew, 1858:341 [1860:117] by synonymy. South Africa.

Atylotus cereolus Bigot, 18920:644 by synonymy. 'Afrique orientale'.

Tabanus antennalis Fain, 1947: 144 by synonymy. Zaire.

Figure 34: Sequential images of *Tabanus par* from top left going clockwise: dorsal, ventral, facial and lateral views.

Morphological diagnosis: A yellow brown species that is approximately 12mm in body length and 11mm in wing length (Figure 34). No distinct pattern, however, the species is adorned with golden hairs on a dark ground colour. The calli are conjoined and resembles and elongated onion shape and is a golden brown in colour. The wings are clear, and the veins and stigma are yellow in colour.

Similar species: *Tabanus antennalis*, distinguish by the lower callus having a higher placement and being more rectangular in shape. The third antennal segment is broader. *Tabanus taeniatus* has uniquely hairy eyes, with indistinct irregular bare patches in the location of both the lower and upper calli and lastly, two grey well defined longitudinal stripes, present on the abdomen. Tabanus donaldsoni is destinguised by the large size (18-19mm) and orange colouration. A clearcut difference is visible dorsally between the orange thorax and abdomen and the white pleura and venter.

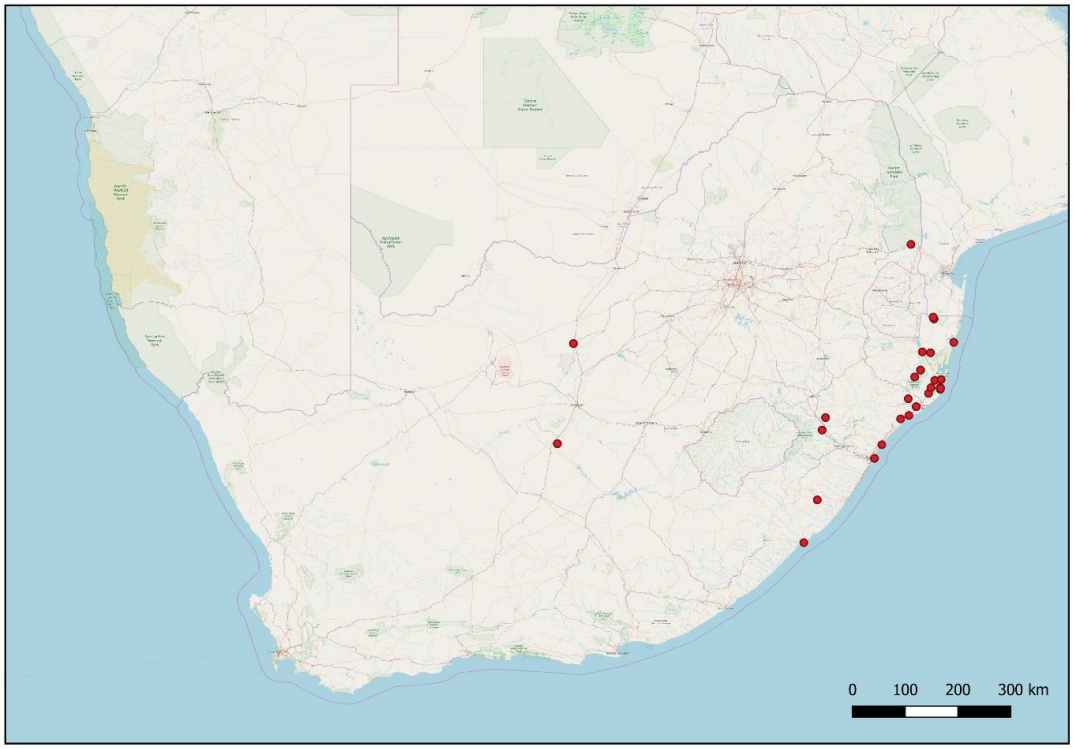

Figure 35: Distribution map representing locations at which *Tabanus par* (red dots) has been collected in South Africa (n=40).

Distribution: Occurs mainly along the eastern coastline and close to water sources (Figure 35). Two samples were collected around the Kimberley areas which is suspect. The coastal occurrence agrees with findings by Usher (1972).

Biology: King (1910) conducted studies on *T. par* from the White Nile from Gebelein southwards. Females hunt animals such as cattle but tend not to continue pursuing the prey if it wanders passed a certain range from the breading site. The females produced three small batches of eggs and laid them on the underside of leaves of a water weed or any vegetation overhanging water. Egg batch placement is slightly uncommon for *Tabanus*; placed vertically and separately, though in a cluster. Eggs are spindle-shaped, about 1.15 mm in length and white in colour, darkening as development occurs. Larvae are predacious and buries themselves in mud. Mature larvae are white with a greyish tint and is roughly 13.3 mm in length. King (1910) found that the larvae have the ability to remain

dormant for at least fifty-seven days in unfavourable conditions. The larvae pupated on the top sand, partially submerged in the water. The pupal colour is yellow, darkening as development occurs, and is roughly 12 to 15mm in length. The average time period for the pupal stage was six to eight days. Males feed on flowering plants.

Seasonality: Occurs during September to March (n=40).

Vector status: *Trypanosoma congolense, Trypanosoma theileri,* and *Babesia bigemina* was detected in Zambia and South Africa by pathogen screening (Taioe, 2017). *Trypanosoma vivax, Trypanosoma evansi* and *Trypanosoma simiae* has been detected in Nigeria by pathogen screening (Odeniran *et al.*, 2019).

Molecular data: Nineteen COI sequences are available from GenBank. In this study, *T. par* formed a monophyletic group comprising two sister clades, all well supported (Figure 24). There is however a *Tabanus taeniatus* grouping within one of the sister clades. The placement of the specimen is suspect and may be due to a misidentification, contamination or low sample size. A similar placement of the specimen, as well as erroneous placement of several other specimens in the phylogeny generated by Taioe (2017) was not discussed in detail. The other three *T. par* sequences published by the same authors with relevance here, A3-ZM, A6-ZM and A2-ZM (see purple arrows in Figure 24) are nonsensically dispersed in several other groupings. AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: Holotypes of both *T. par* and *T. cereolus* can be located at the British Museum. *T. luteolus* is available from Zoologisches Museum der Universität, Berlin. The paratype of *T. antennalis* is available from Tervuren, Belgium.

References: King (1910), Oldroyd (1954), Usher (1972), Taioe *et al.* (2017) and Odeniran *et al.* (2019).

Tabanus pullulus Austen, 1912

http://tabanidae.myspecies.info/taxonomy/term/4186

Tabanus pullulus Austen, 1912:282 by original designation. Malawi; Mozambique, Rhodesia, Tanzania, Zambia,

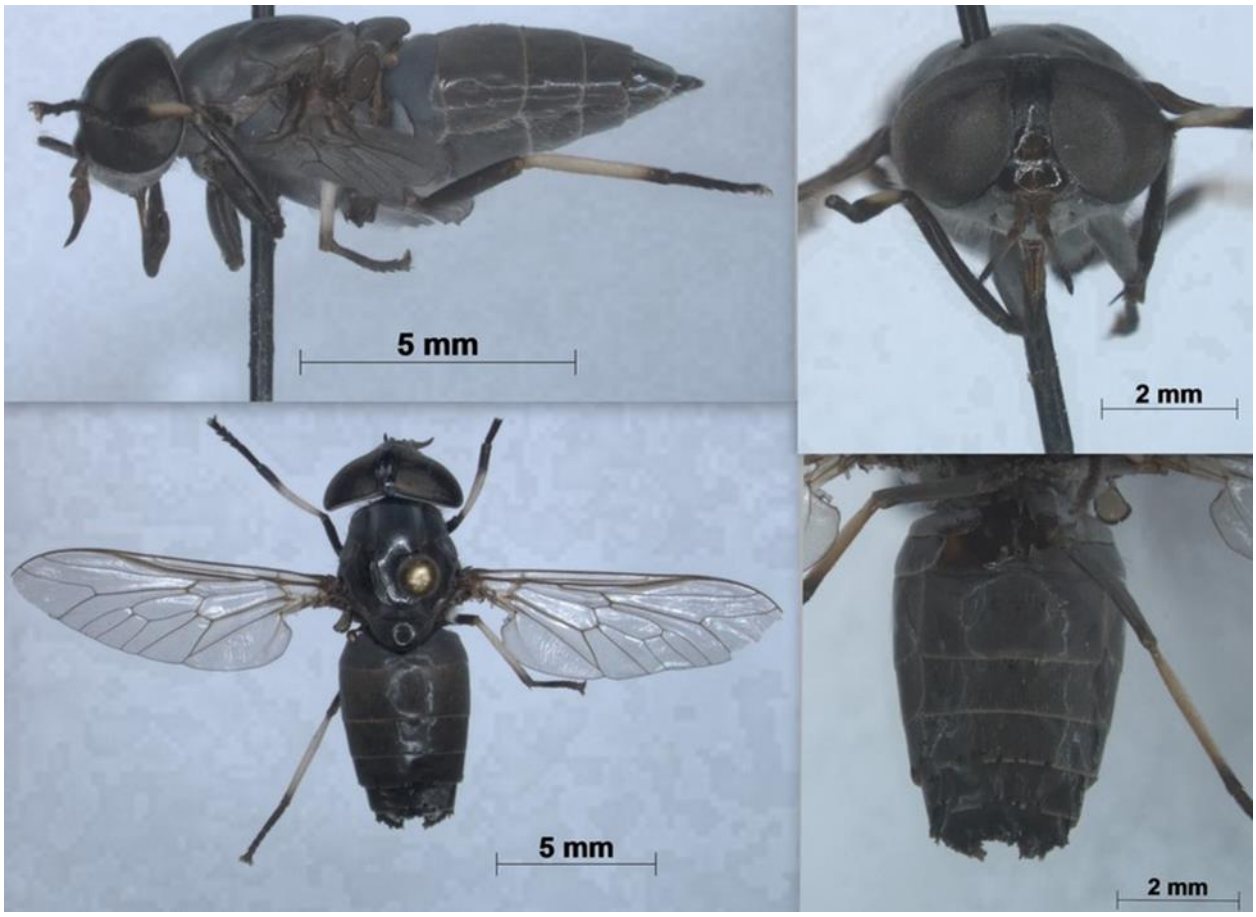

Figure 36: Sequential images of *Tabanus pullulus* from top left going clockwise: lateral, facial, ventral and dorsal views.

Morphological diagnosis: A black species with a purple bloom, estimated body length of 13mm and wing length of 11mm (Figure 36). Calli in the shape of an inversed mitre joined with a short linear extension. Faint narrow yellow hind-margins are visible on all abdominal segments.

Similar species: *Tabanus claritibialis*, distinguished by its dull red abdomen and bronze hairs on the mesonotum.

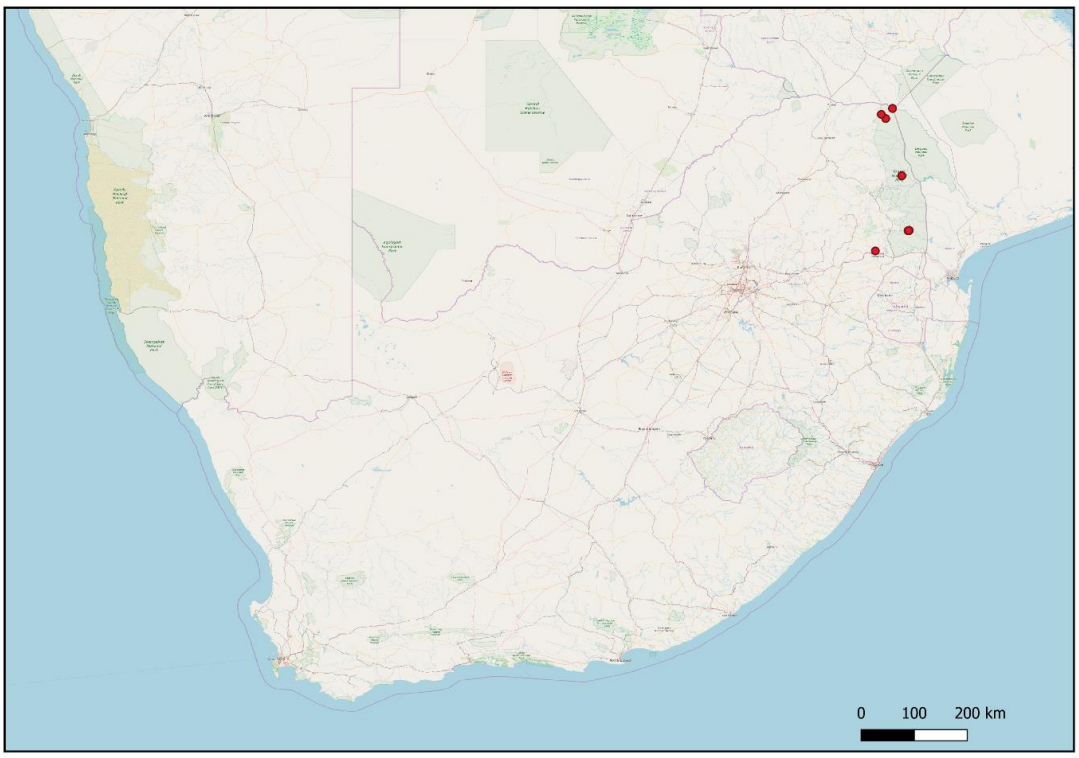

Figure 37: Distribution map representing locations at which *Tabanus pullulus* (red dots) has been collected in South Africa (n=109).

Distribution: Few samples are available, all originating from Kruger National Park (Figure 37). Usher (1972) only reports of specimens obtained in Kruger, agreeing with the current findings.

Biology: Currently no information regarding the life history of *T. pullulus* is known.

Seasonality: Active during November to July (n=109).

Vector status: No studies to date have investigated the vector potential of *T. pullulus.*

Molecular data: No sequences were found on GenBank. In this study *T. pullulus* formed a well-supported clade sister to *T. sericiventris* (Figure 24). AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: A holotype is available at the British Museum.

References: Austen (1911), Oldroyd (1954) and Usher (1971).

Tabanus sericiventris Loew, 1858

http://tabanidae.myspecies.info/taxonomy/term/4296

Tabanus sericiventris Loew, 1858:339 [1860: 110] by original designation. South Africa.

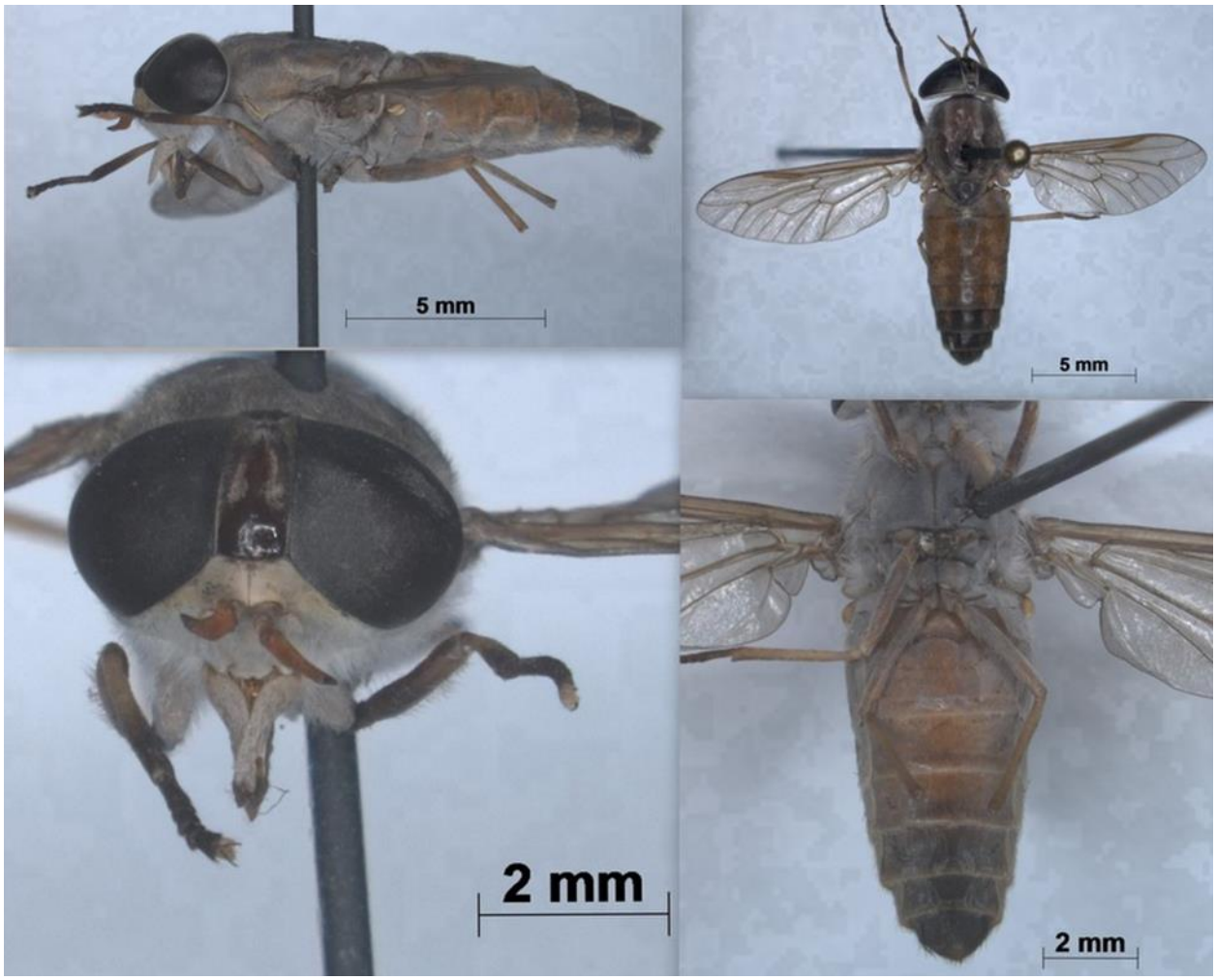

Figure 38: Sequential images of *Tabanus sericiventris* from top left going clockwise: lateral, dorsal, ventral and facial views.

