Species composition of and detection of haemoparasites in horse-flies (Diptera: Tabanidae) in south-eastern Kruger National Park

by

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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

November 2018

SUMMARY

Species composition of and detection of haemoparasites in horse-flies (Diptera: Tabanidae) in south-eastern Kruger National Park

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Keywords: Tabanidae, Haemoparasites, COI, Kruger National Park, Diptera, *Trypanosoma*

Tabanidae, commonly known as horse-flies, is a large family of important pollinators, generally believed to be among the most important brachycerans (Diptera: Brachycera). In addition to their importance in the ecosystem, the females are haematophagous and vectors of pathogens. Despite their importance, the family has been severely neglected by science. The current study was aimed at determining the species composition of tabanids in the south eastern corner of Kruger National Park (KNP), South Africa. Using three different traps, tabanids were sampled in four habitats in south-eastern KNP. Three different traps totalling to 12 traps were used for the sampling. Manitoba trap captured an average of 1.7 flies/trap/day, Ngu-trap 0.7 flies/trap/day and H-trap 2.4 flies/trap/day. A total of 273 flies were captured with the H trap as the most effective. A total of 13 species belonging to the five genera *Haematopota, Tabanus, Philoliche, Chrysops* and *Atylotus* were sampled. A dominant tabanid species, *Tabanus minuscularius*, accounted for 55% (154/273) of the total flies sampled. A total of 60 COI sequences from 12 identified species and two unidentified species were generated and phylogenetically analysed. COI performed adequately as a species-specific identifier. DNA extracted from selected tabanids specimens tested positive for *Trypanosoma theileri* at 0.02%. No detection of *Anaplasma marginale, A. centrale* or *Babesia bigemina* was observed in tested flies. Further research is required to determine the role of tabanids in pathogen transmission in KNP and elsewhere in South Africa.

Reasearch Outputs

Conference

X Mazibuko, LP Snymam, L Lempereur, A Smit, L Neves. Species composition of and detection of haemoparasites in horse-flies diptera: tabanidae in south-eastern Kruger National Park. Oral presentation. 16-18 September 2018, 47th Annual PARSA conference, Tsipise Forever Resort.

Faculty Day

X Mazibuko, LP Snymam, L Lempereur, A Smit, L Neves. Species composition of and detection of haemoparasites in horse-flies diptera: tabanidae in south-eastern Kruger National Park. Poster presentation. 23 August 2018, University of Pretoria Faculty of Veterinary Sciences.

LIST OF ABBREVIATIONS

DVTD	Department of Veterinary Tropical Diseases
KNP	Kruger National Park
RSA	South Africa
GPS	Global Positioning System
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
OBP	Onderstepoort Biological Product
AM	*Anaplasma marginale*
AC	*Anaplasma centrale*
OVI	Onderstepoort Veterinary Institute
BSU	Biosystematic Support Unit
QPCR	Quantitative Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
FOR	Forward
REV	Reverse
UDG	Uracil- DNA Glycosylase
BP	Base pairs
PB	Probe
PAGE	PolyAcrylamide Gel Electrophoresis
TBE	Tris Borate Edta
EDTA	Ethylene-Diamene-Tetra-Acetic Acid
MAFFT	Multiple Alignment Fast Fourier Transform
SANParks	South African National Parks
COI	Cytochrome c oxidase sub unit I
UV	Ultra Violet
V	Voltage

ETHICS STATEMENT

I Xolani Mazibuko declare that I have observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

Student name: Xolani Mazibuko

Signature……

DEDICATIOIN

This dissertation is dedicated to my late mother (may her soul rest in peace), Phumele Zamangwe Ether Mazibuko. I thank her for the encouragement, unconditional love and endless support that has sustained me throughout my life.

I love you Mama !!!

"Confront the dark parts of yourself, and work to banish them with illumination and forgiveness".

ITS ALWAYS SEEMS IMPOSSIBLE UNTIL ITS DONE!

-NELSON MANDELA

ACKNOWLEDGEMENTS

This dissertation was made possible by assistance and guidance of several people from different countries and I offer my sincere gratitude to all of them. I would specifically like to extend my graditude to Ilse Vorster, Dr. Charles Byaruhanga and Marcus Makgabo for the help in the laboratories, without your help this dissertation would not have been possible.

I would like to express my sincere gratitude to my promoter Prof Luis Neves for his continuous support during my studies, for his patience, motivation, and immense knowledge. His guidance helped me at all times.

I thank my co-promoter Dr Laetitia Lempereur for her support, insightful critical comments and corrections of this dissertation.

I would like to show my appreciation to Dr. Louwtjie Snyman for his guidance and knowledge he provided to the study, the inspiration he gave me with his impeccable work ethic and his patience with me during tough times.

I thank the following institutions: The Department of Veterinary Tropical Diseases (DVTD) under the University of Pretoria for not only giving me the opportunity to complete my masters, but also allow me to be part of their research team.

The SANParks (South African National Parks) for granting us permission to collect fly specimens in the Kruger National Park and for providing us with game rangers and state veterinarians to protect us and guide us through the collection process.

I thank my family, friends and fellow students for the words of encouragement, especially Lindani Mazibuko, Mzwandile Mazibuko, Noxolo Mazibuko, Smangele Rachel Mkhize and my beautiful daughter Lwenhle, for the support throughout the duration of this study.

Above all, I praise the almighty God, for providing me with this opportunity and granting me the strength and capability to complete this journey successfully. As stated in

Proverbs 3:5-6, “Trust in the Lord with all your heart. Don't put your confidence in your own understanding. In all your ways acknowledge him, and he will direct your path

This work would not have been possible without the funding support of the Belgium Embassy, South African Agricultural Sector Education and Training Authority and a DST- Scarce skills bursary from the National Research Foundation. This dissertation was in part supported by funding from The National Research Foundation (grant 107861, awarded to Marinda Oosthuizen), Belgian Directorate-General for Development Co-operation Framework Agreement (FA4 DGD-ITM 2017-2021) awarded to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

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CHAPTER 1

1.1 Introduction

It has been several decades since the haematophagous flies of South Africa received major revisory treatment, especially horse-flies (Diptera: Tabanidae). The last major reports, mostly taxonomic in nature, were published by Oldroyd (1952, 1954, 1957) and Usher (1972), supplemented by the works of Chainey (1983, 1987). Tabanidae, commonly known as horse-flies, clegs, marsh-flies and deer-flies, are believed to be one of the most diverse haematophagous insect taxa (Morita 2008). Their diversity is thought to surpass that of fleas (Siphonaptera) (Zhang *et al.* 2011), biting midges (Ceratopogonidae) and mosquitoes (Culicidae) (Harbach 2007). Members of the family are of special interest in South Africa due to the presence of specialist pollinators in the Cape floristic region (Johnson & Morita 2006). The development and adaptive nature of their mouthparts is thought to be associated with that of the diverse angiosperm flowers (Morita 2008, Morita *et al.* 2016).

There are more than 4000 described species of tabanids worldwide (Bitome *et al.* 2015). Over 700 species occur in Africa, occupying a diverse range of habitats (Kirk-Spriggs & Stuckenberg 2009) and are considered harmful to humans and animals, due to their role in transmission of a wide range of pathogens (Foil 1989, Carn 1996). Examples include viruses (malignant catarrhal fever, lumpy skin disease, rift valley fever), bacteria (*Bacillus anthracis*, *Anaplasma marginale*) and parasites (*Besnoitia besnoiti*, *Trypanosoma* spp., *Loa loa*) (Foil 1989; Mihok 2002; Desquesnes & Dia 2003; Baldacchino *et al.* 2014a, b; Taioe *et al.* 2017) (Table 1).

The family Tabanidae comprises four subfamilies, namely Chrysopsinae, Pangoniinae, Scepsidinae and Tabaninae (Bitome *et al.* 2015), of which the Tabaninae, specifically the genera *Haematopota* and *Tabanus*, is generally considered to be the most important medical/veterinary taxon, even though species from other genera, such as *Atylotus* and *Chrysops* are also implicated in disease transmission (Desquesnes & Dia 2003). In addition to their role in disease transmission, tabanids are a nuisance to animals and humans alike, with irritating and painful bites, worsened by their incredible persistence for a blood meal (Foil 1989; Desquesnes & Dia. 2003; Baldacchino *et al.* 2014a). Accordingly, dense tabanid populations may have an important socio-economic impact in countries where they occur (Desquesnes & Dia 2003; Baldacchino *et al.* 2014). Negative

physical and economic impacts on livestock sector include effects such as disturbance, skin lesions, reduction of food intake, stress, blood loss and an immune repressive effect (Mihok 2002; Desquesnes & Dia 2003; Baldacchino *et al.* 2013).

Despite their role as vectors of disease, tabanids have received considerably less attention, both financial and scientific, compared to other Diptera vectors such as the biting midges (Ceratopogonidae) and mosquitoes (Culicidae) of Nematocera (Foil 1989, Taioe *et al.* 2017). The role they play in the transmission of disease-causing agents are thus inadequately studied and the effects thereof unquantifiable. Consequently, this research will investigate aspects of tabanid diversity, ecology, behaviour and their potential role in haemoparasites transmission in the south-eastern Kruger National Park (South Africa).

1.2 Horse-fly morphology and biology

Adult horse-flies are medium to large bodied insects, with a stout like appearance. They normally have bright coloured eyes, the colour of the eyes along with the bands seen on them provide important taxonomic characteristics often used in the identification of the flies (Morita 2008). The colour on the eyes often disappear in dry specimens, which can be a hurdle in morphological identification. Sexes are easily distinguished by the dichoptic eyes of females and holoptic eyes of males (Mullens 2002). Horse-flies are generally diurnal, which is an important feature for locating their host, mates and oviposition sites. Horvath *et al.* (2008) discussed the attractiveness of polarized light in tabanids as both female and male seemed to generally respond well specifically to horizontally polarized light which is a response generated by the ventral eye region of tabanids (Wunderer & Smola 1986; Baldacchino *et al.* 2014b). Despite tabanids responding to polarized light, Horvath *et al.* (2008) did experimentally show the evolution of zebra stripes with brightness and/or polarization modulations disrupting the homogeneous pattern of reflected light, which might be a selective advantage in avoiding attacks from polarotactic tabanids.

1.2.1 Frons

The frons, which are often more or less broad, usually begin to extend from the subcallus in the inner parts of the eye to the vertex at the top of the head (Chvála *et al.* 1972, Teskey, 1990). Normally they are often covered by very fine or thin hairs with varying patterns formed by bare areas termed calli. The presence and shape of which are used for identification. Three distinct ocelli are present on the vertex in both the Pangoniinae and Chrysopsinae. In the Tabaninae, ocelli

are vestigial or absent. In species of the genus *Hybomitra*, a non-functional ocellar tubercle is usually present.