Morphological diagnosis: A yellow-brown species, roughly 14mm in body length and 13mm in wing length (Figure 38). The mesonotum is grey with inconspicuous median and sublateral stripes visible on the anterior section. A broad, dull grey medial stripe is present on the abdomen. Stripes adjacent to the medial one is brown with black hairs, alongside to those are orange spots on the first three abdominal segments and grey ones on the remainder. All segments have orange hind-margins. The wings are characteristically brown, more prominent along the veins.

Similar species: *Tabanus taeniola*, distinguished by clear, well defined thoracic stripes. A slight variation in abdominal pattern, where *T. taeniola* has a narrow well-defined median strip, longer

orange lateral areas and a different shape of the callus. *Tabanus chevalieri* can be differentiated by hairy eyes, however, museum specimens sometimes lack hairy eyes and are then easily confused with this species when the key by Oldroyd (1954) is used.

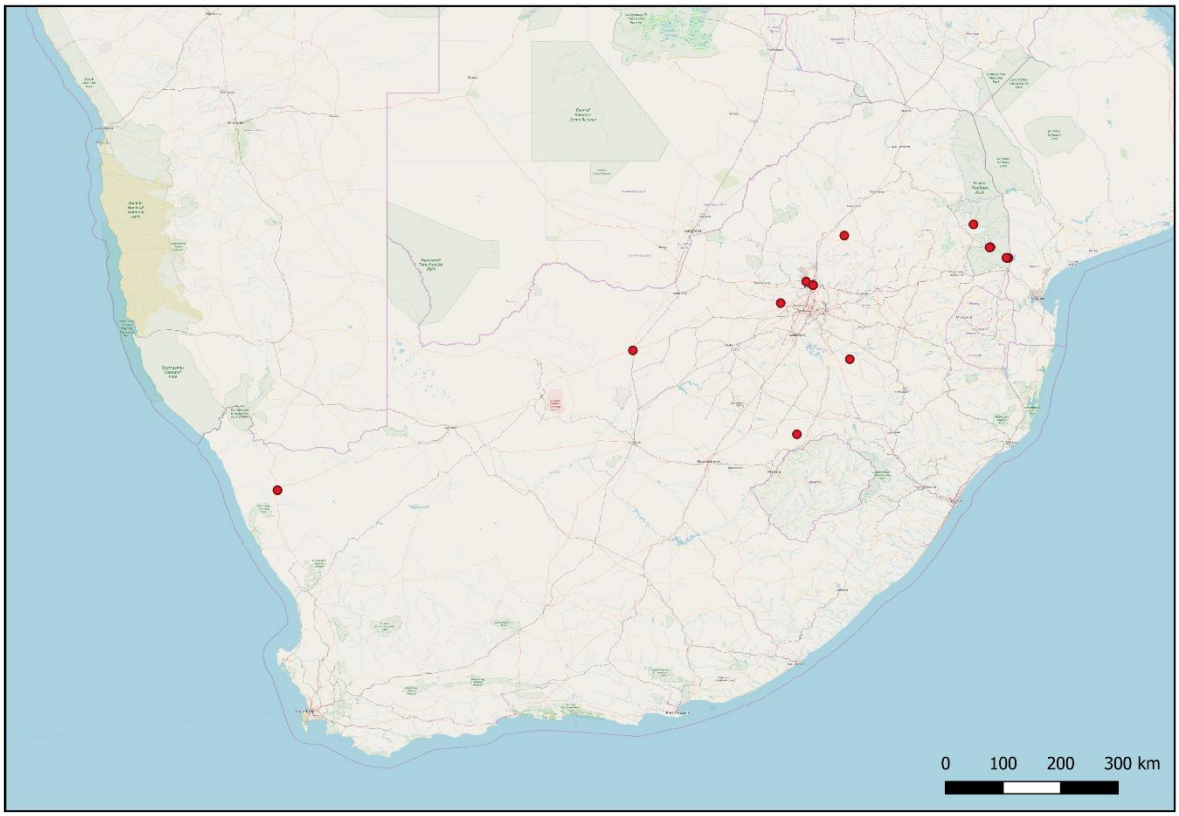

Figure 39: Distribution map representing locations at which *Tabanus sericiventris* (red dots) has been collected in South Africa (n=23).

Distribution: Distribution is scattered; Kruger National Park and Pretoria area has a higher occurrence where a single sample has been collected from the Karoo and two from the Welkom area (Figure 39). This however, does correspond with Usher (1972) and the large spaces between the localities are possibly under sampling.

Biology: Currently no information regarding the life history of *T. sericiventris* is known. Several specimens flew into the fieldwork vehicle used in this study, possibly indicating an attraction to humans.

Seasonality: occurs during November to March (n=23).

Vector status: No studies to date have investigated the vector potential of *T. sericiventris.*

Molecular data: No sequence data is available from GenBank. In this study, *T. sericiventris* formed a monophyletic group, sister to *T. pullulus*, with high bootstrap values (Figure 24). AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: A holotype is available at the Zoologisches Museum der Universität, Berlin.

References: Oldroyd (1954) and Usher (1972).

Tabanus taeniola Palisot de Beauvois, 1806

http://tabanidae.myspecies.info/taxonomy/term/4397

Tabanus taeniola Palisot de Beauvois, 1806:56 by original designation. Nigeria; almost entire Afrotropical region, including Aldabra & Madagascar, mainly riverine in Congo basin.

Tabanus sagittarius Macquart, 1838:127 by synonymy. South Africa.

Tabanus subelongatus Macquart, 1846:159 by synonymy. South Africa.

Tabanus socius Walker, 1848 :160 by synonymy. South Africa.

Tabanus rubicundus Walker, 1848: 161 by synonymy. South Africa.

Tabanus macrops Walker, 1848 :164 by synonymy. Egypt.

Tabanus variatus Walker, 1854:64 by synonymy. [No locality].

Tabanus dorsivitta Walker, 1854:231, see *Tabanus virgatus* Austen, 1906.

Tabanus longitudinalis Loew, 1852:658 [1862:2] by synonymy. Mozambique.

Tabanus serratus Loew, 1858:340 [1860:111] by synonymy. South Africa.

Tabanus exclamationis Girard, 1881:228 by synonymy. Angola.

Tabanus subelongatus var. *proximus* Corti, 1895 :132 by synonymy. Ethiopia.

Tabanus virgatus Austen, 1906:60 by synonymy. Originally a replacement name for *Tabanus dorsivitta* Walker, 1854:231 which is a junior homonym of *Tabanus dorsivitta* Walker, 1850. Gambia.

Tabanus picticeps Becker, 19226 :63 by synonymy. Sudan.

Tabanus hirsuta Tendeiro, 1965: 86 (as form) by synonymy. Guinea-Bissau. [Unavailable name: form name published after 1960.]

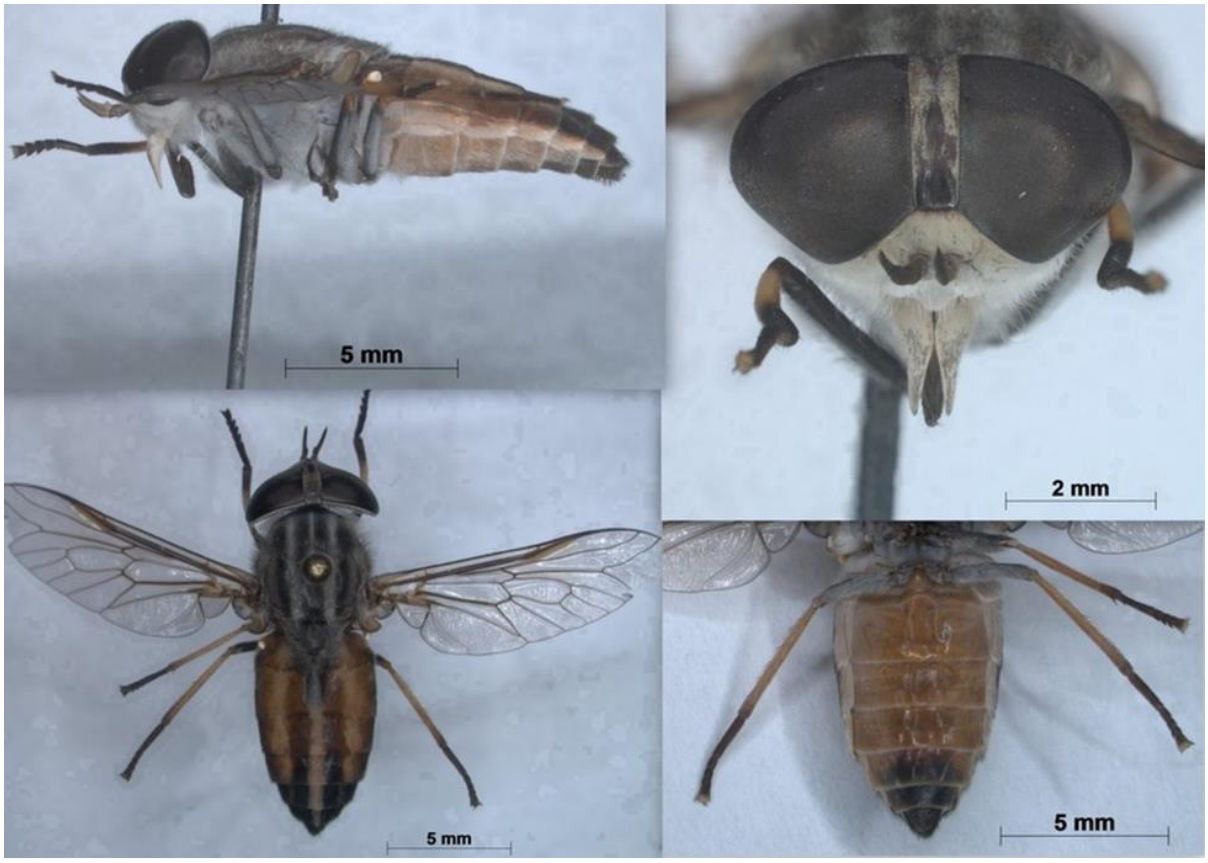

Figure 40: Sequential images of *Tabanus taeniola* from top left going clockwise: lateral, facial, ventral and dorsal views.

Morphological diagnosis: A black, brown and greyish species with and average body length of 16mm and wing length of 16mm (Figure 40). The eyes have no bands. The mesonotum is a reddish base colour with three grey stripes. Scutellum is black with a red tip. The abdomen is boldly patterned with three white longitudinal stripes. The sublateral stripes are broader compared to the narrow medial strip and appears to merge into the lateral margins. The wings are clear, and the stigma is extremely faint, nearly appearing as being non-existent.

Similar species: *Tabanus sericiventris* can be distinguished by indefinite thoracic strips, broader and indistinct median stripe on abdomen, shorter orange lateral areas and differences in the callus structure. Oldroyd (1954) discuss the morphological clines and distribution of the various clines in detail.

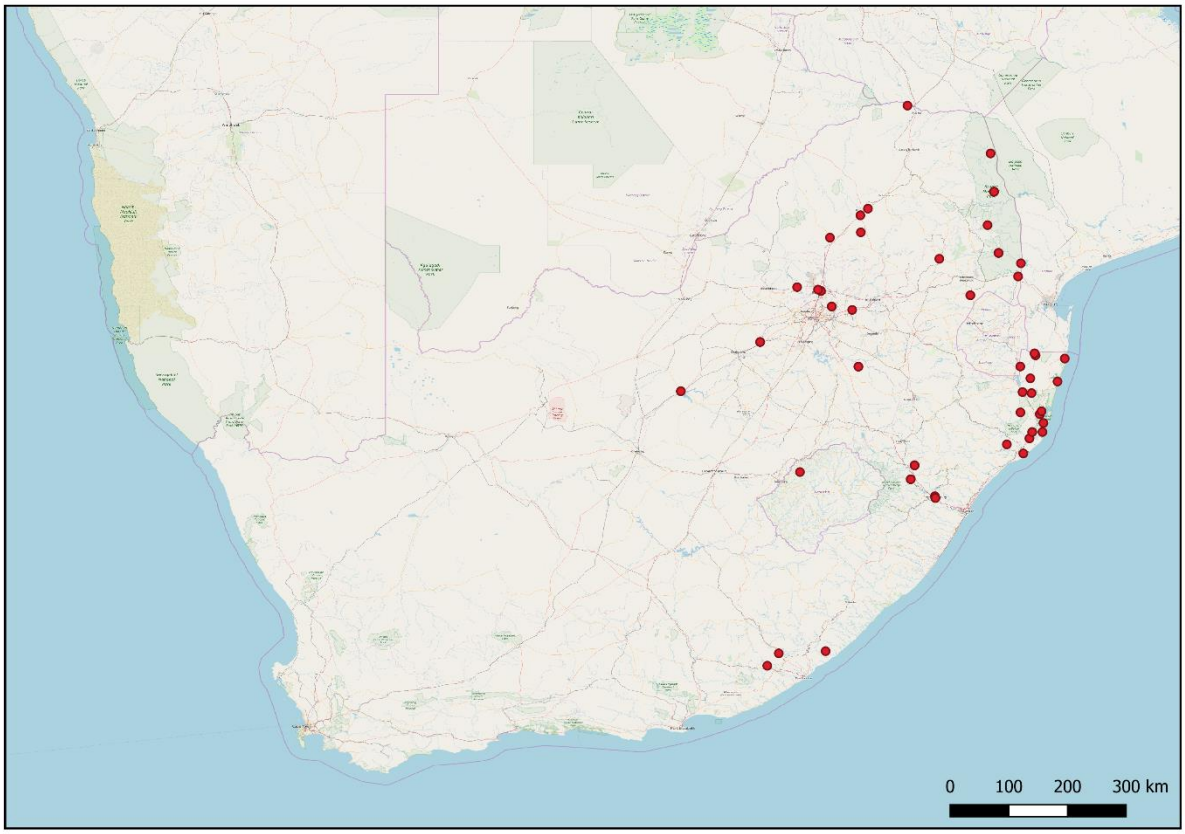

Figure 41: Distribution map representing locations at which *Tabanus taeniola* (red dots) has been collected in South Africa (n=79).

Distribution: This species mainly occur in open areas on the eastern side of South Africa, appearing not to extend beyond Kimberley (Figure 41). Usher (1972) mentions of one sample that was found in the Cape but stated that it was unusual and likely due to errors in the information. For distribution elsewhere in Africa, see Oldroyd (1954).

Biology: King (1910) conducted studies on the bionomics of *T. taeniola* and found that females are persistent feeders and will pursue pray for long distances. Eggs are laid closely together and is left bare on vegetation overhanging a body of water. *T. taeniola* eggs are 1.75mm in length, spindle shape, white and do not hatch at a relative humidity of lower than 50%. Inaoka *et al.* (1988) conducted studies on larval development and concluded that *T. taeniola* larvae has an average length of 29 to 35 mm. The larvae are a whitish cream colour with a finely striated cuticle. This species has between six to eight instars. King (1910) found that the larvae in captivity are more ferocious and have cannibalistic tendencies. Larvae pupate in mud cylinders that is formed below the ground (Drees, 1987). Hafez *et al.* (1970) concluded that *T. taeniola* completes its whole life cycle within 10 to 11 weeks when the temperature remains constant and high (32–35°C). *T. taeniola*

has been documented as a noisy attacker which disrupts the cattle more than *A. agrestis* (Usher, 1972)

Seasonality: Occurs during August to May (n=74).

Vector status: *Babesia bigemina, Theileria parva Trypanosoma evansi, Trypanosoma congolense* and *Trypaosoma theileri* was detected by pathogen screening in Zambia and South Africa (Taioe, 2017). *Trypanosoma evansi* and *Trypaosoma vivax* was detected by pathogen screening in Nigeria (Odeniran *et al.*, 2019). *Trypanosoma soudanense* was detected by vector screening (Shoukry and Morsy, 2011)

Molecular data: 22 COI sequences, one gGAPDH sequence and one 5.8S rRNA gene, ITS2 and large subunit rRNA gene sequence is available on GenBank. In this study, *T. taeniola* formed a well-supported monophyletic group, constituting four clades (Figure 24). Within one of these clades, a *T. par* (A3-ZM) has been located while one *T. taeniola* (B4-SA) grouping with other mismatched *T. par* samples. AATS sequences has been obtained for this species and both COI and AATS sequences will be submitted to GenBank.

Depositories: A holotype of *T. taeniola* is available at the Museum National d'Histoire Nuturelle, Paris.

References: King (1910), Oldroyd (1954), Usher (1972), Ouhelli and Dakkak (1987), Inaoka *et al.* (1988), Shoukry and Morsy (2011), Taioe (2017) and Odeniran *et al.* (2019).

Discussion

Little work has been conducted on the overall species composition of tabanids in the whole of South Africa (SA), where the only papers are restricted to Usher (1972), Esterhuizen (2006), Taioe et al. (2017) and Mazibuko (2018). To improve knowledge on Tabanidae in South Africa, this study was conducted to identify, describe and barcode tabanid species found in the Kruger National Park (KNP). The Kruger National Park, roughly two million hectares in size, is located in the lowveld of South Africa, expanding over Limpopo and Mpumalanga. According to Usher (1972), 27 species of Tabanids are located in the lowveld region of southern Africa of which 10 are thought to be endemic. Overall, 16 morphologically unique tabanid species were identified belonging to five genera which included: *Atylotus*, *Chrysops*, *Haematopota*, *Philoliche* and *Tabanus*. Two of the identified species, *Philoliche medialis* and *Haematopota daveyi*, has not been recorded within South Africa prior to this study, however, both occur in neighbouring regions and the new distributions are unsurprising but useful.

According to Morita *et al.* (2016) the taxonomy of the Tabanidae remains difficult despite the ubiquity of the family, leaving the systematics of the group poorly understood, especially from a phylogenetic perspective. With the limited molecular data available, new DNA barcodes could assist in a more precise and quick identification of tabanid species in South Africa (Votýpka *et al.*, 2019). During this study, alanyl-tRNA-synthetase (AATS) and cytochrome oxidase I (COI) barcode data for nearly all species were generated. COI topologies of the tribes were constructed. Out of the 16 species that were identified, only five (*Philoliche rondanii*, *Atylotus agrestis*, *Tabanus gratus*, *Tabanus par* and *Tabanus taeniola*) had sequence data available from GenBank, placing an emphasis on the need for studies on this family.

Within the tribe Chrysopini, only one species was captured, *Chrysops obliquefasciata*. According to Usher (1972), eight species of Chrysops are present in South Africa, however, *C. obliquefasciata* are the sole species occurring in the lowveld. Chrysops seems to be either not abundant or not easily collected in South Africa with studies from Esterhuizen (2006) in the Lowveld biome of KwaZulu Natal, with seemingly similar low number of *Chrysops* species and specimens present in his study. *C. obliquefasciata* clustered with *Chrysops distinctipennis*, a member of the "*distinctipennis*" group. This contradicts the original placement made by Oldroyd (1957), where *C. obliquefasciata* forms part of the "*longicornis*" group. However, the phylogenetic placement here supports the transference of *C. obliquefasciata* to the "*distinctipennis*" group by Usher (1972). To date, no studies on *C. obliquefasciata* has been conducted, thus making comparisons based on literature impossible. It is

also worthy to note that the tribe, Rhinomyzini, which forms part of the Chrysopsinae sub-family grouped alongside that of the Philolichini and not with the Chrysopini. Morita *et al.* (2016) found that the Rhinomyzini was sister to Tabaninae and that both Chrysopini and Rhinomyzini were not well supported. Usher (1972) identified commonality between the Philolichini and Rhinomyzini, in which both tribes are common bee-mimics and thus more alike in appearance than Rhinomyzini is to Chrysopini. Morita *et al.* (2016) did state that the Chrysopinae need to be redefined, which the findings here support.

The subfamily Pangoniinae constitutes of four tribes Pangoniini, Philolichini, Scionini and Mycteromyiini, where each genus is further subdivided into subgenera. The phylogenetic analysis indicated that all the *Philoliche Buplex* sequences obtained during the study clustered together alongside the undescribed morphologically darker *Philoliche* sequences (DVTD 8 and DVTD 59). These morphologically darker samples lack the unique longitudinal tiger stripes on the mesonotum, replaced by a completely eclipsed black mesonotum. This lead to complications when using the identification key provided by Oldroyd (1957) since no other species within the Philolichini had this type of pattern and colouration. The variability in the coloration of these samples requires further investigation. The only available *P*. (*Buplex*) *suavis* sequences to compare the "dark samples" too, were the sequences obtained during this study. The strong grouping of the unidentified *Philoliche* samples with that of *P.* (*Buplex*) *suavis* may indicate that they are a darker morph of the same species. The small sample size as well as specimens from limited localities does however not allow a more robust hypothesis and the presence of morphologically darker samples within or alongside *P. sauvis* remain a mystery and require further scrutiny. Oney *et al.* (2013) states that broader ranged species have a higher intraspecific morphological variation and occupies a large niche, however, Torres and Miranda-Esquivel (2016) found that the opposite was true with the tabanids. Torres and Miranda-Esquivel (2016) found that the widest-ranged species had the lowest intraspecific morphological variation. Morphological variation may be attributed to a geographical split in species or a mutation that occurred in the phenotypic DNA. Whether the unidentified species are *P.* (*Buplex*) *suavis* with intraspecific morphological variation or are a sub-species, requires further investigation. Some of the reference sequences within the Philolichini, specifically *P.* (*Ommatiosteres*) *angulate*, *P.* (*Maverica*) *bukamensis* and *P.* (*Philoliche*) *rondani*, did not cluster with their respective groupings as expected. *P.* (*Ommatiosteres*) *angulate* clustered within the *P.* (*Dorcaloemus*) subgenus instead of the *P.* (*Ommatiosteres*) subgenus. This could be the result of a morphological misidentification of the misplaced samples. Misidentification is a well occurring problem in the identification of Tabanidae (Usher, 1972, Krolow et al., 2015). *P.* (*Maverica*) *bukamensis* clustered with the *P.* (*Philoliche*)

subgenus and since no other sequences of *P.* (*Maverica*) species were available, little inferences can be made. This is further emphasized by the shorter *P.* (*Maverica*) *bukamensis* sequence, which was 168 nucleotides shorter than the matrix. This difference in the length of the sequence available for *P.* (*Maverica*) *bukamensis* may have resulted into little distinguishing difference in the sequences for an accurate placement.

Morita (2008) found that the former *aethiopica* complex could be divided into two distinct clades: the "*aethiopica*" and the "*rondani*" groups. During this analysis, separation between the "*aethiopica*" and "*rondani*" groups was not well supported or recovered as in Morita (2008). The Morita study, however, had a more representative dataset, which was meant to be published, but seemingly never were. Unfortunately, the sequence data was also never uploaded to a molecular depository such as GenBank making reanalysis of the data impossible. The *P.* (*Philoliche*) *rondani* (DQ983528) was recovered in the "*rondani*" clade but did not group with the remaining *P.* (*Philolich*) *rondani* sequences from this study. Mugasa *et al.* (2018) reported that some identical species with slight morphological variation, did not cluster together with COI barcoding but still formed a monophyletic group, maybe indicative of the limits of the resolution ability of COI. The locality detail of the specimen from which the sequence was obtained, could not be obtained. This is unfortunate, because the specimen could possibly be from elsewhere in southern Africa and a simple issue of sample size and missing data might be responsible for the peculiar grouping.

During this study, *Haematoporta daveyi*, *Haematopota decora* and *Haematopota vittata* clustered into their respective groups as classified by Oldroyd (1952). *H. vittata* formed a monophyletic group with *Haematopota albihita*, which is also an Afrotropic species, within the vittata group. The division between *H. vittata* and *H. albihita,* however, was not well supported by bootstrap value. With the little sequence data to compare to it appears that COI was able to distinguish between all the *Haematopota* species, placing each in their described group.

Several phylogenetic incongruences were observed with the Tabaninae topology, reaffirming the dire need to revise this hyper-diverse cosmopolitan genus. The taxonomic groups suggested by Oldroyd (1954) could not be supported and the genus was rendered paraphyletic by *Atylotus*, albeit with support lacking. All African *Atylotus* sequences clustered together and formed a monophyletic group. This trend was also observed by Mugasa *et al.* (2018), who hypothesized that the observations may be due to cryptic speciation. The clustering may also indicate that the African *Atylotus* are not true species but rather one species with either sub-species or simply a case of

polymorphism. Alternatively, *Atylotus* might have only recently occupied the Afrotropics and diversified rapidly and a case of weak signal from COI due to coalescence is seen. Perhaps markers from the nuclear genome might show better species delimiting power. Either way, this genus deserves re-evaluation and modern scientific scrutiny.

Some support for the higher classification as proposed by Oldroyd (1954) was recovered here. The author was well aware of the possible problems his classification hypotheses might encountered down the line and stated that the "grouping" within Tabanus should rather be viewed as a system for breaking the diverse genus down to facilitate identification and should not be viewed as a system with an evolutionary foundation. *Tabanus atrimanus*, *Tabanus leucostomus* and the *Tabanus gratus* cluster together and formed a well-supported monophyletic group, all members of the "*insignis*" group. Unfortunately, beyond the "*insignis*" group, little support for the Oldroyd groupings could be found and the addition of at least another marker as well as revisiting the morphology, could perhaps lead to a more robust and stable classification within an evolutionary paradigm.

Tabanus gratus formed a monophyletic group with two sister clades. This separation into clades was also observed by Mugasa *et al.* (2018), who found slight morphological and genetical variation within the well supported monophyly. The clades were separated according to the geographical location at which the samples were located. The sequences obtained in this study, however, grouped with both the Tanzania and Kenya *T. gratus* sequences in the sister clades. The South African *T. gratus* reference sequence uploaded by Taioe *et al.* (2017), however, did not form part of this monophyly. Taioe *et al.* (2017) study had nonsensical groupings and caution should be taken when analysing data from the study. Nonetheless, the two clades of *T. gratus* as recovered in Mugasa *et al.* (2018) and reaffirmed here, may require further investigation due to the possibility of a sub species separation or another from or genetic variation.

Neither of the *Tabanus minuscularius* samples grouped with each other and formed a polyphyletic group. One *T. minuscularius* samples clustered with *Euancala irrorata*. The morphological difference between the species are substantial; *T. minuscularius* being a dull minimalistically patterned species, where *E. irrorate* is a brightly coloured species with countless spots on the eyes, wings, thorax and abdomen. *T. minuscularius* as a species requires further evaluation with more reference sequences, which may aid in a proper clustering of the species and very little inferences can be made from the analysis produced here.

Tabanus par formed a monophyletic group with two sister clades of which South African and Zamibia sequences were separated from the Central African Republic sequences. The sequence obtained during this study clustered with the SA and Zambia samples, however all the SA and Zambia sequences (including three *T. par* sequences that did not cluster with the monophyletic group) were uploaded as unverified identifications, also originating from the Taioe *et al.* (2017) study. The three misplaced *T. par* sequences may indicate that COI is mostly able to differentiate; however, the species may need revision. Another incongruency is the placement of the *Tabanus taeniatus* within the *T. par* monophyly. Other than a misidentification, other complications that may have occurred includes incorrect sequencing, incorrect sequence editing and accidental upload of the wrong sequencing data or under the wrong species name.

Both *Tabanus pullulus* and *Tabanus sericiventris* formed monophyletic groups with high bootstrap values, sister to each other. According to Oldroyd (1954), *T. pullulus* is in the pullulus group where *T. sericiventris* is in the patterned group. No reference sequences are available for either of these species.

Tabanus taeniola formed a monophyletic group with four clades separated by geographic regions. One clade is from Kenya/Tanzania, one from SA, one from Gabon and the last from the Central African Republic. This implies genetic variation due to species separation by geographical location, possibly the same as seen with *T. gratus* and *T. par* (Taioe, 2017, Mugasa *et al.*, 2018). The South African reference sequences, however, did not cluster within the monophyly. The South African reference sequences, uploaded by Taioe *et al.* (2017), were uploaded as a sequence from a specimen with unverified identification which may be a result of the several possibilities aforementioned and may imply a need for re-evaluation of the GenBank sequences rather than the species.

The COI barcode indicated that some species are very uniform such as *Tabanus pullulus* while others such as *T. taeniola* and *T. gratus* forms co-occurring haplotypes. COI, however, alone was not sufficient in distinguishing between all the species within the South African Tabanidae, indicative of its limitations as a barcode for delimiting dipteran species which has already been covered by Meier *et al.* (2006). While the topologies were congruent with the classification, no statistical support has been obtained for these topologies and ultimately, no definite inferences can be made. Currently very little COI barcodes are available from the 4455 known tabanid species (Mugasa *et al.*, 2018). The use of several molecular markers is ideal for barcoding to provide sufficient signal (Botero-

Castro *et al.*, 2013, Wang and Wu, 2013). The two markers used here were both protein coding genes but where COI is a mitochondrial gene, AATS is was a nuclear gene. The combination of genes from several different cell sections may provide different phylogenetic signals and by extension different topologies (Meiklejohn et al., 2013). It is therefore advised to utilise more than one maker when discussing phylogenies and ideally produce phylogenies using several methods and several gene regions, especially for resolution of deeper branches. During the analysis, AATS sequences were not used. Complications occurred during the amplification process with all *Philoliche* samples. The inability to amplify the *Philoliche* with the AATS primers may be a result of substantial differences in the annealing location on the isolated DNA. The DNA may also have been segmented, interrupting the annealing location. Further investigation will be conducted on the probable causes. The AATS sequences that were obtained were not used in the phylogenetic analysis due to the time constraints. The analysis will however be completed for publication purposes.

To conclude, this study is one of few that provides a full description on selected South African tabanid species alongside morphological data. The South Africa tabanids however, are still in need of revision. COI was not sufficient in distinguishing between all species collected but still had some utility in other species and or species groups. The incongruencies that were observed may be resolved with the use of more markers. The addition of the COI and AATS sequences obtained during this study broaden the little information that is currently known and will aid studies on this crucial family in the future.

Detection of *Besnoitia besnoiti* from tabanids from Skukuza in Kruger National Park, South Africa

Abstract

Bovine besnoitiosis, caused by *Besnoitia besnoiti*, is a generally non-fatal disease with a significant impact on the economic output of cattle farmers. With the recent spread of the pathogen in Europe, emphasis on the identification of the transmission method as well as the definitive host of the pathogen has been stressed. This study aimed to determine the presence of *B. besnoiti* in Tabanidae from Skukuza in Kruger National Park, thereby clarifying the role they might possess in transmission. Samples were collected from KNP using baited traps, after which they were identified and subjected to DNA extractions. Samples were pooled and screened with *B. besnoiti* ITS1 specific primers. In total 561 tabanids divided into 61 pools were screened for *B. besnoiti*, with no positives obtained. Further investigations should be conducted on this pathogen and the role of tabanids in its transmission.

Key words: ITS1, Protozoan, Tabanidae, elephant-skin disease, vector, horsefly, pathogen screening

Introduction

The genus *Besnoitia* entails obligatory intracellular coccidian protozoan parasites of the family Sarcocystidae within the phylum Apicomplexa (Gutiérrez-Expósito *et al.*, 2012, Cortes *et al.*, 2014). Currently, 10 species are recognized worldwide of which most are recorded from marsupials and small mammals (Gutiérrez-Expósito *et al.*, 2012, Cortes *et al.*, 2014). These species include*: B. akadoni, B. bennetti, B. besnoiti, B. caprae, B. darlingi, B. jellisoni, B. neotomofelis, B. oryctofelisi, B. tarandi, and B. wallacei* (Gutiérrez-Expósito *et al*., 2012). To date, only four of the species, *B. oryctofelisi, B. darling, B. neotomofelis, and B. wallacei,* has well documented life cycles, where the definitive host of the remaining six has been elusive, resulting in little information on the aetiology of these pathogens (Sharif *et al.*, 2019). The means of transmission is also under investigation, where blood-sucking arthropods, mainly tabanids, are believed to be among the main contributors (Bigalke, 1967, Sharif *et al.*, 2019).

Besnoitia besnoiti is the causative agent of bovine besnoitiosis in cattle (Bigalke *et al.*, 1967, Diesing *et al.*, 1988, Liénard *et al.*, 2011, Frey *et al.*, 2016, Ryan *et al.*, 2016). Bovine besnoitiosis is a severe but generally non-fatal disease, first described in France (Muñoz-Caro *et al.*, 2014). It has a high economic impact on many countries within Sub-Saharan Africa, Asia and southwestern Europe. However, geographical spread has been observed to Portugal, Spain, Germany and Italy and as of 2010 bovine besnoitiosis has been classified as an emerging disease in the EU by the European Food Safety Authority (EFSA) (EFSA, 2010, Muñoz-Caro *et al.*, 2014).

Bovine besnoitiosis presents in two sequential clinical stages; the acute and the chronic stage. The acute stage occurs 11 to 13 days after infection and lasts between six to 10 days, this is followed by the chronic stage which is lifelong and develops in the time frame at which the animals recover from the acute stage (Pols, 1960, Bigalke, 1968). The acute stage is characterized by a high parasitaemia, hyperthermia, photophobia, ocular and nasal discharge, lymphadenitis, subcutaneous oedema (anasarca), lameness, orchitis and severe respiratory disorders (EFSA, 2010, Cortes *et al.*, 2011, García-Lunar *et al.*, 2013, Cortes *et al.*, 2014). The chronic stage, subsequently, is characterized by visible tissue cysts which develops within the cell of the cutaneous and subcutaneous connective tissue within the sclera and conjunctiva and vulval regions (EFSA, 2010, Cortes *et al.*, 2014). With the fading of the oedema; a progressive thickening, hardening and wrinkling or folding of the skin occurs in areas around the neck, shoulders and hindquarters accompanied by hyperkeratosis, hyperpigmentation and alopecia (Pols, 1960, Cortes *et al.*, 2011, García-Lunar *et al.*, 2013). All breeds, ages and sexes of cattle are affected by *Besnoitia besnoiti*. The economic losses are

attributed to the sterility in bulls (caused by necrotizing orchitis), abortions, weight decline, reduction in milk production, mortality, lower value of slaughter animals and decreased hide quality (Pols, 1960, Liénard *et al.*, 2013).