1.2.2 Antennae

Tabanids have structures called the antennae which have a three part division, namely: the pedicel, the scape and the flagellum. Usually there are four to eight flagellomeres, dependant on species. Antennae are deemed very important for classification at all taxonomic levels. In the genus Chrysops and Haematopota, the lower or basal flagellomere is thin and elongates whereas in the Tabanini, it is big/enlarged and laterally compressed, generally with a conspicuous developed dorsal protrusion/tooth; the pedicel is short in both Tabanini and Haematopotini. The pedicel is thin in Chrysops. The scape is short in Tabanini whereas it is long in both Haematopini and Chrysops. The antennae have sensilla present which are structures known to be the main organs for smell in many insects and signals which are usually chemcals are involved in the host location by female tabanids. The most of sensilla appear to be present in the first segment of the flagellum (Ivanov, 2007; Parashar *et al.* 1994). Some olfactory sensilla are also present on the maxillary palps.

1.2.3 Mouthparts

Mouthparts of female tabanids normally have an unpaired structure termed the dorsal labrum. There is also an unpaired hypopharynx, paired mandibles, paired maxillae which are composed of the palps and the laciniae, and the unpaired ventral labium with its distal labellum (Stoffolano & Yin. 1983; McKeever & French 1999). The labium and palps are not for biting. During blood-feeding, both the mandibles and maxillary laciniae function to cut into the skin of the host (Lehane, 2005; Baldacchino *et al.* 2014b). Together with the labrum and hypopharynx, they penetrate by cutting through the skin and subcutaneous capillaries to create a wound. Saliva enters the wound via the salivary duct opening at the tip of the hypopharynx and blood is sucked up into the mouth or food canal (Mullens 2002). This is formed by a deep gutter in the labrum. This feeding method is known as pool feeding or telmophagy, in contrast to the vessel feeding or solenophagy used by mosquitoes (Mullens 2002). The genus *Philoliche*, a persistent blood feeder on livestock in Africa, has elongated mouthparts that allow it also to probe for nectar in deep-throated flowers (Morita 2008).

1.2.4 Thorax

The thorax consists of lobes and strong wing muscles, which greatly assists in the very fast flying ability of tabanids (Baldacchino *et al.* 2014a, b). Tabanids are kown to have a very high dispersal ability, flying up to 2 km per day (Cooksey & Wright, 1987). The thorax of tabanids appears to be as broad as the abdomen (depending on the species), anteriorly compressed and usually with a distinguishing colour or pattern. Wings have a constant venation within the horseflies. Mostly tabanid species normally have clear wings or with a grayish or brownish tinge, however some wings are partially or totally infuscated, which provides useful features for identification. The wings of *Chrysops* and *Ancala* have distinct darkened bands, whereas the wings of *Haematopota* have patterns of spots or rosettes, all useful for species delimitation (Oldroyd 1952, 1954).

1.2.5 Legs

The legs differ amongst species and bear useful characters for identification. Tabanid species particularly of the genus *Chrysops* have spurs on the hind tibiae. The fore-tibia of the genus *Ancala* are bulbous, and the appearance or colour of the legs is an important feature for identification (Oldroyd 1952, 1954, 1957).

1.3 Life cycle and reproduction of tabanids

Tabanid reproduction has been studied in South Africa by both Crosskey *et al.* (1980) and Foil (1989). To summarise (Figure 1), Tabanidae breeding sites are generally wet places adjacent to water bodies such as lakes and rivers. Tabanid adults feed on nectar and other plant products for general metabolic processes, however, females of most species require a blood-meal in order to produce eggs subsequent to mating. *Chrysops* spp. are an exception and are capable of laying eggs before a blood meal through a process called autogeny (Mullens 2002). In some genera, particularly *Chrysops*., the larvae are scavengers and predatory, they feed mainly on detritus as well as dead or decaying animal or plant matter. Considering the difference among species, following a blood-meal, egg development normally begins and takes from 3 to 11 days.