Currently no effective treatment is available for bovine besnoitiosis, however, a live vaccine has been developed in South Africa. The vaccine was developed from a cultured strain obtained from a blue wildebeest which presented with the viscerotropic form and was of low virulence to cattle (Bigalke *et al.*, 1974). The vaccine protects against clinical besnoitiosis although sub-clinical infections could still be observed (Bigalke *et al.*, 1974, Cortes *et al.*, 2014).

In southern Africa, Bigalke (1967) found *B. besnoiti* circulating in impala and blue wildebeest within the vicinity of the Skukuza area of Kruger National Park. However, the last study conducted on tabanids role in the transmission of *Besnoitia besnoiti* was conducted by Taioe *et al.* (2017). Taioe *et al.* (2017), nevertheless, only tested 370 flies from South Africa from areas that are not commonly associated with *Besnoitia besnoiti*. Thus, we aim to determine the presence of *Besnoitia besnoiti* in tabanids captured in the Skukuza area of Kruger National Park (South Africa), to clarify their role in the transmission of this pathogen in the area.

Material and Methods

Samples

Samples were collected and identified from Kruger National Park as mentioned in the previous manuscript. Only the Skukuza samples were used for the screening of *Besnoitia besnoiti*.

Extraction method

Extraction of the whole tabanid was conducted as mentioned in the previous manuscript, using the Chelex 100 resin method and stored at -20°C until screening. Samples were pooled, in which 5 uL of DNA template from each individual, maximum of 10 individuals, of the same species were placed in the same Eppendorf tube and given a respective pool code.

Screening

All pools were screened with *B. besnoiti* specific primers targeting the ITS1 region. The primers were obtained from Cortes *et al.* (2007) (Table 5). A 20 uL total volume reaction was prepared constituting of 4 uL ddH_2O, 10uL Phusion Flash PCR Master Mix (final concentration of x1) and 0.5 uL of both the forward and reverse primers (final concentration of 0.5uM each). This master mix was aliquoted, 15

µL in each tube, after which 5 µL of the pooled DNA was added. The PCR conditions were as follows; Initial denaturalization at 98°C for 10 seconds, followed by 40 cycles of amplification using 98°C for 1 second, 56°C for 5 seconds and 72°C for 15 seconds, elongation at 70°C for 1 minute and ended with a 4°C hold. A *B. besnoiti* positive sample alongside a ddH_2O negative sample was used as controls during all screening to validate results.

Table 5: Summarized information on the *Besnoitia besnoiti* primers used for amplification including: primer names, length, sequence, target size and reference.

Target region	Primer Names	Primer Length	Primer Sequence (5'-3')	Target Size	Reference
ITS1 (*B. besnoiti*)	ITS1F	24-mer	TGA CAT TTA ATA ACA ATC AAC CCT T	231 bp	Cortes *et al.* (2007)
	ITS1R	26-mer	GGT TTG TAT TAA CCA ATC CGT GA		

Gel electrophoreses

To prepare the amplified products for visualization, 2 uL of amplified DNA was added to 2 µL of 6x TriTrack DNA Loading Dye. The amplified products were then visualized by agarose gel electrophoresis (1.5 % (w/v) agarose, 120V for 60 minutes) in 1 x Tris-acetate EDTA (TAE) electrophoresis buffer. The gel was analysed using the ChemiDoc XRS+ System with corresponding image lab software. A 100 bp molecular marker was used for reference.

When a positive band was observed, the pool was noted and the individual constituting the pool was screened individually following the same methodology. If the individual positive was confirmed, the amplified product was sent to Inqaba Biotec™ (Pretoria, South Africa) for Sanger sequencing.

Results

A total of 561 tabanids were identified and stratified into 11 species within five genera, including: *Atylotus, Chrysops, Haematopota, Philoliche* and *Tabanus*. In total 61 pools were screened for *B. besnoiti*. Of the 61 pools screened, all results were negative except for a single inconclusive result. During the pool screening, pool 2 tested positive (Figure 42). This pool constituted of six *Atylotus agrestis* individuals. However, when the six individuals were screened separately, they all tested negative (Figure 43).

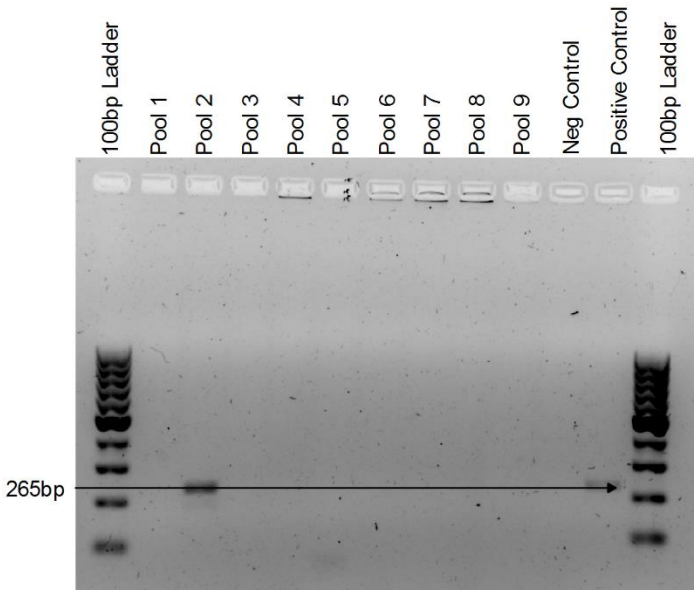

Figure 42: Gel image, where lane 1 and 13 contains 100bp Ladders, lane 11 contained the negative control, lane 12 contains the positive control and lane 3 is pool 2 that tested positive.

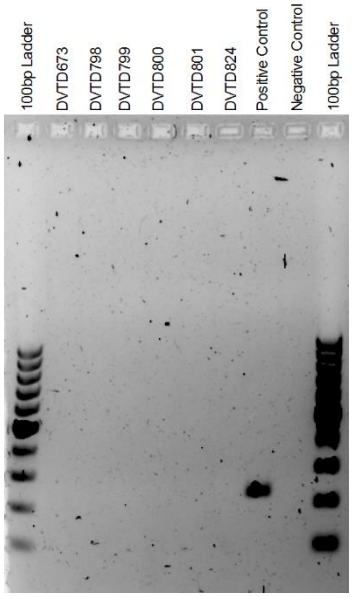

Figure 43: Individual samples that constituted pool 2. All of which tested negative. Lane 1 and 10 contained the 100bp ladder, 8 contained the negative control and 9 contains the positive control.

Discussion

Spreading of *Besnoitia besnoiti* within and between cattle herds has remained poorly understood (Sharif *et al.*, 2019). Mechanical transmission of *B. besnoiti* by tabanids and other blood sucking arthropods has been experimentally proven (Bigalke, 1967, Bigalke, 1968). During the experimental studies, Bigalke (1968) found that tabanids are more effective vectors compared to *Stomoxys calcitrans*, which was confirmed by Sharif *et al.* (2019).

The single positive result could have been due to contamination from the positive control. The inconclusive pool was, however, from the *Atylotus* genus which according to Bigalke (1968) is among the main transmitters of *B. besnoiti* alongside that of *Tabanus*. Thus, our results are not entirely dismissible and possibly warrant further evaluation.

The absence of *B. besnoiti* in the samples, however, does not exclude tabanids as being mechanical vectors of the pathogen. There are several factors that influence the detection of such pathogens in mechanical vectors, including: 1) titre of the pathogen in hosts, 2) density of the hosts, 3) synchronization of hosts and vectors and 4) time of year that the vector and host interacts (Foil and Gorham, 2004, Baldacchino *et al.*, 2014a). Another factor which may influence the detection of pathogens, is the unlikelihood of a fully engorge female to be attracted to the octenol baited traps. This lowers the collection of flies that has been in contact with infected hosts; however this is yet to be fully investigated.

It is to be noted that the presentation of *B. besnoiti* in the Skukuza area is in the viscerotropic form (Bigalke *et al.*, 1967). In this type of presentation, the cysts are found internally during the chronic stage and not on the external skin. This limits the time in which the tabanids are able to obtain the pathogen from the host to the acute stage where the tachyzoites are circulating in the bloodstream. Thus, the seasonality is of foremost importance. Little research is available on tabanid seasonality in Southern Africa. According to Liénard *et al.* (2011), most clinical cases are generally observed during the summer, thus, the acute phase is generally found during the spring season. The samples in this study were collected in mid-summer (January), possibly decreasing the likelihood of detecting *B. besnoiti*.

To conclude, our study on the detection of *B. besnoiti* in tabanids of the Skukuza area of Kruger National Park resulted in no positives. There is still a gap in knowledge regarding this pathogen, not only in Southern Africa but worldwide. This gives the potential for several studies, including research on the seasonality, the lifecycle as well as identification of a definitive host.

Concluding discussion

This study served as one of few comprehensive species reports on South African Tabanidae and the detection of *Besnoitia besnoiti* within them. The overall aim was the comparison of the traditional alpha-taxonomic approach to tabanid species delimitation with molecular methods using two gene regions, mitochondrial cytochrome oxidase I (COI) and nuclear alanyl-tRNA-synthetase (AATS). Additionally, the study aimed to detect the presence of *Besnoitia besnoiti* in the collected tabanids to hypothesize on their role as vectors. A prior component to the main study was a short evaluation on DNA extraction method efficiency.

Current comparisons of DNA extraction methods have excluded the classical salting out methods, disregarding the lower cost advantages (Chacon-Cortes et al., 2012). These comparisons have also neglected the use of arthropods as samples (Lienhard and Schäffer, 2019). During this evaluation, Tabanidae, Ixodidae and Culicidae were compared based on the quantity and quality of extracted DNA, time per extraction as well as cost per extraction. Chelex 100 Resin produced a significantly higher quantity of DNA compared to the remaining methods; however, the quality of the DNA amongst all methods had little variation and was not significant. Whilst each method had its own advantages and disadvantages, Chelex 100 resin had proven to be a quick and affordable method that was selected as the preferred method of DNA extraction for the remainder of this study.

During the identification of the tabanid specimens, 16 morphologically unique species were identified of which the *Tabanus* genus was the most predominant constituting nearly 90% of the overall catches. With *Tabanus* comprising nearly 3200 out of the approximate 4450 described species worldwide, this was to be expected (Morita *et al.*, 2016). Two described species, *Philoliche medialis* and *Haematopota daveyi*, was not reported by Usher (1972) as occurring in South Africa and should be regarded as new to the region. With the limited molecular information of tabanids worldwide, molecular analysis proved to be challenging (Mugasa *et al.*, 2018). Several incongruences were detected in all the topologies excluding that of the *Haematopota*. During analysis, some species were found to be uniform such as *Chrysops obliquefasciata* and *Tabanus atrimanus*; others formed co-occurring haplotypes such as *Tabanus gratus*, *Tabanus taeniola* and *Tabanus par*. The haplotypes could sometimes be separated based on geographical location of the collected samples, in other instances not. Several reference sequences, all from a single study by Taioe *et al.* (2017) were found not to cluster with the obtained sequences in this study and the reference sequences of Morita (2008), Morita *et al.* (2016), Mugasa *et al.* (2018) and Votýpka *et al.* (2019). This may be the result of misidentification of the species or indicative of taxonomic instability; however, human error

is likely to be the result of these incongruences. Misidentifications could result of intraspecific morphological variation; where a species is genetically identical but morphological differentiation has occurred and may be subtle or extreme (Oney *et al.*, 2013). This can be seen with the *Philoliche* (*Bulex*) *suavis* and the *Philoliche* (*Buplex*) "undescribed" sequences that formed a well-supported monophyletic group but had a similar but variable morphological appearance. Sub-species may also explain some of the co-occurring haplotypes separated by regions but with the little morphological data that is currently available, conclusive discussions cannot be made. Human error such as incorrect sequencing, over sequence editing or wrongfully uploading the incorrect sequences may lead to the unusual clustering of reference sequences. In the present study, the sole use of the COI gene did not provide enough phylogenetic signal to differentiate between all species of the Tabanidae of South Africa. Unfortunately, the AATS analysis could not be completed in the timeframe which may have aided in clarifying some incongruences that were observed. The combination of genes in several different cell locations provides different variations of the topologies which may have different phylogenetic signals (Meiklejohn *et al.*, 2013).

Tabanidae has not just been neglected in molecular studies, but in vector studies as well (Baldacchino *et al.*, 2014a). Tabanids are known to be mechanical and biological vectors of a wide variety of pathogens including protozoa, nematodes, viruses and bacteria (Krinsky, 1976, Foil and Hogsette, 1994, Foil and Gorham, 2004, Baldacchino *et al.*, 2014a). The vector ability of tabanids in South Africa has only been investigated by two studies Taioe *et al.* (2017) and Mazibuko (2018). The study conducted by Taioe *et al.* (2017) was the only one that included South Africa and *Besnoitia besnoiti* screening, however, they did not include areas that *B. besnoiti* are generally thought to circulate in. *B. besnoiti* has been found to circulate in the Skukuza area within impala and blue wildebeest (Bigalke *et al.*, 1967). While this study focused on the indicated area, no tabanids tested positive for *B. besnoiti*. The absence of the pathogen does not exclude tabanids as vectors, external factors may have skewed the chances of obtaining positives, such as: 1) the collection of tabanids outside of the *B. besnoiti* circulating season, 2) low titter of the pathogens in the hosts, 3) disease presenting in the viscerotropic form as compared to the epidermis form and 4) the engorgement state of the female (fully engorge female are less likely to be attracted to the octenol baited traps).

In conclusion, this study determined that the use of the COI gene by itself does not provide sufficient phylogenetic signal to differentiate at the species level for all South African tabanid species. The family Tabanidae, does however, require phylogenetic revision which will be aided by an increase in availability of barcodes of more species. No *B. besnoiti* was detected in the tabanids from Kruger

National Park. With the gap in knowledge of *B. besnoiti's* life cycle, further investigation must be conducted on the role tabanids play as intermediate hosts.

References

AL DHAFER, H. M., DAWAH, H. A. & ABDULLAH, M. A. 2009. Tabanidae (Diptera) of Saudi Arabia. *Saudi journal of biological sciences,* 16**,** 77-83.

ANTHONY, D. W. 1962. Tabanidae as disease vectors. *Biological transmission of disease agents***,** 93-107.

ANTHONY, D. W. 1977. Pathogens of Tabanidae (horseflies). *Bulletin of the World Health Organization,* 55**,** 239-41.

ATHANASIO, C. G., CHIPMAN, J. K., VIANT, M. R. & MIRBAHAI, L. 2016. Optimisation of DNA extraction from the crustacean Daphnia. *PeerJ,* 4**,** e2004.

AUSTEN, E. E. 1911. New African species of Tabanus.—Part I. *Bulletin of entomological research,* 2**,** 279-290.

AUSUBEL, F., BRENT, R., KINGSTON, R., MOORE, D., SEIDMAN, J., SMITH, J. & STRUHL, K. 1995. Phenol extraction and ethanol precipitation of DNA. *Short Protocols in Molecular Biology, 3rd Ed.; John Wiley and Sons, Inc.: Canada***,** 2-3.

AXTELL, R. C. 1976. Coastal horse flies and deer flies (Diptera: Tabanidae). *Marine insects***,** 415.

BAGHDADI, N., MALLET, C. & ZRIBI, M. 2018. *QGIS and Generic Tools,* John Wiley & Sons.

BALDACCHINO, F., DESQUESNES, M., MIHOK, S., FOIL, L. D., DUVALLET, G. & JITTAPALAPONG, S. 2014a. Tabanids: neglected subjects of research, but important vectors of disease agents! *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases,* 28**,** 596-615.

BALDACCHINO, F., PUECH, L., MANON, S., HERTZOG, L. R. & JAY-ROBERT, P. 2014b. Biting behaviour of Tabanidae on cattle in mountainous summer pastures, Pyrenees, France, and effects of weather variables. *Bulletin of entomological research,* 104**,** 471-479.

BANERJEE, D., KUMAR, V., MAITY, A., GHOSH, B., TYAGI, K., SINGHA, D., KUNDU, S., LASKAR, B. A., NASKAR, A. & RATH, S. 2015. Identification through DNA barcoding of Tabanidae (Diptera) vectors of surra disease in India. *Acta tropica,* 150**,** 52-58.

BARKER, K. 1998. At the bench. *A Laboratory navigator. Cold spring Laboratory*.

BASSON, P., MCCULLY, R. & BIGALKE, R. 1970. Observations on the pathogenesis of bovine and antelope strains of Besnoitia besnoiti (Marotel, 1912) infection in cattle and rabbits.

BAYLESS, K. M. 2012. Evolutionary Studies in Tabanomorpha (Diptera) with New Classifications of Xylophagidae and Tabaninae.

BELTRÁN, M., JIGGINS, C. D., BULL, V., LINARES, M., MALLET, J., MCMILLAN, W. O. & BERMINGHAM, E. 2002. Phylogenetic discordance at the species boundary: comparative gene genealogies among rapidly radiating Heliconius butterflies. *Molecular biology and evolution,* 19**,** 2176-2190.

BIGALKE, R. 1967. The artificial transmission of Besnoitia besnoiti (Marotel, 1912) from chronically infected to susceptible cattle and rabbits.

BIGALKE, R., VAN NIEKERK, J., BASSON, P. & MCCULLY, R. 1967. Studies on the relationship between Besnoitia of blue wildebeest and impala, and Besnoitia besnoiti of cattle. *Onderstepoort Journal of Veterinary Research,* 34**,** 7-28.

BIGALKE, R. D. 1968. New concepts on the epidemiological features of bovine besnoitiosis as determined by laboratory and field investigations. *Onderstepoort Journal of Veterinary Research,* 35.

BIGALKE, R. D. 1981. Besnoitiosis and globidiosis. *Diseases of Cattle in the Tropics.* Springer.

BIGALKE, R. D., SCHOEMAN, J. H. & MCCULLY, R. M. 1974. Immunization against bovine besnoitiosis with a live vaccine prepared from a blue wildebeest strain of Besnoitia besnoiti grown in cell cultures. 1. Studies on rabbits.

BIMBOIM, H. C. & DOLY, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids research,* 7**,** 1513-1523.

BOTERO-CASTRO, F., TILAK, M.-K., JUSTY, F., CATZEFLIS, F., DELSUC, F. & DOUZERY, E. J. 2013. Next-generation sequencing and phylogenetic signal of complete mitochondrial genomes for resolving the evolutionary history of leaf-nosed bats (Phyllostomidae). *Molecular phylogenetics and evolution,* 69**,** 728-739.

BOYD, K. E. & FARNHAM, P. J. 1997. Myc versus USF: discrimination at the cad gene is determined by core promoter elements. *Molecular and Cellular Biology,* 17**,** 2529-2537.

BURNETT, A. M. & HAYS, K. L. 1977. Seasonal and diurnal distributions of adult female horse flies (Diptera, Tabanidae) at Gold Hill, Alabama.

CASQUET, J., THEBAUD, C. & GILLESPIE, R. G. 2012. Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. *Molecular Ecology Resources,* 12**,** 136-141.

CASTALANELLI, M. A., SEVERTSON, D. L., BRUMLEY, C. J., SZITO, A., FOOTTIT, R. G., GRIMM, M., MUNYARD, K. & GROTH, D. M. 2010. A rapid non-destructive DNA extraction method for insects and other arthropods. *Journal of Asia-Pacific Entomology,* 13**,** 243-248.

CHACON-CORTES, D., HAUPT, L. M., LEA, R. A. & GRIFFITHS, L. R. 2012. Comparison of genomic DNA extraction techniques from whole blood samples: a time, cost and quality evaluation study. *Molecular biology reports,* 39**,** 5961-5966.

CHAINEY, J. 2017. Tabanidae *In:* EDITING, S. G. (ed.) *Manual of Afrotropical Diptera.* Pretoria: Suricata.

CHEN, H., RANGASAMY, M., TAN, S. Y., WANG, H. & SIEGFRIED, B. D. 2010. Evaluation of five methods for total DNA extraction from western corn rootworm beetles. *PLoS one,* 5**,** e11963.

CHENG, L. 1976. Marine insects.

CONNAL, A. M. R. I. N. & CONNAL, S. L. M. 1922. The development of Loa Loa (Guyot) in Chrysops Silacea (Austen) and in Chrysops Dimidiata (van der Wulp). *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 16**,** 81-80.

COOKSON, D. M. 1967. Some habits of male horse-flies in Rhodesia (Diptera: Tabanidae). *Annals of the Natal Museum,* 18**,** 647-654.

CORTES, H., LEITAO, A., GOTTSTEIN, B. & HEMPHILL, A. 2014. A review on bovine besnoitiosis: a disease with economic impact in herd health management, caused by Besnoitia besnoiti (Franco and Borges, 1916). *Parasitology,* 141**,** 1406-1417.

CORTES, H., MULLER, N., BOYKIN, D., STEPHENS, C. E. & HEMPHILL, A. 2011. In vitro effects of arylimidamides against Besnoitia besnoiti infection in Vero cells. *Parasitology,* 138**,** 583-592.

CORTES, H. C., REIS, Y., GOTTSTEIN, B., HEMPHILL, A., LEITÃO, A. & MÜLLER, N. 2007. Application of conventional and real-time fluorescent ITS1 rDNA PCR for detection of Besnoitia besnoiti infections in bovine skin biopsies. *Veterinary parasitology,* 146**,** 352-356.

CYWINSKA, A., HANNAN, M., KEVAN, P., ROUGHLEY, R., IRANPOUR, M. & HUNTER, F. 2010. Evaluation of DNA barcoding and identification of new haplomorphs in Canadian deerflies and horseflies. *Medical and veterinary entomology,* 24**,** 382-410.

DAHM, R. 2005. Friedrich Miescher and the discovery of DNA. *Developmental biology,* 278**,** 274-288.

DESQUESNES, M. & DIA, M. L. 2003a. Mechanical transmission of Trypanosoma congolense in cattle by the African tabanid Atylotus agrestis. *Experimental Parasitology,* 105**,** 226-231.

DESQUESNES, M. & DIA, M. L. 2003b. Trypanosoma vivax: mechanical transmission in cattle by one of the most common African tabanids, Atylotus agrestis. *Experimental Parasitology,* 103**,** 35-43.

DIAS, J. T. S. 1960. The discovery of a new species of the genus Adersia, Austen, 1912 (Diptera: Tabanidae) found in the coastal region of Mozambique. *South African Journal of Science,* 56**,** 115-118.

DIESING, L., HEYDORN, A., MATUSCHKA, F., BAUER, C., PIPANO, E., DE WAAL, D. & POTGIETER, F. 1988. Besnoitia besnoiti: studies on the definitive host and experimental infections in cattle. *Parasitology research,* 75**,** 114-117.

DILLON, N., AUSTIN, A. & BARTOWSKY, E. 1996. Comparison of preservation techniques for DNA extraction from hymenopterous insects. *Insect Molecular Biology,* 5**,** 21-24.

DITTRICH-SCHRODER, G., WINGFIELD, M. J., KLEIN, H. & SLIPPERS, B. 2012. DNA extraction techniques for DNA barcoding of minute gall-inhabiting wasps. *Molecular Ecology Resources,* 12**,** 109-115.

DREES, B. M. 1987. Mud cylinders of Tabanidae (Diptera) from Texas. *Annals of the entomological Society of America,* 80**,** 51-54.

DUBEY, J., VAN WILPE, E., BLIGNAUT, D., SCHARES, G. & WILLIAMS, J. 2013. Development of early tissue cysts and associated pathology of Besnoitia besnoiti in a naturally infected bull (Bos taurus) from South Africa. *The Journal of parasitology,* 99**,** 459-466.

EFSA 2010. Bovine besnoitiosis: An emerging disease in Europe. *EFSA Journal,* 8**,** 1499.

ELLIS, J. T., HOLMDAHL, O. J. M., RYCE, C., NJENGA, J. M., HARPER, P. A. & MORRISON, D. A. 2000. Molecular phylogeny of Besnoitia and the genetic relationships among Besnoitia of cattle, wildebeest and goats. *Protist,* 151**,** 329-336.

ESTERHUIZEN, J. 2006. Seasonal abundance of horse flies (Diptera: Tabanidae) from two conservation areas in northeastern KwaZulu-Natal Province, South Africa. *African Entomology,* 14**,** 395-397.

FERGUSON-MILLER, S., BRAUTIGAN, D. & MARGOLIASH, E. 1978. Definition of cytochrome c binding domains by chemical modification. III. Kinetics of reaction of carboxydinitrophenyl cytochromes c with cytochrome c oxidase. *Journal of Biological Chemistry,* 253**,** 149-159.

FOIL, L. & HOGSETTE, J. 1994. Biology and control of tabanids, stable flies and horn flies. *Revue scientifique et technique-Office international des épizooties,* 13**,** 1125-1158.

FOIL, L. D. & GORHAM, J. R. 2004. Mechanical transmission of disease agents by arthropods. *Medical entomology*. Springer.

FOLMER, O., HOEH, W., BLACK, M. & VRIJENHOEK, R. 1994. Conserved primers for PCR amplification of mitochondrial DNA from different invertebrate phyla. *Molecular marine biology and biotechnology,* 3**,** 294-299.

FREY, C. F., REGIDOR-CERRILLO, J., MARREROS, N., GARCÍA-LUNAR, P., GUTIÉRREZ-EXPÓSITO, D., SCHARES, G., DUBEY, J. P., GENTILE, A., JACQUIET, P. & SHKAP, V. 2016. Besnoitia besnoiti lytic cycle in vitro and differences in invasion and intracellular proliferation among isolates. *Parasites & vectors,* 9**,** 115.

FUJIWARA, H. & ISHIKAWA, H. 1986. Molecular mechanism of introduction of the hidden break into the 28S rRNA of insects: implication based on structural studies. *Nucleic acids research,* 14**,** 6393-6401.

GARCÍA-LUNAR, P., ORTEGA-MORA, L., SCHARES, G., GOLLNICK, N., JACQUIET, P., GRISEZ, C., PREVOT, F., FREY, C., GOTTSTEIN, B. & ÁLVAREZ-GARCÍA, G. 2013. An inter-laboratory comparative study of serological tools employed in the diagnosis of Besnoitia besnoiti infection in bovines. *Transboundary and emerging diseases,* 60**,** 59-68.

GILBERT, M. T. P., MOORE, W., MELCHIOR, L. & WOROBEY, M. 2007. DNA extraction from dry museum beetles without conferring external morphological damage. *PLoS one,* 2**,** e272.

GILLESPIE, J., JOHNSTON, J., CANNONE, J. & GUTELL, R. 2006. Characteristics of the nuclear (18S, 5.8 S, 28S and 5S) and mitochondrial (12S and 16S) rRNA genes of Apis mellifera (Insecta: Hymenoptera): structure, organization, and retrotransposable elements. *Insect Molecular Biology,* 15**,** 657-686.

GLASEL, J. 1995. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *Biotechniques,* 18**,** 62-63.

GODDARD, J. 2008. Dynamics of arthropod-borne diseases. *Infectious Diseases and Arthropods.* Springer.

GOLLNICK, N., SCHARR, J., SCHARES, S., BÄRWALD, A., SCHARES, G. & LANGENMAYER, M. 2018. Naturally acquired bovine besnoitiosis: Disease frequency, risk and outcome in an endemically infected beef herd. *Transboundary and emerging diseases*.

GUILIANI, N., BENGRINE, A., BORNE, F., CHIPPAUX, M. & BONNEFOY, V. 1997. Alanyl-tRNA synthetase gene of the extreme acidophilic chernolithoautotrophic Thiobacillus ferrooxidans is highly homologous to alaS genes from all living kingdoms but cannot be transcribed from its promoter in Escherichia coli. *Microbiology,* 143**,** 2179-2187.

GUIMARAES, R. R., JÚNIOR, R. R. G., HARLAN-RODRIGUES, R. S., GUIMARÃES, R. R., CARVALHO, R. W. & DE XEREZ, R. 2017. Seasonality and Relative Abundance of tabanids (Diptera, Tabanidae) on Marambaia Island, Rio de Janeiro, Brazil. *EntomoBrasilis,* 10**,** 82-88.

GUIMARÃES, R. R., RODRIGUES, H. R. S. & JÚNIOR, R. R. G. 2017. Tabanids in South America. *Insect Physiology and Ecology***,** 55.

GUTIÉRREZ-EXPÓSITO, D., ARNAL, M. C., MARTÍNEZ-DURÁN, D., REGIDOR-CERRILLO, J., REVILLA, M., DE LUCO, D. L. F., JIMÉNEZ-MELÉNDEZ, A., CALERO-BERNAL, R., HABELA, M. A. & GARCÍA-BOCANEGRA, I. 2016. The role of wild ruminants as reservoirs of Besnoitia besnoiti infection in cattle. *Veterinary parasitology,* 223**,** 7-13.

GUTIÉRREZ-EXPÓSITO, D., ORTEGA-MORA, L. M., GAJADHAR, A. A., GARCÍA-LUNAR, P., DUBEY, J. P. & ÁLVAREZ-GARCÍA, G. 2012. Serological evidence of Besnoitia spp. infection in Canadian wild ruminants and strong cross-reaction between Besnoitia besnoiti and Besnoitia tarandi. *Veterinary parasitology,* 190**,** 19-28.

HACKENBERGER, B. K., JARIĆ, D. & KRČMAR, S. 2009. Distribution of tabanids (Diptera: Tabanidae) along a two-sided altitudinal transect. *Environmental entomology,* 38**,** 1600-1607.

HAFEZ, M., EL-ZIADY, S. & HEFNAWY, T. 1970. Biological studies of the immature stages of Tabanus taeniola P. de B. in Egypt (Diptera: Tabanidae). *Bulletin de la Societe Entomologique d'Egypte,* 54**,** 465-493.

HEBERT, P. D., CYWINSKA, A. & BALL, S. L. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B: Biological Sciences,* 270**,** 313-321.

HERCZEG, T., BLAHÓ, M., SZÁZ, D., KRISKA, G., GYURKOVSZKY, M., FARKAS, R. & HORVÁTH, G. 2014. Seasonality and daily activity of male and female tabanid flies monitored in a Hungarian hill-country pasture by new polarization traps and traditional canopy traps. *Parasitology research,* 113**,** 4251-4260.

HERCZEG, T., SZÁZ, D., BLAHÓ, M., BARTA, A., GYURKOVSZKY, M., FARKAS, R. & HORVÁTH, G. 2015. The effect of weather variables on the flight activity of horseflies (Diptera: Tabanidae) in the continental climate of Hungary. *Parasitology research,* 114**,** 1087-1097.

HULTMAN, T., STÅHL, S., HORNES, E. & UHLÉN, M. 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucleic acids research,* 17**,** 4937-46.

INAOKA, T., HORI, E., OGUNBA, E. O., WATANABE, M., YAMAGUCHI, K., YONEYAMA, Y., AMANO, T., TAKAOKA, M. & MAEDA, R. 1988. Morphological characteristics of the anal segment in the identification of the larvae of Tabanus-species (Diptera, Tabanidae) collected from Nigeria. *Medical Entomology and Zoology,* 39**,** 277-282.

JERSÁKOVÁ, J., JÜRGENS, A., ŠMILAUER, P. & JOHNSON, S. D. 2012. The evolution of floral mimicry: identifying traits that visually attract pollinators. *Functional Ecology,* 26**,** 1381-1389.

JOHNSON, S. 2006. Pollination by long-proboscid flies in the endangered African orchid Disa scullyi. *South African Journal of Botany,* 72**,** 24-27.

KAROLYI, F., COLVILLE, J. F., HANDSCHUH, S., METSCHER, B. D. & KRENN, H. W. 2014. One proboscis, two tasks: Adaptations to blood-feeding and nectar-extracting in long-proboscid horse flies (Tabanidae, Philoliche). *Arthropod structure & development,* 43**,** 403-413.

KIM, Y.-T., CHOI, E.-H., SON, B.-K., SEO, E.-H., LEE, E.-K., RYU, J.-K., HA, G.-W., KIM, J.-S., KWON, M.-R. & NAM, J.-H. 2012. Effects of Storage Buffer and Temperature on the Integrity of Human DNA. *대한임상검사과학회지 (Korean Journal of Clinical Laboratory Science),* 44**,** 24-30.

KING, H. H. 1910. Some observations on the bionomics of Tabanus par , Walker, and Tabanus tæniola , Pal. de Beauv. *Bulletin of entomological research,* 1**,** 99-104.

KIT, S., TOKUNO, S., NAKAJIMA, K., TRKULA, D. & DUBBS, D. 1970. Temperature-sensitive simian virus 40 mutant defective in a late function. *Journal of virology,* 6**,** 286-294.

KNIEPERT, F.-W. 1980. Blood-feeding and nectar-feeding in adult Tabanidae (Diptera). *Oecologia,* 46**,** 125-129.

KOUAM, M. K. & KAMGNO, J. 2017. The African Chrysops. *Biological Control of Pest and Vector Insects***,** 285.

KRCMAR, S., MERDIC, E. & KOPI, M. 2005. Diurnal periodicity in the biting activity of horsefly species in the Kopacki rit Nature Park, Croatia (Diptera: Tabanidae). *Entomologia generalis,* 28**,** 139.

KRINSKY, W. L. 1976. Animal disease agents transmitted by horse flies and deer flies (Diptera: Tabanidae). *Journal of Medical Entomology,* 13**,** 225-275.

LANGENMAYER, M., GOLLNICK, N., MAJZOUB-ALTWECK, M., SCHARR, J., SCHARES, G. & HERMANNS, W. 2015. Naturally acquired bovine besnoitiosis: histological and immunohistochemical findings in acute, subacute, and chronic disease. *Veterinary pathology,* 52**,** 476-488.

LAWSON, F. S., CHARLEBOIS, R. L. & DILLON, J. 1996. Phylogenetic analysis of carbamoylphosphate synthetase genes: complex evolutionary history includes an internal duplication within a gene which can root the tree of life. *Molecular biology and evolution,* 13**,** 970-977.

LESSARD, B., CAMERON, S., BAYLESS, K., WIEGMANN, B. M. & YEATES, D. K. 2013. The evolution and biogeography of the austral horse fly tribe Scionini (Diptera: Tabanidae: Pangoniinae) inferred from multiple mitochondrial and nuclear genes. *Molecular phylogenetics and evolution,* 68**,** 516-540.

LI, Y., ZHOU, X., FENG, G., HU, H., NIU, L., HEBERT, P. D. & HUANG, D. 2010. COI and ITS2 sequences delimit species, reveal cryptic taxa and host specificity of fig-associated Sycophila (Hymenoptera, Eurytomidae). *Molecular Ecology Resources,* 10**,** 31-40.