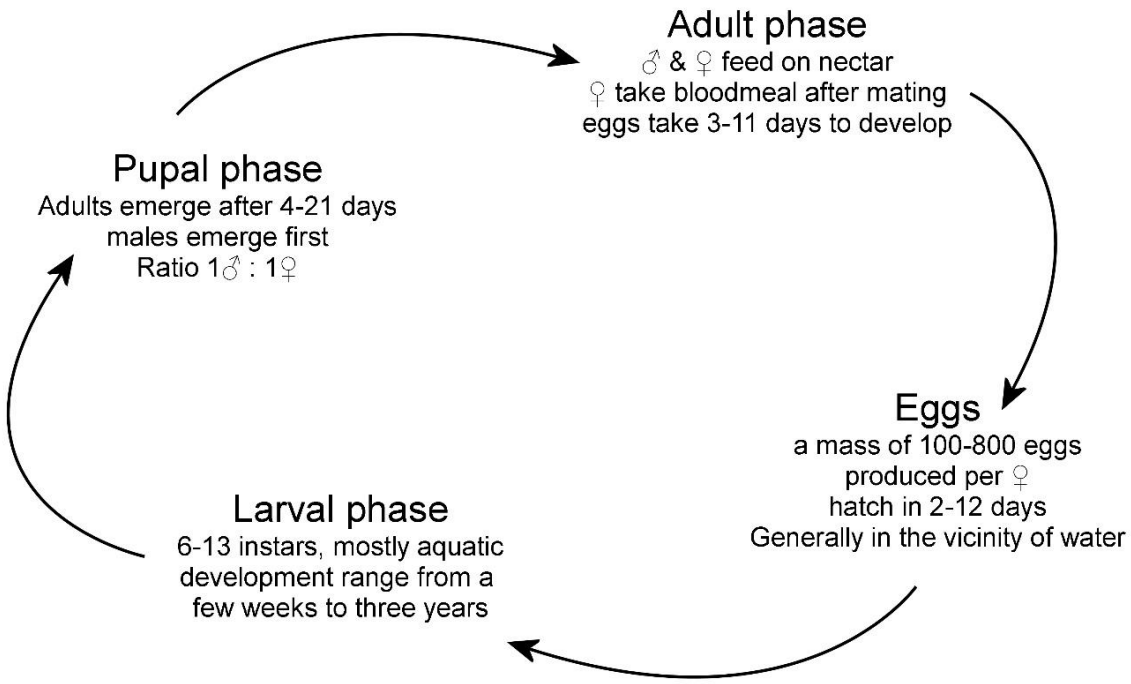


Figure 1: Illustration of the life cycle of tabanids (adapted from Baldacchino *et al.* (2014a))

Approximately 100 to 800 eggs are oviposited in large untidy "clusters", generally on foliage in close proximity to water. The number of eggs is variable and depend on the species as well as the physical condition of the female (Baldacchino *et al.* 2014a). Temperatures and humidity fluctuations also affect the hatching process (Mullen 2002; Baldacchino *et al.* 2014a). According to Mullen (2002) it takes 2 to 12 days for the eggs to hatch. There are 6 to 13 larval-instars that develop near water and are generally semi-aquatic, even though terrestrial and aquatic species exist (Baldacchino *et al.* 2014a). Pupation occurs underground and the pupa is positioned vertically. Before pupation, the larvae of some African tabanid flies make hollow mud cylinders underground. By doing this, the pupa is protected from being exposed when cracks form in the mud and also enables the pupa to move deeper in the ground away from the surface when temperatures are too high (Chainey 1993). Normally, it takes one to three weeks for a larva to complete metamorphosis (Roberts & Janovy, 2009; Baldacchino *et al.* 2014a). The pupa stage takes approximately three weeks to complete (Baldacchino *et al.* 2014a).

1.4 Horse-fly phylogeny

Several aspects of tabanid phylogeny remains unstudied, especially in the tropical regions of the world, including the Afrotropics (Morita 2008, Turcatel 2014). According to Morita (2008), regular changes have been seen in the classification of Tabanidae during the past half century, indicating the need for taxonomic work utilising both classical and molecular techniques. The simplified morphology of tabanids and the usual scarcity of males add to the complexity of studying their taxonomy (Morita 2008). Morphological characteristics described by Oldroyd (1954) like the callus, wing colour and frons can be quite confusing for the entomologists to work with identifying tabanids as the distinction can be very small when compared with other closely related species (Taioe *et al.* 2017). This creates some difficulty in the identification. Female genitalia are also not useful at genus or species level due to low intraspecific variation (Coscarón & Philip 1967, Krolow *et al.* 2015). These are sometimes useful in higher classification, *e.g.* in Mycteromyiini (Coscarón & Papavero 1993). Character traits such as colour features of the head or thorax are considered as secondary sexual traits and can thus only be observed in one sex. Additionally, the appearance of these traits can be variable and may be based on age and/or preservation method (Morita 2008). As a result, the difficult morphology present and the little information on the taxonomy in the family emphasises the importance of developing molecular methods to aid in elucidating the taxonomy and/or systematics of the group, as well as aid in practical applications, such as molecular identification of species (Godfray 2002).

1.5 COI as a barcode

Considering the identification problems with the use of traditional taxonomic tools for horseflies, molecular tools may play a pivotal role for accurate species identification (Banerjee *et al.* 2015). The identification of pestiferous mechanical vector species with the use of molecular tools is not affected by sex or a reproductive stage of the targeted taxa. Another advantage seen with the use of molecular tools is its ability to reveal genetic diversity which can solve species complexes (Banerjee *et al.* 2015).

DNA barcoding has thus been afforded the attention as a tool to resolve taxonomic difficulties seen in most invertebrates especially tabanids at species level. It employs the use of Cytochrome c oxidase sub unit I gene (COI) as a standard method for identification of species of most invertebrates (Hebert *et al.* 2003). Folmer *et al.* (1994) described the COI gene on 11 diverse

invertebrates in a short communication, the gene which was postulated to be the most conservative protein coding gene in the mitochondria of animals and therefore an appropriate barcoding gene region (Brown & Armstrong 1985).

Ever since, the described primers of COI have been used to supplement morphological identification of horse-flies (Taioe *et al.* 2017). DNA barcoding based on the mitochondrial cytochrome c oxidase subunit 1 (COI) gene sequences has been used to discover new or previously unknown biodiversities due to its ability to differentiate diverse arthropod species including insect pests such as tussock moths, Lepidoptera: Lymantriidae (Ball *et al.* 2005; Mugasa *et al.* 2018).

Molecular methods certainly have an edge in solving identification difficulties seen in not only tabanids but many other taxa, ranging from plants to mammals. However, similar studies are largely wanting, not only in South Africa but in other African countries (Taioe *et al.* 2017). In addition, there are very little studies related to higher level phylogenetic relationships in the family (Wiegman *et al.* 2000, 2003). Even though barcode data has been successfully employed in the identification and discrimination of tabanids and in other families of the order Diptera, including Culicidae (Harbach 2007). Its utility in southern Africa is still in its infancy as there are about 250 species reported in South Africa of which approximately 10 have barcodes in the BOLD system (Accessed: June 2018: http://www.boldsystems.org). As a result, one of the objectives of this study is to generate barcode data of morphologically identified tabanid species occurring in south eastern Kruger National Park.

1.6 Disease agents transmitted by horseflies

Published reports of tabanid transmission of pathogenic agents of animals (including human) have been reviewed in the past. Krinsky *et al.* (1976), indicated that tabanids are essential for the biological transmission of *Haemoproteus metchnikovi, Trypanosoma. theileri*, *Loa loa*, *Dirofilaria roemeri*, and *Elaeophora schneideri* and that tabanids are mechanical vectors of viruses causing equine infectious anemia, vesicular stomatitis, hog cholera, and rinderpest; bacteria (*Anaplasma marginale, Bacillus anthracis, Clostridium. chauvoei, Pasteurella multocida, Francisella tularensis, Brucella* spp., *Listeria monocytogenes, Ehrlichia. rhusiopathiae*; and protozoa such as *Besnoitia besnoiti, Trypanosoma evansi, T. equiperdum, T. congolense, T. brucei brucei, T. brucei gambiense rhodesiense*). However, the extent to which tabanids are important in the natural and mechanical transmission of most of these agents is not fully known in southern Africa especially

areas between Mpumalanga and Limpopo provinces such as the Kruger National Park. Studies on *Tryapanosoma, Babesia* and *Theileria* parasites have been done in the Afrotropics, including South Africa (Zumpt 1949), but the reports were focused on either the affected vertebrate hosts or known vectors such as ticks or *Glossina* species rather than horseflies (Taioe *et al.* 2017).

1.7 Diseases and parasites associated with Tabanidae in SA

1.7.1 Trypanosomosis

African animal trypanosomosis (AAT) also known as nagana is a deadly disease caused by protozoan parasites of the genus *Trypanosoma* which are mainly cyclically transmitted to wild and domestic animals by tsetse flies (*Glossina* spp.) (OIE 2013; Votýpka *et al.* 2015; Taioe *et al.* 2017). Tsetse flies are limited to sub-Saharan Africa and occur in only the north-eastern parts of KwaZulu-Natal Province and possibly along the Limpopo province border in South Africa (Leak 1999; Service 2008). Trypanosomosis in Africa is caused, in part, by *Trypanosoma congolense, T. vivax, T. brucei brucei* whereas *T. equiperdum* and *T. evansi* causes dourine and surra in equines respectively (Leak, 1999; OIE, 2013). Unlike the other trypanosome parasites, *T. equiperdum, T. evansi, T. theileri* and *T. vivax* are not restricted to the African continent only and have wider distribution extending to Asia and some parts of Europe as well as South America (Desquesnes *et al.* 2013). *Trypanosoma equiperdum* is venereally transmitted and therefore does not require an arthropod vector like the other trypanosome parasites (Taylor & Authié 2004). In turn, *T. evansi* require arthropod vectors to be transmitted to susceptible hosts. Interestingly, *Trypanosoma theileri* is a nonpathogenic haemoprotozoon extensively distributed in livestock specifically cattle (Villa *et al.* 2008). *T. theileri* enters tabanids during blood feeding and subsequent cyclopropagative development in the posterior part of the insect's digestive tract, known as a stercorarian trypanosome, occurs (Hoare 1972). Infective metacyclic stages were identified in the gut and in the feces of tabanids such as *Haematopota pluvialis, Haematopota italica, Hybomitra micans* and *Tabanus bromius* (Böse *et al.* 1987). These stages can penetrate the intact oral mucosa (Kraneveld 1931) of their mammalian hosts. Böse et al, (1987) suggested that oral contamination of mucosal membranes could take place while cattle defend themselves against tabanids. In addition, a sporozoite form of the parasite has been found in the salivary glands of *Chrysops callidus* and was seen to give rise to gametocyte forms after being inoculated to turtles (DeGiusti *et al.* 1973). Taioe *et al.* (2017) produced the sole study reporting a relationship between horseflies

and this parasite in Africa. Futhermore, studies by Desquesnes & Dia (2003; 2004) have experimentally demonstrated that mechanical transmission of *T. vivax* and *T. congolense* is possible by the tabanid fly species *Atylotus agrestis* and *A. fuscipes*. *Trypanosoma evansi* on the other hand is known to be mechanically transmitted by biting flies including *Stomoxys* and tabanid flies (Sumba *et al.* 1998; Gutierrez *et al.* 2010; Desquesnes *et al.* 2013). As a result, the current study aims to detect *Trypanosoma* species from tabanid flies collected from south-eastern Kruger National Park South Africa.