LIÉNARD, E., SALEM, A., GRISEZ, C., PRÉVOT, F., BERGEAUD, J., FRANC, M., GOTTSTEIN, B., ALZIEU, J., LAGALISSE, Y. & JACQUIET, P. 2011. A longitudinal study of Besnoitia besnoiti infections and seasonal abundance of Stomoxys calcitrans in a dairy cattle farm of southwest France. *Veterinary parasitology,* 177**,** 20-27.

LIÉNARD, E., SALEM, A., GRISEZ, C., PRÉVOT, F., BERGEAUD, J. P., FRANC, M., GOTTSTEIN, B., ALZIEU, J. P., LAGALISSE, Y. & JACQUIET, P. 2011. A longitudinal study of Besnoitia besnoiti infections and seasonal abundance of Stomoxys calcitrans in a dairy cattle farm of southwest France. *Veterinary parasitology,* 177**,** 20-7.

LIÉNARD, E., SALEM, A., JACQUIET, P., GRISEZ, C., PRÉVOT, F., BLANCHARD, B., BOUHSIRA, E. & FRANC, M. 2013. Development of a protocol testing the ability of Stomoxys calcitrans (Linnaeus, 1758)(Diptera: Muscidae) to transmit Besnoitia besnoiti (Henry, 1913)(Apicomplexa: Sarcocystidae). *Parasitology research,* 112**,** 479-486.

LIENHARD, A. & SCHÄFFER, S. 2019. Extracting the invisible: obtaining high quality DNA is a challenging task in small arthropods. *PeerJ,* 7**,** e6753.

LORENZ, M. G. & WACKERNAGEL, W. 1987. Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. *Appl. Environ. Microbiol.,* 53**,** 2948-2952.

LUDWIG, W. & SCHLEIFER, K.-H. 2005. Molecular phylogeny of bacteria based on comparative sequence analysis of conserved genes. *Microbial phylogeny and evolution, concepts and controversies***,** 70-98.

LUNT, D., ZHANG, D. X., SZYMURA, J. & HEWLTT, O. 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. *Insect Molecular Biology,* 5**,** 153-165.

MACKERRAS, I. 1955. The classification and distribution of tabanidae (Diptera) 3. Subfamilies scepsidinae and chrysopinae. *Australian Journal of Zoology,* 3**,** 583-633.

MACKERRAS, I. M. 1954. Classification and distribution of Tabanidae (Diptera). *Australian Journal of Zoology,* 2**,** 431.

MARCHAND, R. W. 1920. *The Early Stages of Tabanidæ (horse-flies): By Werner Marchand, PH. D,* Rockefeller Institute for medical research.

MARGAM, V. M., GACHOMO, E. W., SHUKLE, J. H., ARIYO, O. O., SEUFFERHELD, M. J. & KOTCHONI, S. O. 2010. A simplified arthropod genomic-DNA extraction protocol for polymerase chain reaction (PCR)-based specimen identification through barcoding. *Molecular biology reports,* 37**,** 3631-3635.

MAZIBUKO, X. 2018. *Species composition of and detection of haemoparasites in horse-flies (Diptera: Tabanidae) in south-eastern Kruger National Park.* University of Pretoria.

MCALPINE, J., PETERSON, B., SHEWELL, G., TESKEY, H., VOCKEROTH, J. & WOOD, D. 1981. Manual of Nearctic Diptera. Volume 1. Agriculture Canada Monograph No. 27. Research Branch. *Agriculture Canada, Ottawa, ON*.

MCKEEVER, S. & FRENCH, F. E. 1997. Fascinating, beautiful blood feeders. *American Entomologist,* 43**,** 217-226.

MEDINA, M. & WALSH, P. J. 2000. Molecular systematics of the order Anaspidea based on mitochondrial DNA sequence (12S, 16S, and COI). *Molecular phylogenetics and evolution,* 15**,** 41-58.

MEIER, R., SHIYANG, K., VAIDYA, G. & NG, P. K. L. 2006. DNA Barcoding and Taxonomy in Diptera: A Tale of High Intraspecific Variability and Low Identification Success. *Systematic Biology,* 55**,** 715-728.

MEIKLEJOHN, K. A., WALLMAN, J. F., PAPE, T., CAMERON, S. L. & DOWTON, M. 2013. Utility of COI, CAD and morphological data for resolving relationships within the genus Sarcophaga (sensu lato)(Diptera: Sarcophagidae): a preliminary study. *Molecular phylogenetics and evolution,* 69**,** 133-141.

MORITA, S. I. 2008. A phylogeny of long-tongued horse flies (Diptera: Tabanidae: Philoliche) with the first cladistic review of higher relationships within the family. *Invertebrate Systematics,* 22**,** 311-327.

MORITA, S. I., BAYLESS, K. M., YEATES, D. K. & WIEGMANN, B. M. 2016. Molecular phylogeny of the horse flies: a framework for renewing tabanid taxonomy. *Systematic Entomology,* 41**,** 56-72.

MOULTON, J. K. & WIEGMANN, B. M. 2004. Evolution and phylogenetic utility of CAD (rudimentary) among Mesozoic-aged Eremoneuran Diptera (Insecta). *Molecular phylogenetics and evolution,* 31**,** 363-378.

MUGASA, C. M., VILLINGER, J., GITAU, J., NDUNGU, N., CIOSI, M. & MASIGA, D. 2018. Morphological re-description and molecular identification of Tabanidae (Diptera) in East Africa. *ZooKeys***,** 117.

MULLEN, G. R. & DURDEN, L. A. 2002. Medical and veterinary entomology. Amsterdam ;: Academic Press.

MÜLLER, G. C., HOGSETTE, J. A., REVAY, E. E., KRAVCHENKO, V. D., LESHVANOV, A. & SCHLEIN, Y. 2011. New records for the horse fly fauna (Diptera: Tabanidae) of Jordan with remarks on ecology and zoogeography. *Journal of vector ecology,* 36**,** 447-450.

MUÑOZ-CARO, T., SILVA, L. M., RITTER, C., TAUBERT, A. & HERMOSILLA, C. 2014. Besnoitia besnoiti tachyzoites induce monocyte extracellular trap formation. *Parasitology research,* 113**,** 4189-4197.

NAGANUMA, M., SEKINE, S.-I., FUKUNAGA, R. & YOKOYAMA, S. 2009. Unique protein architecture of alanyl-tRNA synthetase for aminoacylation, editing, and dimerization. *Proceedings of the National Academy of Sciences,* 106**,** 8489-8494.

NEAVE, S. A. 1915. The Tabanidae of Southern Nyasaland with Notes on their Life-histories. *Bulletin of entomological research,* 5**,** 287-320.

NISHIGUCHI, M. K., DOUKAKIS, P., EGAN, M., KIZIRIAN, D., PHILLIPS, A., PRENDINI, L., ROSENBAUM, H. C., TORRES, E., WYNER, Y. & DESALLE, R. 2002a. DNA isolation procedures. *Techniques in molecular systematics and evolution.* Springer.

NISHIGUCHI, M. K., DOUKAKIS, P., EGAN, M., KIZIRIAN, D., PHILLIPS, A., PRENDINI, L., ROSENBAUM, H. C., TORRES, E., WYNER, Y., DESALLE, R. & GIRIBET, G. 2002b. *DNA Isolation Procedures* Springer Science & Business Media.

NSUBUGA, A. M., ROBBINS, M. M., ROEDER, A. D., MORIN, P. A., BOESCH, C. & VIGILANT, L. 2004. Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. *Molecular ecology,* 13**,** 2089-2094.

ODENIRAN, P., MACLEOD, E., ADEMOLA, I. & WELBURN, S. 2019. Molecular identification of bloodmeal sources and trypanosomes in Glossina spp., Tabanus spp. and Stomoxys spp. trapped on cattle farm settlements in southwest Nigeria. *Medical and veterinary entomology,* 33**,** 269-281.

OLDROYD, H. 1952. The Horse-Flies (Diptera: Tabanidae) of the Ethiopian Region. Vol. I. Haematopota and Hippocentrum. *British Museum of Natural History*.
OLDROYD, H. 1954. The Horse-flies of the Ethiopian Region. Vol. II. Tabanus and related genera. *British Museum of Natural History*.
OLDROYD, H. 1957. The horseflies of the Ethiopian region. Vol. III. Subfamiles Chrysopsinae, Scepsidinae and Pangoniinae, and a revised classification. *British Museum of Natural History*.
ONEY, B., REINEKING, B., O'NEILL, G. & KREYLING, J. 2013. Intraspecific variation buffers projected climate change impacts on Pinus contorta. *Ecology and Evolution,* 3**,** 437-449.
OUHELLI, H. & DAKKAK, A. 1987. Protozoal diseases of dromedaries. *Rev Sci Techn Off Int Epiz,* 6**,** 417-422.
POLS, J. 1960. *Studies on bovine besnoitiosis with special reference to the aetiology*. University of Pretoria.
PSIFIDI, A., DOVAS, C. I. & BANOS, G. 2010. A comparison of six methods for genomic DNA extraction suitable for PCR-based genotyping applications using ovine milk samples. *Molecular and Cellular Probes,* 24**,** 93-98.
RAJENDRAN, V., KALITA, P., SHUKLA, H., KUMAR, A. & TRIPATHI, T. 2018. Aminoacyl-tRNA synthetases: Structure, function, and drug discovery. *International journal of biological macromolecules,* 111**,** 400-414.
REN, D. 1998. Flower-associated Brachycera flies as fossil evidence for Jurassic angiosperm origins. *Science,* 280**,** 85-88.
ROGNESS, N., STEPHENSON, P., STEPHENSON, P. & MOORE, D. S. 2002. *SPSS Manual: For Introduction to the Practice of Statistics 4e*, Macmillan.
RYAN, E., LEE, A., CARTY, C., O'SHAUGHNESSY, J., KELLY, P., CASSIDY, J., SHEEHAN, M., JOHNSON, A. & DE WAAL, T. 2016. Bovine besnoitiosis (Besnoitia besnoiti) in an Irish dairy herd. *Vet Rec,* 178**,** 608.
SANG, T. 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. *Critical Reviews in Biochemistry and Molecular Biology,* 37**,** 121-147.
SARASTE, M. 1990. Structural features of cytochrome oxidase. *Quarterly reviews of biophysics,* 23**,** 331-66.
SASAKI, H. 2001. Comparison of capturing tabanid flies (Diptera: Tabanidae) by five different color traps in the fields. *Applied Entomology and Zoology,* 36**,** 515-519.
SCHARES, G., BASSO, W., MAJZOUB, M., ROSTAHER, A., SCHARR, J., LANGENMAYER, M., SELMAIR, J., DUBEY, J., CORTES, H. & CONRATHS, F. 2011a. Evaluation of a commercial ELISA for the specific detection of antibodies against Besnoitia besnoiti. *Veterinary parasitology,* 175**,** 52-59.
SCHARES, G., LANGENMAYER, M., SCHARR, J., MINKE, L., MAKSIMOV, P., MAKSIMOV, A., SCHARES, S., BÄRWALD, A., BASSO, W. & DUBEY, J. 2013. Novel tools for the diagnosis and differentiation of acute and chronic bovine besnoitiosis. *International Journal for Parasitology,* 43**,** 143-154.
SCHARES, G., MAKSIMOV, A., BASSO, W., MORÉ, G., DUBEY, J., ROSENTHAL, B., MAJZOUB, M., ROSTAHER, A., SELMAIR, J. & LANGENMAYER, M. 2011b. Quantitative real time polymerase chain reaction assays for the sensitive detection of Besnoitia besnoiti infection in cattle. *Veterinary parasitology,* 178**,** 208-216.
SCHIMMEL, P. 1993. Alanine transfer RNA synthetase: structure-function relationships and molecular recognition of transfer RNA. *Advances in enzymology and related areas of molecular biology,* 63**,** 233-233.
SCHULTZ, J., MAISEL, S., GERLACH, D., MÜLLER, T. & WOLF, M. 2005. A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. *Rna,* 11**,** 361-364.
SERVICE, M. & SERVICE, M. 1980. Horseflies, deerflies and clegs (Order Diptera: Family Tabanidae). *A Guide to Medical Entomology***,** 88-94.
SHARIF, S., JACQUIET, P., PREVOT, F., GRISEZ, C., RAYMOND-LETRON, I., SEMIN, M., GEFFRÉ, A., TRUMEL, C., FRANC, M. & BOUHSIRA, É. 2019. Stomoxys calcitrans, mechanical vector of virulent Besnoitia besnoiti from chronically infected cattle to susceptible rabbit. *Medical and veterinary entomology,* 33**,** 247-255.

SHKAP, V., PIPANO, E. & WARON, H. U. 1987. Beisnoitia besnoiti: chemotherapeutic trials in vivo and in vitro. *Revue d'élevage et de médecine vétérinaire des pays tropicaux,* 40**,** 259-264.

SHOUKRY, N. M. & MORSY, T. A. 2011. Arthropod borne diseases at Toshka, Upper Egypt. *World J Zool,* 6**,** 126-133.

SIMON, C., FRATI, F., BECKENBACH, A., CRESPI, B., LIU, H. & FLOOK, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the entomological Society of America,* 87**,** 651-701.

SMITH, H. O. 1980. Recovery of DNA from gels. *Methods in Enzymology.* Elsevier.

SMITH, K., DIGGLE, M. & CLARKE, S. 2003. Comparison of commercial DNA extraction kits for extraction of bacterial genomic DNA from whole-blood samples. *Journal of Clinical Microbiology,* 41**,** 2440-2443.

SONE, N., YOKOI, F., FU, T., OHTA, S., METSO, T., RAITIO, M. & SARASTE, M. 1988. Nucleotide sequence of the gene coding for cytochrome oxidase subunit I from the thermophilic bacterium PS3. *The Journal of Biochemistry,* 103**,** 606-610.

SUTCLIFFE, W. H. & SHARP, J. 1968. Measurement of deoxyribonucleic acid in the ocean and its ecological significance. *Limnology and Oceanography,* 13**,** 507-514.

TAIOE, M. O. 2017. *Molecular characterization of horse flies (Diptera: Tabanidae) and determination of their role in transmission of haemoparasites in southern Africa.* North-West University (South Africa), Potchefstroom Campus.

TAIOE, M. O., MOTLOANG, M. Y., NAMANGALA, B., CHOTA, A., MOLEFE, N. I., MUSINGUZI, S. P., SUGANUMA, K., HAYES, P., TSILO, T. J. & CHAINEY, J. 2017. Characterization of tabanid flies (Diptera: Tabanidae) in South Africa and Zambia and detection of protozoan parasites they are harbouring. *Parasitology***,** 1-17.

TAN, S. C. & YIAP, B. C. 2009. DNA, RNA, and protein extraction: the past and the present. *BioMed Research International,* 2009.

TORRES, A. & MIRANDA-ESQUIVEL, D. R. 2016. Wing shape variation in the taxonomic recognition of species of Diachlorus Osten-Sacken (Diptera: Tabanidae) from Colombia. *Neotropical entomology,* 45**,** 180-191.

TURCATEL, M. 2014. A Molecular Phylogeny of Deer Flies and their Closest Relatives.

USHER, P. J. 1970. Descriptions of two new species of Amanella, and a new record of Tabanus minuscularius Austin (Diptera: Tabanidae). *Annals of the Natal Museum,* 20**,** 329-339.

USHER, P. J. 1971. New and little-known South African species of Cydistomyia and Tabanus (Diptera: Tabanidae). *Annals of the Natal Museum,* 21**,** 1-15.

USHER, P. J. 1972. A review of the South African horsefly fauna (Diptera: Tabanidae). *Annals of the Natal Museum,* 21**,** 459-507.

VEER, V. 1999. Notes on Tabanidae (Diptera) that are Surra vectors or pestiferous with description of a new species of Tabanus from India. *Oriental Insects,* 33**,** 247-266.

VENTER, F. & GERTENBACH, W. 1986. A cursory review of the climate and vegetation of the Kruger National Park. *Koedoe,* 29**,** 139-148.

VINK, C. J., THOMAS, S. M., PAQUIN, P., HAYASHI, C. Y. & HEDIN, M. 2005. The effects of preservatives and temperatures on arachnid DNA. *Invertebrate Systematics,* 19**,** 99-104.

VOTÝPKA, J., BRZOŇOVÁ, J., JEŽEK, J. & MODRÝ, D. 2019. Horse flies (Diptera: Tabanidae) of three West African countries: a faunistic update, barcoding analysis and trypanosome occurrence. *Acta tropica***,** 105069.

WALSH, P. S., METZGER, D. A. & HIGUCHI, R. 2013. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques,* 54**,** 134-139.

WANG, Y., ENGEL, M. S., RAFAEL, J. A., DANG, K., WU, H., WANG, Y., XIE, Q. & BU, W. 2013. A unique box in 28S rRNA is shared by the enigmatic insect order Zoraptera and Dictyoptera. *PLoS one,* 8**,** e53679.

WANG, Z. & WU, M. 2013. A phylum-level bacterial phylogenetic marker database. *Molecular biology and evolution,* 30**,** 1258-1262.

WATTS, P. C., THOMPSON, D. J., ALLEN, K. A. & KEMP, S. J. 2007. How useful is DNA extracted from the legs of archived insects for microsatellite-based population genetic analyses? *Journal of Insect Conservation,* 11**,** 195-198.

WAUGH, J. 2007. DNA barcoding in animal species: progress, potential and pitfalls. *BioEssays,* 29**,** 188-197.

WENDEL, J. F. & DOYLE, J. J. 1998. Phylogenetic incongruence: window into genome history and molecular evolution. *Molecular systematics of plants II.* Springer.

WILFINGER, W. & CHOMCZYNSKI, M. 1997. P 260/280 and 260/230 Ratios NanoDrop® ND-1000 and ND-8000 8-Sample Spectrophotometers. *Biotechniques,* 22**,** 474-481.

WILFINGER, W. W., MACKEY, K. & CHOMCZYNSKI, P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques,* 22**,** 474-481.

WOODMAN, H. & BOKHARI, A. 1941. Studies on Loa loa and the first report of Wuchereria bancrofti in the Sudan. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 35**,** 77-92.

YANG, L., TAN, Z., WANG, D., XUE, L., GUAN, M.-X., HUANG, T. & LI, R. 2014. Species identification through mitochondrial rRNA genetic analysis. *Scientific reports,* 4**,** 4089.

ZAMBATIS, N. 2003. *Determinants of grass production and composition in the Kruger National Park.*

ZARDOYA, R. & MEYER, A. 1996. Evolutionary relationships of the coelacanth, lungfishes, and tetrapods based on the 28S ribosomal RNA gene. *Proceedings of the National Academy of Sciences,* 93**,** 5449-5454.

Annexure A

Table 1: Sequences from this study as well as sequences obtained from GenBank for analysis. The subfamily, tribe, genus, subgenus and epithet are noted where available including the GenBank accession number and sequence voucher. The country of origin is listed if it was available.

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Chrysopsinae	Chrysopsini	Chrysops		madagascarensis	GMMDA071_15	Madagascar
Chrysopsinae	Chrysopsini	Chrysops		madagascarensis	GMMDF172_15	Madagascar
Chrysopsinae	Chrysopsini	Chrysops		madagascarensis	GMMDK155_15	Madagascar
Chrysopsinae	Chrysopsini	Chrysops		madagascarensis	GMMDL224_15	Madagascar
Chrysopsinae	Chrysopsini	Chrysops		distinctipennis	KX946519	Uganda
Chrysopsinae	Chrysopsini	Chrysops		brucei	KX946516	Uganda
Chrysopsinae	Chrysopsini	Chrysops		distinctipennis	KX946518	Uganda
Chrysopsinae	Chrysopsini	Chrysops		distinctipennis	KX946517	Uganda
Chrysopsinae	Chrysopsini	Chrysops		obliquefasciata	DVTD00193	RSA
Chrysopsinae	Chrysopsini	Chrysops		obliquefasciata	DVTD00194	RSA
Chrysopsinae	Chrysopsini	Chrysops		obliquefasciata	DVTD00852	RSA
Chrysopsinae	Chrysopsini	Chrysops		silaceus	MK396276	Central African Republic
Chrysopsinae	Chrysopsini	Chrysops		silaceus	MK396277	Central African Republic
Chrysopsinae	Chrysopsini	Chrysops		longicornis	MK396273	Gabon
Chrysopsinae	Chrysopsini	Chrysops		longicornis	MK396274	Gabon
Chrysopsinae	Chrysopsini	Chrysops		longicornis	MK396266	Central African Republic
Chrysopsinae	Chrysopsini	Chrysops		dimidiata	MK396265	Central African Republic
Chrysopsinae	Chrysopsini	Chrysops		longicornis	MK396267	Central African Republic
Chrysopsinae	Chrysopsini	Chrysops		longicornis	MK396275	Liberia
Chrysopsinae	Chrysopsini	Chrysops		longicornis	MK396268	Central African Republic
Chrysopsinae	Chrysopsini	Chrysops		silaceus	MK396278	Central African Republic
Chrysopsinae	Chrysopsini	Chrysops		coquilletti	DQ983512	
Chrysopsinae	Chrysopsini	Silvius		gigantulus	DQ983531	
Chrysopsinae	Chrysopsini	Chrysops		madagascariensis	KM243500	
Tabaninae	Diachlorini	Diachlorini		sp.	DQ983513	
Tabaninae	Haematopotini	Haematopota		duttoni	KX946522	Kenya

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Tabaninae	Haematopotini	Haematopota		duttoni	KX946521	Kenya
Tabaninae	Haematopotini	Haematopota		duttoni	KX946523	Kenya
Tabaninae	Haematopotini	Haematopota		fenestralis	KX946528	Kenya
Tabaninae	Haematopotini	Haematopota		fenestralis	KX946524	Kenya
Tabaninae	Haematopotini	Haematopota		fenestralis	KX946525	Kenya
Tabaninae	Haematopotini	Haematopota		duttoni	KX946520	Kenya
Tabaninae	Haematopotini	Haematopota		fenestralis	KX946527	Kenya
Tabaninae	Haematopotini	Haematopota		fenestralis	KX946526	Kenya
Tabaninae	Haematopotini	Haematopota		americana	KM285558	Canada
Tabaninae	Haematopotini	Haematopota		daveyi	DVTD00033	RSA
Tabaninae	Haematopotini	Haematopota		decora	DVTD00048	RSA
Tabaninae	Haematopotini	Haematopota		daveyi	DVTD00094	RSA
Tabaninae	Haematopotini	Haematopota		decora	DVTD00197	RSA
Tabaninae	Haematopotini	Haematopota		daveyi	DVTD00291	RSA
Tabaninae	Haematopotini	Haematopota		vittata	DVTD00668	RSA
Tabaninae	Haematopotini	Haematopota		pandazisi	LT903697	Italy
Tabaninae	Haematopotini	Haematopota		pandazisi	LT903698	Italy
Tabaninae	Haematopotini	Haematopota		pandazisi	LT903699	Italy
Tabaninae	Haematopotini	Haematopota		biorbis	MF144222	Thailand
Tabaninae	Haematopotini	Haematopota		cilipes	MF144209	Thailand
Tabaninae	Haematopotini	Haematopota		cilipes	MF144210	Thailand
Tabaninae	Haematopotini	Haematopota		cilipes	MF144207	Thailand
Tabaninae	Haematopotini	Haematopota		cilipes	MF144208	Thailand
Tabaninae	Haematopotini	Haematopota		torquens	MK396280	Liberia
Tabaninae	Haematopotini	Haematopota		brucei	MK396285	Gabon
Tabaninae	Haematopotini	Haematopota		brucei	MK396286	Gabon
Tabaninae	Haematopotini	Haematopota		albihirta	MK396281	Gabon

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Tabaninae	Haematopotini	Haematopota		albihirta	MK396282	Gabon
Tabaninae	Haematopotini	Haematopota		bowdeni	MK396283	Gabon
Tabaninae	Haematopotini	Haematopota		furians	MK396289	Liberia
Tabaninae	Haematopotini	Haematopota		furians	MK396290	Liberia
Tabaninae	Haematopotini	Haematopota		guineensis	MK396294	Liberia
Tabaninae	Haematopotini	Haematopota		griseicoxa	MK396291	Central African Republic
Tabaninae	Haematopotini	Haematopota		griseicoxa	MK396292	Central African Republic
Tabaninae	Haematopotini	Haematopota		griseicoxa	MK396293	Central African Republic
Tabaninae	Haematopotini	Haematopota		ciliatipes	MK396287	Central African Republic
Tabaninae	Haematopotini	Haematopota		ciliatipes	MK396288	Central African Republic
Tabaninae	Haematopotini	Haematopota		brucei	MK396284	Central African Republic
Tabaninae	Haematopotini	Haematopota		glenni	MF144213	Thailand
Tabaninae	Haematopotini	Haematopota		glenni	MF144214	Thailand
Tabaninae	Haematopotini	Haematopota		glenni	MF144235	Thailand
Tabaninae	Haematopotini	Haematopota		glenni	MF144236	Thailand
Tabaninae	Haematopotini	Haematopota		gracilicornis	MF144215	Thailand
Tabaninae	Haematopotini	Haematopota		gracilicornis	MF144216	Thailand
Tabaninae	Haematopotini	Haematopota		gracilicornis	MF144234	Thailand
Tabaninae	Haematopotini	Haematopota		howarthi	MF144219	Thailand
Tabaninae	Haematopotini	Haematopota		javana	MF144212	Thailand
Tabaninae	Haematopotini	Haematopota		javana	MF144237	Thailand
Tabaninae	Haematopotini	Haematopota		javana	MF144211	Thailand
Tabaninae	Haematopotini	Haematopota		libera	MF144221	Thailand
Tabaninae	Haematopotini	Haematopota		lata	MF144204	Thailand
Tabaninae	Haematopotini	Haematopota		pallida	MF144223	Thailand
Tabaninae	Haematopotini	Haematopota		pachycera	MF144232	Thailand

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Tabaninae	Haematopotini	Haematopota		pachycera	MF078332	Thailand
Tabaninae	Haematopotini	Haematopota		pachycera	MF144205	Thailand
Tabaninae	Haematopotini	Haematopota		procyon	MF144224	Thailand
Tabaninae	Haematopotini	Haematopota		procyon	MF144228	Thailand
Tabaninae	Haematopotini	Haematopota		procyon	MF144229	Thailand
Tabaninae	Haematopotini	Haematopota		pachycera	MF144230	Thailand
Tabaninae	Haematopotini	Haematopota		singularis	MF144226	Thailand
Tabaninae	Haematopotini	Haematopota		singularis	MF144227	Thailand
Tabaninae	Haematopotini	Haematopota		tenasserimi	MF144217	Thailand
Tabaninae	Haematopotini	Haematopota		tenasserimi	MF144218	Thailand
Tabaninae	Haematopotini	Haematopota		whartoni	MF144220	Thailand
Tabaninae	Haematopotini	Haematopota		whartoni	MF144238	Thailand
Tabaninae	Haematopotini	Haematopota		whartoni	MF144239	Thailand
Tabaninae	Haematopotini	Haematopota		pluvialis	KC192969	
Tabaninae	Haematopotini	Haematopota		sp.	KM243541	RSA
Tabaninae	Haematopotini	Haematopota		rara	KM243540	
Tabaninae	Haematopotini	Haematopota		howarthi	KM243539	
Tabaninae	Haematopotini	Haematopota		latifascia	KM243523	
Tabaninae	Haematopotini	Haematopota		pachycera	KM243504	
Tabaninae	Haematopotini	Haematopota		personata	KM243505	
Tabaninae	Haematopotini	Haematopota		singularis	KM243524	
Tabaninae	Haematopotini	Hippocentrum		versicolor	KM243525	
Tabaninae	Haematopotini	Haematopota		sp.	DQ983519	
Tabaninae	Haematopotini	Haematopota		javana	KM111712	India
Pangoniinae	Pangoniini	Asaphomyia		floridensis	DQ983521	
Pangoniinae	Philolichini	Philoliche	Ommatiosteres	atricornis	DQ983504	

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Pangoniinae	Philolichini	Philoliche	Dorcaloemus	compacta	DQ983502	
Pangoniinae	Philolichini	Philoliche	Philoliche	gulosa	DQ983516	
Pangoniinae	Philolichini	Philoliche	Philoliche	gulosa	DQ983517	
Pangoniinae	Philolichini	Philoliche	Phara	bivirgulata	DQ983508	
Pangoniinae	Philolichini	Philoliche	Philoliche	rubiginosa	DQ983526	
Pangoniinae	Philolichini	Philoliche	Philoliche	rubiginosa	DQ983536	
Pangoniinae	Philolichini	Philoliche	Philoliche	gulosa	DQ983518	
Pangoniinae	Philolichini	Philoliche	Philoliche	rostrata	DQ983529	
Pangoniinae	Philolichini	Philoliche	Philoliche	rostrata	DQ983530	
Pangoniinae	Philolichini	Philoliche	Philoliche	umbratipennis	DQ983527	
Pangoniinae	Philolichini	Philoliche	Philoliche	elegans	DQ983514	
Pangoniinae	Philolichini	Philoliche	Philoliche	elegans	DQ983515	
Pangoniinae	Philolichini	Philoliche	Philoliche	aethiopica	DQ983511	
Pangoniinae	Philolichini	Philoliche	Philoliche	rondani	DQ983528	
Pangoniinae	Philolichini	Philoliche	Buplex	sp.	DVTD00008	RSA
Pangoniinae	Philolichini	Philoliche	Buplex	sauvis	DVTD00046	RSA
Pangoniinae	Philolichini	Philoliche	Buplex	sp.	DVTD00059	RSA
Pangoniinae	Philolichini	Philoliche	Dorcaloemus	medialis	DVTD00186	RSA
Pangoniinae	Philolichini	Philoliche	Philoliche	rondani	DVTD00303	RSA
Pangoniinae	Philolichini	Philoliche	Buplex	suavis	DVTD00359	RSA
Pangoniinae	Philolichini	Philoliche	Philoliche	rondani	DVTD00371	RSA
Pangoniinae	Philolichini	Philoliche	Stenophara	zonata	DVTD01000	RSA
Pangoniinae	Philolichini	Philoliche		gravoti	MK396295	Liberia
Pangoniinae	Philolichini	Philoliche	Subpangonia	gravoti	MK396295	Liberia
Pangoniinae	Philolichini	Philoliche	Subpangonia	gravoti	MK396296	Liberia
Pangoniinae	Philolichini	Philoliche	Subpangonia	gravoti	MK396297	Liberia
Pangoniinae	Philolichini	Philoliche	Subpangonia	gravoti	MK396298	Liberia
Pangoniinae	Philolichini	Philoliche	Subpangonia	gravoti	MK396299	Liberia
Pangoniinae	Philolichini	Philoliche	Buplex	subfascia	DQ983501	
Pangoniinae	Philolichini	Philoliche	Maverica	bukamensis	DQ983503	

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Pangoniinae	Philolichini	Philoliche	Ommatiosteres	lateralis	DQ983505	
Pangoniinae	Philolichini	Philoliche	Phara	andrenoides	DQ983506	
Pangoniinae	Philolichini	Philoliche	Ommatiosteres	angulata	DQ983507	
Pangoniinae	Philolichini	Philoliche	Phara	quinquemaculata	DQ983509	
Pangoniinae	Philolichini	Philoliche	Philoliche	rostrata	KM243449	
Pangoniinae	Philolichini	Philoliche	Returneria	alternans	KM243530	
Pangoniinae	Philolichini	Philoliche	Buplex	basalis	KM243448	
Chrysopsinae	Rhinomyzini	Tabanocella		longirostris	KM243550	
Chrysopsinae	Rhinomyzini	Tabanocella		denticornis	DQ983532	
Tabaninae	Tabanini	Atylotus		sublunaticornis	KR669647	Canada
Tabaninae	Tabanini	Atylotus		sublunaticornis	KR659602	Canada
Tabaninae	Tabanini	Atylotus		sublunaticornis	KR655213	Canada
Tabaninae	Tabanini	Ancala		sp.	KX946500	Uganda
Tabaninae	Tabanini	Ancala		sp.	KX946504	Uganda
Tabaninae	Tabanini	Tabanus		thoracinus	KX946580	Uganda
Tabaninae	Tabanini	Ancala		sp.	KX946505	Uganda
Tabaninae	Tabanini	Ancala		sp.	KX946499	Uganda
Tabaninae	Tabanini	Ancala		sp.	KX946501	Uganda
Tabaninae	Tabanini	Ancala		sp.	KX946503	Uganda
Tabaninae	Tabanini	Ancala		sp.	KX946497	Uganda
Tabaninae	Tabanini	Ancala		sp.	KX946502	Uganda
Tabaninae	Tabanini	Ancala		sp.	KX946498	Uganda
Tabaninae	Tabanini	Tabanus		thoracinus	KX946579	Uganda
Tabaninae	Tabanini	Tabanus		thoracinus	KX946552	Uganda
Tabaninae	Tabanini	Atylotus		agrestis	KX946496	Uganda
Tabaninae	Tabanini	Tabanus		taeniola	KX946538	Tanzania
Tabaninae	Tabanini	Tabanus		gratus	KX946534	Tanzania
Tabaninae	Tabanini	Tabanus		gratus	KX946536	Tanzania
Tabaninae	Tabanini	Atylotus		nigromaculatus	KX946515	Kenya
Tabaninae	Tabanini	Atylotus		nigromaculatus	KX946513	Tanzania

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Tabaninae	Tabanini	Atylotus		nigromaculatus	KX946509	Kenya
Tabaninae	Tabanini	Atylotus		diurnus	KX946507	Kenya
Tabaninae	Tabanini	Atylotus		diurnus	KX946506	Kenya
Tabaninae	Tabanini	Tabanus		taeniola	KX946550	Kenya
Tabaninae	Tabanini	Tabanus		taeniola	KX946551	Kenya
Tabaninae	Tabanini	Tabanus		donaldsoni	KX946531	Kenya
Tabaninae	Tabanini	Tabanus		donaldsoni	KX946530	Kenya
Tabaninae	Tabanini	Tabanus		donaldsoni	KX946532	Kenya
Tabaninae	Tabanini	Atylotus		diurnus	KX946508	Kenya
Tabaninae	Tabanini	Atylotus		nigromaculatus	KX946510	Kenya
Tabaninae	Tabanini	Atylotus		nigromaculatus	KX946511	Tanzania
Tabaninae	Tabanini	Tabanus		gratus	KX946533	Tanzania
Tabaninae	Tabanini	Tabanus		subangustus	KX946537	Kenya
Tabaninae	Tabanini	Atylotus		nigromaculatus	KX946514	Kenya
Tabaninae	Tabanini	Atylotus		nigromaculatus	KX946514	Kenya
Tabaninae	Tabanini	Tabanus		gratus	KX946535	Kenya
Tabaninae	Tabanini	Atylotus		nigromaculatus	KX946512	Kenya
Tabaninae	Tabanini	Tabanus		sp.	KX946529	Uganda
Tabaninae	Tabanini	Tabanus		par	KY555738	Zambia
Tabaninae	Tabanini	Tabanus		par	KY555737	Zambia
Tabaninae	Tabanini	Tabanus		par	KY555741	Zambia
Tabaninae	Tabanini	Tabanus		par	KY555739	Zambia
Tabaninae	Tabanini	Atylotus		calcar	KM285423	Canada
Tabaninae	Tabanini	Atylotus		diurnus	KY555749	Zambia
Tabaninae	Tabanini	Atylotus		diurnus	KY555745	Zambia
Tabaninae	Tabanini	Tabanus		taeniola	KY555755	RSA
Tabaninae	Tabanini	Atylotus		diurnus	KY555750	Zambia
Tabaninae	Tabanini	Atylotus		thoracius	MF830268	Canada
Tabaninae	Tabanini	Atylotus		thoracius	MF828652	Canada
Tabaninae	Tabanini	Atylotus		thoracius	KR980762	Canada