1.7.2 Babesiosis

Bovine babesiosis is a disease often found in tropical and subtropical regions of the world, known to be biologically transmitted by ticks (Mtshali *et al.* 2014). It is caused by protozoan parasites of the genus *Babesia* (Apicomplexa: Piroplasmida) (OIE, 2010; Iseki *et al.* 2007; Mtshali *et al.* 2014). The disease is caused by *Babesia bovis* and *Babesia bigemina* in susceptible mammalian hosts, mainly cattle in Africa, Asia, Australia, and Central and South America. *B. divergens* is the economically most important species in Europe (OIE, 2010). *B. bovis* and *B. bigemina* are transmitted by *Rhipicephalus microplus, R. annulatus, R. decoloratus, R. geigyi* and *R. evertsi evertsi* ticks to susceptible mammalian hosts. *B. divergens* in turn, is transmitted by *Ixodes ricinus* (Hunfeld & Hildebrandt 2008; OIE, 2010). Humans and domestic animals get infected during a blood meal from an infected tick. Humans usually are dead-end hosts. Human-to-human transmission is also reported to occur via contaminated blood transfusions (OIE, 2010). Molecular and serological assays have been conducted to detect the disease from the host animals as well as in the tick vectors in affected nations around the world (Hunfeld *et al.* 2008; Ica *et al.* 2007; Iseki *et al.* 2007; Mtshali *et al.* 2014; Sivakumar *et al.* 2014). There is, however, no information on any association of *Babesia* parasites and flies, including tabanids. Hence one of this study aims was to determine if there is any link or relationship between the two organisms in the south-eastern Kruger National Park.

1.7.3 *Anaplasmosis*

A. marginale (Rickettsiales), is one of the aetiological agents of bovine anaplasmosis, an infectious but non-contagious disease (Aubry & Geale, 2011). Bovine anaplasmosis occurs in tropical and subtropical regions worldwide including Africa, America, the United States, southern Europe, Asia and Australia. *A. marginale* is known to be transmitted mechanically by biting flies, usually horse-

flies, and biologically by ticks (Kocan *et al.* 2010; Baldacchino *et al.* 2014a). Biological transmission by ticks is considered to be more efficient than mechanical transmission and can result in enzootic stability. Interestingly, previous studies on mechanical transmission have clearly demonstrated that horse-flies are efficient mechanical vectors of *A. marginale*, (Foil 1989) but useful comparisons of vectorial efficiency are limited by differences in donor and recipient animals among experiments (Krinsky, 1976).

Natural transmission between cattle has been demonstrated with *Tabanus* spp. (Howell *et al.* 1941, Lotze, 1944, Wilson and Meyer, 1966). Krinsky (1976) conducted a thorough review of transmission of *A. marginale* by different horse flies. Many studies used naturally infected cattle and adult cows as recipients, and mechanical transmission was consistently demonstrated. Hawkins *et al.* (1982) showed that transmission of *A. marginale* from acutely infected splenectomized calves to susceptible splenectomized calves can be accomplished with as few as ten horse fly bites. Horse-flies were shown to remain mechanically infective for at least two hours.

Table 1: Disease agents associated with tabanids compiled from (Baldacchino *et al.* 2014a, Taioe *et al.* 2017). Legend: ET: ET, NT: Natural Transmission, Iso: Isolation, Dev: Development.

Disease Agents	Vectors	Geographic occurrence	Transmission	Association	References
Equine infectious anemia virus	*Tabanus* spp., *Hybomitra* spp., *Chrysops* spp.	Worldwide	Mechanical	ET, NT, Iso	Foil *et al.* (1983), Hawkins *et al.* (1973), Kemen *et al.* (1978), Scott (1922) and Stein *et al.* (1942)
Bovine leukosis virus	*Tabanus* spp.	Worldwide	Mechanical	ET, NT	Buxton *et al.* (1985), Foil *et al.* (1988, 1989), Hasselschwert *et al.* (1993) and Perino *et al.* (1990)
Bovine viral diarrhea virus Hog cholera virus	*Haematopota pluvialis* *Tabanus* spp.	Worldwide	Mechanical	ET, NT	Tarry *et al.* (1991), Tidwell *et al.* (1972)
Trypanosoma congolense	*Atylotus agrestis*, *Tabanus* spp.	Africa	Mechanical	NT	Desquesnes & Dia (2003), Taioe *et al.* (2017)
Trypanosoma brucei	*Tabanus par*	Africa	Mechanical	NT	Taioe *et al.* (2017)
Rinderpest virus	*Tabanus orientis*	Eradicated worldwide	Mechanical	NT	Bhatia (1935)
Trypanosoma evansi	*Tabanus* spp., *Haematopota* spp., *Chrysops* spp.,*Ancala* spp., *Atylotus spp*	South America, Africa, Asia, Europe	Mechanical	ET, NT	Taioe et al 2017, Collier (1928), Gruvel & Balis (1965), Mitzmain (1913), Mohler & Thompson (1911), Nieschulz (1925, 1926, 1927a,b), Nieschulz & Ponto (1927), Sergent & Sergent (1905), Yutuc (1949)