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Tabaninae	Tabanini	Atylotus		sublunaticornis	MG168140	Canada
Tabaninae	Tabanini	Atylotus		sublunaticornis	MG168807	Canada
Tabaninae	Tabanini	Atylotus		insuetus	MF832957	Canada
Tabaninae	Tabanini	Atylotus		insuetus	MF835595	Canada
Tabaninae	Tabanini	Tabanus		gratus	KY555751	RSA
Tabaninae	Tabanini	Tabanus		minuscularius	DVTD00003	RSA
Tabaninae	Tabanini	Tabanus		minuscularius	DVTD00006	RSA
Tabaninae	Tabanini	Atylotus		agrestis	DVTD00023	RSA
Tabaninae	Tabanini	Tabanus		atrimanus	DVTD00097	RSA
Tabaninae	Tabanini	Tabanus		atrimanus	DVTD00100	RSA
Tabaninae	Tabanini	Atylotus		agrestis	DVTD00104	RSA
Tabaninae	Tabanini	Tabanus		gratus	DVTD00109	RSA
Tabaninae	Tabanini	Tabanus		atrimanus	DVTD00138	RSA
Tabaninae	Tabanini	Tabanus		sericiventris	DVTD00139	RSA
Tabaninae	Tabanini	Tabanus		gratus	DVTD00190	RSA
Tabaninae	Tabanini	Tabanus		taeniola	DVTD00262	RSA
Tabaninae	Tabanini	Tabanus		pullulus	DVTD00301	RSA
Tabaninae	Tabanini	Tabanus		pullulus	DVTD00360	RSA
Tabaninae	Tabanini	Atylotus		agrestis	DVTD00673	RSA
Tabaninae	Tabanini	Tabanus		sericiventris	DVTD00712	RSA
Tabaninae	Tabanini	Tabanus		taeniola	DVTD01115	RSA
Tabaninae	Tabanini	Tabanus		donaldsoni	DVTD01116	RSA
Tabaninae	Tabanini	Tabanus		biguttatus	DVTD01125	RSA
Tabaninae	Tabanini	Tabanus		leucostomus	DVTD01138	RSA
Tabaninae	Tabanini	Atylotus		agrestis	DVTD01140	RSA
Tabaninae	Tabanini	Tabanus		par	DVTD01141	RSA
Tabaninae	Tabanini	Tabanus		leucostomus	DVTD01143	RSA
Tabaninae	Tabanini	Atylotus		agrestis	MG587915	Bangladesh
Tabaninae	Tabanini	Atylotus		agrestis	KY555746	Zambia
Tabaninae	Tabanini	Atylotus		agrestis	KY555747	Zambia

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Tabaninae	Tabanini	Ancala		fasciata	MK396261	Central African Republic
Tabaninae	Tabanini	Tabanus		secedens	MK396333	Central African Republic
Tabaninae	Tabanini	Tabanus		secedens	MK396336	Central African Republic
Tabaninae	Tabanini	Tabanus		secedens	MK396351	Gabon
Tabaninae	Tabanini	Tabanus		secedens	MK396356	Liberia
Tabaninae	Tabanini	Tabanus		secedens	MK396357	Liberia
Tabaninae	Tabanini	Tabanus		secedens	MK396342	Gabon
Tabaninae	Tabanini	Tabanus		secedens	MK396337	Gabon
Tabaninae	Tabanini	Tabanus		taeniola	MK396361	Gabon
Tabaninae	Tabanini	Tabanus		taeniola	MK396358	Central African Republic
Tabaninae	Tabanini	Ancala		fasciata	MK396262	Gabon
Tabaninae	Tabanini	Tabanus		fraternus	MK396304	Central African Republic
Tabaninae	Tabanini	Tabanus		secedens	MK396347	Gabon
Tabaninae	Tabanini	Tabanus		fraternus	MK396308	Central African Republic
Tabaninae	Tabanini	Tabanus		par	MK396326	Gabon
Tabaninae	Tabanini	Tabanus		par	MK396327	Gabon
Tabaninae	Tabanini	Tabanus		obcurehirtus	MK396314	Central African Republic
Tabaninae	Tabanini	Tabanus		taeniola	MK396362	Gabon
Tabaninae	Tabanini	Tabanus		par	MK396328	Gabon
Tabaninae	Tabanini	Tabanus		variabilis	MK396375	Central African Republic
Tabaninae	Tabanini	Tabanus		triquetrornatus	MK396374	Central African Republic
Tabaninae	Tabanini	Euancala		irrorata	MK396279	Gabon
Tabaninae	Tabanini	Tabanus		rufipes	MK396329	Liberia
Tabaninae	Tabanini	Tabanus		rufipes	MK396330	Liberia
Tabaninae	Tabanini	Tabanus		rufipes	MK396331	Liberia
Tabaninae	Tabanini	Tabanus		rufipes	MK396332	Liberia
Tabaninae	Tabanini	Tabanus		secedens	MK396352	Liberia
Tabaninae	Tabanini	Tabanus		boueti	MK396301	Liberia
Tabaninae	Tabanini	Tabanus		denshamii	MK396302	Liberia
Tabaninae	Tabanini	Tabanus		argenteus	MK396300	Liberia

Subfamily	Tribe	Genus	Subgenus	Epithet	Acc. Num.	Country of Origin
Tabaninae	Tabanini	Tabanus		ianthinus	MK396309	Liberia
Tabaninae	Tabanini	Tabanus		ianthinus	MK396313	Liberia
Tabaninae	Tabanini	Tabanus		thoracinus	MK396372	Liberia
Tabaninae	Tabanini	Tabanus		thoracinus	MK396373	Liberia
Tabaninae	Tabanini	Tabanus		secedens	MK396343	Liberia
Tabaninae	Tabanini	Tabanus		secedens	MK396344	Liberia
Tabaninae	Tabanini	Tabanus		par	MK396317	Central African Republic
Tabaninae	Tabanini	Tabanus		par	MK396318	Central African Republic
Tabaninae	Tabanini	Tabanus		obcurehirtus	MK396315	Central African Republic
Tabaninae	Tabanini	Tabanus		obcurehirtus	MK396316	Central African Republic
Tabaninae	Tabanini	Tabanus		taeniola	MK396359	Central African Republic
Tabaninae	Tabanini	Tabanus		fraternus	MK396303	Central African Republic
Tabaninae	Tabanini	Tabanus		secedens	MK396346	Gabon
Tabaninae	Tabanini	Tabanus		taeniola	MK396360	Gabon
Tabaninae	Tabanini	Tabanus		thoracinus	MK396365	Gabon
Tabaninae	Tabanini	Tabanus		taeniatu	KY555744	Zambia
Tabaninae	Tabanini	Tabanus		laverani	KY555754	RSA
Tabaninae	Tabanini	Atylotus		agrestis	KY555748	RSA
Tabaninae	Tabanini	Hybomitra		rhombica	DQ983520	
Tabaninae	Tabanini	Tabanus		aegrotus	DQ983533	
Tabaninae	Tabanini	Tabanus		biguttatus	DQ983534	
Tabaninae	Tabanini	Atylotus		miser	KT225291	
Tabaninae	Tabanini	Atylotus		miser	NC030000	
Tabaninae	Tabanini	Ancala		fasciata	KM243527	
Tabaninae	Tabanini	Ancala		africana	KM243526	
Tabaninae	Tabanini	Atylotus		horvathi	KF966580	South Korea
Tabaninae	Tabanini	Tabanus		par	KY555740	RSA
Tabaninae	Tabanini	Tabanus		par	KY555742	RSA
Tabaninae	Tabanini	Tabanus		par	KY555743	RSA
Tabaninae	Tabanini	Atylotus		agrestis	KM111665	India
Athericidae		Atherix		variegata	KM243490	

Annexure B

DECLARATION OF ORIGINALITY

UNIVERSITY OF PRETORIA

The Department of Veterinary Tropical Diseases places great emphasis upon integrity and ethical conduct in the preparation of all written work submitted for academic evaluation.

Academics teach you about referencing techniques and how to avoid plagiarism; it is your responsibility to act on this knowledge. If you are at any stage uncertain as to what is required, you should speak to your lecturer before any written work is submitted.

You are guilty of plagiarism if you copy something from another author's work (e.g. a book, an article or a website) without acknowledging the source and pass it off as your own. In effect you are stealing something that belongs to someone else. This is not only the case when you copy work word-for-word (verbatim) but also when you submit someone else's work in a slightly altered form (paraphrase) or use a line of argument without acknowledging it.

Students who commit plagiarism will not be given any credit for plagiarised work. The matter may also be referred to the Disciplinary Committee (Students) for a ruling. Plagiarism is regarded as a serious contravention of the University's rules and can lead to expulsion from the University.

The declaration which follows must accompany all written work submitted while you are a student of the Department of Veterinary Tropical Diseases No written work will be accepted unless the declaration has been completed and submitted.

Full names and surname of student: Andeliza Smit

Student number: 14023190

Topic of work: Abundance, composition and barcoding of Tabanidae in Kruger National Park and screening for Besnoitia besnoiti

Declaration

1. I understand what plagiarism is and am aware of the University's policy in this regard.

2. I declare that this dissertation (e.g. essay, report, project, assignment, dissertation, thesis, etc) is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

SIGNATURE

02/12/2019
DATE

Annexure C

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**The abundance, composition and barcoding of Tabanidae within Kruger National Park and their role in the transmission of Besnoitia besnoiti**
PROJECT NUMBER	**REC057-189**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Miss A Smit**

DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Luis Neves**

APPROVED	Date **22 July 2019**
CHAIRMAN: UP Research Ethics Committee	Signature M. M. Duncan

ISSUE DATE 02 March 2018

To develop, expand, manage and promote a system of sustainable national parks that represent biodiversity and heritage assets, through innovation and best practice for the just and equitable benefit of current and future generations.

South African NATIONAL PARKS

Research Permit: Kruger,Mapungubwe National Park, Ais-Ais/Richtersveld

REFERENCE: NEVL1497

addo elephant
agulhas
augrabies
bontebok
camdeboo
golden gate highlands
karoo
kglalagadi transfrontier
knysna lake area
kruger
mapungubwe
marakele
mokala
mountain zebra
namaqua
table mountain
tankwa karoo
tsitsikamma
richtersveld
west coast
wilderness

Scientific Services
Kruger National Park, Private Bag X402, Skukuza, 1350

Neves L	Seasonal responses of haematophagous flies across a non-transformed and livestock-transformed gradient: the potential effect on disease transmission (Diptera: Tabanidae, Culicidae)
Co-Workers	Snyman LP,Lempereur L, Mazibuku X,Smit A, van Schalkwyk L, Dekker A

Herewith the permit for your research project valid from 01 January 2018 until 31 December 2018. The approval is subject to the following conditions. The Park Management staff must be contacted prior to entry into the park (see website for contact details).

Standard Conditions:
PLEASE CONTACT THE PARK MANAGEMENT STAFF IF RESTRICTED AREAS NEED TO BE ACCESSED.
No damage shall be permitted to any natural vegetation, environment or property.
Uncontrolled vehicle access and parking could cause damage to vegetation and soil erosion and therefore only the use of approved vehicles routes and parking areas is allowed.
Fires can cause loss of vegetation, soil erosion and life and therefore fires, and braai's are not permitted unless in dedicated braai areas.
Other visitors to the area and or neighbours may not be hindered in any way.
No pollution or excessive noise is permitted.
Your permit must be retained and kept on your person at all times, and produced on request.

The areas under the control of SANParks are used entirely at your own risk. South African National Parks, its Board, directors, employees and agents are not liable for any loss or damage to the property or possession of any guest or participant (or accompanying minor) whether such damage is caused by the negligent act or omission of South African National Parks; arising from death or any bodily injuries of whatsoever nature sustained by a guest or participant (or accompanying minor) whether such injuries are caused by the negligent act or omission by South African National Parks, and/or by the defective functioning of any apparatus. The guest or participant and/or his/her/their estate hereby indemnifies South African National Parks against any claim, action, judgment, costs and/or expenses which may be made against South African National Parks and as may in any way be related to the above. The onus lies with the company or applicant to ensure that they are adequately insured.
Please note that you (your delegates, staff etc) are subject to the conditions set in terms of Section 86(1) of the National Environmental Management Act (107 of 1998) and the National Environmental Act: Protected Areas Act (Act 57 of 2003) for the duration of your stay in the National Park. Your attention is specifically drawn to sections 64(1) (a), (b) (c) which refers to penalties in terms of the Act.
SANParks staff's instructions, notices, regulations and signs must be complied with.
The activity shall be restricted to the area applied for.
Gate and operating times to be complied with.
NO PETS ALLOWED

Special Conditions:
Any contraventions of the above will result in your permit being revoked
SIgned on behalf of SANParks

643 Leyds Street
Muckleneuk
Pretoria

PO Box 787
Pretoria
0001

tel: 012 426 5000
fax: 012 343 0905

central reservations: 012 428 9111
reservations@parks.co.za
www.parks-sa.co.za

South African NATIONAL PARKS

PERMIT TO COLLECT NATURAL RESOURCES MATERIAL IN THE KRUGER NATIONAL PARK

In compliance with section 4(1) of the National Environmental Management: Protected Areas Act No. 57 of 2003, permission is herby granted to the person named below to collect natural resources material in the Kruger National Park, as detailed below.

Permit No: SKZ 113

TOPS No:

Title: Prof Initials and Surname: L. Neves ID or Passport No: 10AA96235

Institution (or residential address, if collecting in personnal capacity) U.P

DESCRIPTION OF MATERIAL	QUANTITY
Haematophagos flies	1000 Per site

In (area of the KNP): Entire KNP

For the purpose of: Project NEVL1497

This permit is valid until (date): 31 Dec 2019

Conditions under which this permit is issued:

1. *This permit must always be available for presentation, should it be requested by any Section Ranger or any other SANParks official.*
2. *This permit is non-transferable, i.e. may not be used by any person other than the person whose name appears above.*
3. *This permit is not valid without an official stamp and the signature of the permit-holder.*
4. *Unless specifically authorised to do so, no live material may be collected and removed from the KNP.*
5. *The material may only be utilised for the purposes agreed to with SANParks.*
6. *The material may not be used for the purposes of propagation or enhancement in any manner whatsoever, without the written approval of SANParks.*
7. *All intellectual property rights relating to the material remain the property of SANParks, unless otherwise agreed.*
8. *Misuse of the permit will result in its withdrawl and cancellation, and may also result in criminal prosecution.*
9. *The authorization of the State Veterinary Office in Skukuza is a statutory requirement for the removal of material from the KNP.*

Additional conditions:

Official stamp

I understand and accept the above conditions and any other conditions which may have been set for the collection of the above material. Furthermore, I certify that the information provided by me for the issuing of this permit is true and correct.

KRUGER NATIONAL PARK 2019 -02- 27

Signature of permit-holder

Date:

Authorised by (name): pp Nawashni Govender.

Signature:

Date: 27/2/2019.

GENERAL MANAGER: CONSERVATION MANAGEMENT KRUGER NATIONAL PARK

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

Ref: V059-18

30 July 2018

Prof L Neves
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
(luis.neves@up.ac.za)

Dear Prof Neves

Project V059-18
The abundance, composition and phylogenetical analysis of Tabanidae within Kruger National Park (South Africa) and their role in the transmission of Besnoitia besnoiti (A Smit)

The application was discussed by the Animal Ethics Committee of the University of Pretoria at the May 2018 meeting. Since no vertebrates are involved in the study, the committee had no concerns with the study.

If you have any question, please feel free to contact the committee.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Miss Andeliza Smit
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Dear Miss Smit

Your fax / memo / letter/ Email dated 23 July 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. No live flies may be transported. Flies must be frozen until dead and stored in 100% ethanol;
4. Flies may only leave the trapping locations after having obtained movement permits from the responsible state veterinarians;

5. Flies to be transported must be packaged in compliance with the National Road Traffic Act, 1996 (Act No 93 of 1996);
6. An accredited waste management company must be used for waste disposal;
7. Extracted tabanid DNA may be stored at the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria;
8. This Section 20 approval is valid until 30 September 2020.

Title of research/study: The abundance, composition and barcoding of Tabanidae within Kruger National Park and their role in the transmission of *Besnoitia besnoiti*
Researcher (s): Miss Andeliza Smit
Institution: DVTD, Faculty of Veterinary Science, UP
Your Ref./ Project Number:
Our ref Number: 12/11/1/1/6

Kind regards,

Maja

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -08- 24

SUBJECT: RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)