Trypanosoma vivax	*Tabanus* spp., *Atylotus* spp., *Cryptotylus unicolor*	South America, Africa	Mechanical	NT	Desquesnes & Dia (2003, 2004), Ferenc *et al.* (1990), Otte & Abuabara (1991), ParraHenao *et al.* (2008), Raymond (1990)
Trypanosoma theileri	*Haematopota* spp., *Tabanus* spp., *Hybomitra spp*	Worldwide	Mechanical	ET, NT; Iso, Dev	Böse *et al.* (1987), Davies & Clark (1974), Dirie *et al.* (1990), Kraneveld (1931), Nöller (1916, 1925), Packchanian (1957)
Elaeophora schneideri	*Hybomitra* spp., *Tabanus* spp.	North America, Europe	Biological	ET, Iso Dev	Clark & Hibler (1973), Couvillion *et al.* 1986), Hibler *et al.* (1971), Weinmann *et al.* (1973)
Dirofilaria repens	*Haematopota variegate*	Africa, Asia, Europe	Biological	Dev	Coluzzi (1964)
Dirofilaria roemeri	*Tabanus* spp., *Dasybasis spp*	Australia	Biological	ET, Iso Dev	Spratt (1974)
Loa loa	*Chrysops* spp.	Central Africa	Biological	ET, NT; Iso, Dev	Connal & Connal (1922), Duke (1972), Gordon & Crewe (1953), Kleine (1915), Lavoipierre (1958), Leiper (1913), Noireau *et al.* (1990), Orihel & Lowrie (1975), Williams (1960)
Babesia bigemina	*Atylotus* spp., *Haematopota* spp., *Tabanus* spp.	Zambia	Mechanical	ET, NT, Iso, Dev	Taioe *et al.* (2017),
Besnoitia sp.	*Tabanus conformis*	Zambia	Mechanical	ET, NT, Iso, Dev	Taioe *et al.* (2017),
Theileria parva	*Tabanus taeniola*	Zambia	Mechanical	ET, NT, Iso, Dev	Taioe *et al.* (2017),
Trypanosoma congolense	*Tabanus taeniola*	South Africa	Mechanical	ET, NT, Iso, Dev	Taioe *et al.* (2017)

Trypanosoma theleiri	*Tabanus par*	South Africa	Mechanical	ET, NT, Iso, Dev	Taioe *et al.* (2017)
Trypanosoma theleiri	*Tabanus taeniola*	South Africa	Mechanical	ET, NT, Iso, Dev	Taioe *et al.* (2017)
Trypanosoma congolense	*Tabanus par*	South Africa	Mechanical	ET, NT, Iso, Dev	Taioe *et al.* (2017)

1.8 Trapping and the use of attractants

Traps on their own are sometimes of limited efficiency in capturing haematophagous flies including tabanids (Kappmeier & Nevill 1999). This often happens during the dry season when fly activity begins to decline. Apart from seasonality affecting trapping efficiency, olfactory lures and colour have been seen affect trapping efficiency as well (Foil 1989). Fly traps are used for the control and surveillance of target species and the use of attractants generally exploit the sensorial characteristics of the species. This is done not only by the use of olfactory attractants, but the incorporation of colour and sound may have a major effect (Kappmeier *et al.* 1999) as well as movement on the attractiveness of the trap (Thorsteinson 1958). Trap designs and their differences stem from observing fly behaviour and hosts behaviour (Thorsteinson 1958). Several fly traps have been designed especially for *Glossina* species such as the Nzi-, Ngu- and H traps, with only the Nzi-trap designed for general haematophagous flies, including tabanids and *Stomoxys* species (Mihok 2002).

African variations of traps are made from blue (pthalogen) and black fabrics, known to illicit a response from biting flies (Kappmeier *et al.* 1999). Over the years some changes to these designs were added to maximise the trapping for different fly species (Kappmeier *et al.*1999; Kappmeier *et al.* 2007).

A plethora of examples of tabanid and other haematophagous fly traps include the biconical-trap, Vavoua-trap, Epsilon-trap, F3-trap, the pyramidal-trap and the tetra-trap (Challier, & Laveissiere 1973, Laveissiere & Grebaut 1990). Worldwide, the most effective trap for tabanids seems to be the Nzi-trap (Figure 2), it has proved effective in Africa, North America, Australia and parts of Europe (Kappmeier & Nevill 2000; Baldacchino *et al.* 2013). The absolute efficiency of an unbaited Nzi trap, defined as the proportion of flies caught from the total attracted to the trap, was evaluated at 45 % for tabanids in Sudan (Mohamed-Ahmed *et al.* 2007). This efficiency increased to 91 % when the Nzi trap was baited with octenol. The Ngu trap, often used in southern Africa, resembles that of the Nzi trap with some modifications (Figure 3). There is practical evidence of the Ngu-trap capturing over 2000 tabanids in Mozambique (pers. comm. Dr. FC Mulandane). Apart from this report, the capturing efficiency of the Ngu trap is still not known in other African countries.

The horizontal trap or H-trap, also often used in southern Africa, has a design of two collection chambers situated on either side of the trap, was originally designed for the capturing

and controlling of *G. brevipalpis* and *G. austeni* populations in southern Africa. Kappmeier *et al.* (1998) and Kappmeier & Nevill (2000), described the horizontal trap as means to do away with the top cone system of the previous design, as these *Glossina* species were difficult to trap, and this was seen after a thorough re-evaluation of their behaviour (Kappmeier *et al.* 2007).

Thorsteinson (1958) described a trap for horse-flies formerly known as a helio-thermal trap. The trap, now referred as the Manitoba trap, was designed with a shiny and heat generating body to stimulate or mimic the mammalian body heat of the host (Figure 5). Later the value of using a shiny, large black object reflecting polarized light in tabanid traps of various designs was demonstrated and is a common practice (Thorsteinson *et al.* 1966). However, it is only very recently that the underlying basis for this observation has been elucidated in detail. It has been said that the attraction of female tabanids to black, shiny objects of a certain shape, height, orientation, *etc*. requires a high degree of polarization of reflected light (Egri *et al.* 2012) As a result the attractiveness of the trap was key to lure tabanids on to the trap with the movement of the black heat generating body. The trap has since been highly efficient in tabanid capture in Europe (Lempereur *et al.* 2018).

Figures 2-5: Clockwise from top left, Nzi-trap, Ngu-trap, Manitoba trap, Horizontal trap.

To date very few researchers have ever measured trap efficiency, with even fewer studies on how well trap collections reflect the species attracted to hosts (Muzari *et al.* 2010; Baldacchino *et al.* 2014b). Hence the current study aims to evaluate the efficiency of the different traps used to collect tabanids in south-eastern Kruger National Park.

1.9 Tabanids of South Africa

According to Usher (1972), with amendments by Chainey (1983, 1987), Manning (1991) and Morita (2008), South Africa (RSA) has a relatively rich diversity of tabanids with approximately 200 species described from the region. A review by Usher (1972) was the first to document tabanids of southern Africa as well as their distribution. In southern Africa, there are reports of approximately 410 species of tabanid flies with approximately 64 species occurring in KwaZulu-Natal Province (KZN), which accounts for the highest species diversity in the country (Usher 1972). Twelve species are endemic to KZN coastal climatic region which contributes 16% of the total endemic tabanid species to South Africa (Esterhuizen, 2006). The Cape floristic region, well known for its unique biodiversity, especially flowering plants, seems to be an important region regarding tabanid diversity, as well as the rest of the coastal regions (south coast, east coast and garden route) (Usher 1972). Even though the lowveld savanna (29 spp.) and northern savanna (29 spp.) are not as rich in species as other parts of the country, these regions are considered to be very relevant regarding disease transmission (Kappmeier & Nevill 1999). This might be due to several factors, one which is the reported disease spillover from neighboring African countries such as Mozambique and Zimbabwe, which are central to the matter (Kappmeier & Nevill 1999).

The lack of information of tabanids in other regions of South Africa adds to the neglect and better understanding of these important vectors. As this provides largely limited data on the whole fauna of tabanids present in not only South Africa but other African countries (Mugasa et al 2018). To start solving this problem, Taioe *et al.* (2017) suggested that further studies on possible transmission of parasites by tabanid flies in South Africa and other African countries are still required, as well as wider surveys where the capturing of these flies is not only limited in game reserves but also includes capturing these flies in other areas like livestock farming areas.

1.10 Justification of the study

South African tabanids have received very little attention, especially compared to other haematophagous flies. This is despite their documented ability of biological and mechanical transmission of parasites, bacteria and viruses (Mihok 2002). Recently, *T. congolense* have been recorded from cattle near the eastern border of the Kruger National Park (Pers. comm., directorate of animal sciences, Mozambique). The vector of *T. congolense* are generally considered to be *Glossina* species, but in Lowveld Savanna of southern Africa, where these do not occur, tabanids might be the responsible vector for the population maintenance of the disease-causing agent (Baldacchino *et al.* 2014a). Due to lack of studies conducted in southern Africa especially the Kruger National Park, the threat that these vectors pose in this area in terms of mechanical transmission of different parasites has not been adequately explored. Hence this study sought to investigate the presence of these flies in the area as well as the role they play in pathogen transmission.

1.11 Project Aims

a) To determine the species composition and presence of horse-flies in south-eastern Kruger National Park South Africa.

b) Investigate the possible role of horse-flies in the transmission of *Trypanasoma*, *Babesia* and *Anaplasma* parasites in the region

1.12 Objectives:

a) Evaluation of the capture efficiency of the different traps for tabanid flies in south eastern Kruger National Park.

b) Determination of species composition and presence of tabanids by morphological characteristics and molecular methods

c) To investigate presence of haemoparasites in tabanid samples by means of species-specific PCR.

1.13 Key Questions

1. What is the capture efficiency of the different traps deployed for tabanids in KNP?

2. What is the species composition and presence of tabanids in south-eastern Kruger Park?

3. Which haemoparasites are present in tabanid samples, and in which species of tabanids is showing the presence of haemoparasite DNA?

Chapter 2

2.1 Materials and Methods

2.1.1 Study site

The study was conducted in south-eastern Kruger National Park (South Africa) using seven sites (Table 2, Figure 6).

Table 2: Collection sites and trapping duration details.

Site name	Coordinates	Site Description	Trapping duration
Moamba river	S25,250005^0 E31,592331^0	Seasonal river bed with pools throughout the year, large trees (Jackalberry tree - *Diospyros mespiliformus*) provide ample shade, riverine bushveld.	09/03/2018 - 29/03/2018
Sabie drainage	S25,177872^0 E31,975624^0	Natural drainage system dominated by grassland between two rocky outcrops, few trees, seasonal water pools present.	09/03/2018 – 20/04/2018
Sabie matrix	S25,181522^0 E32,002162^0	Flat open lowveld savannah, shrubs, grass and small flowering plants dominate the landscape	09/03/2018 – 20/04/2018
Sabie reeds	S25,179097^0 E31,963002^0	Natural drainage system with seasonal water, riverine vegetation dominated by sedges (Cyperaceae).	09/03/2018 – 20/04/2018
Sabie bucket	S25,184580^0 E32,008112^0	Open lowveld savannah, shrubs, grass and small flowering plants dominate the landscape, habitat on an incline	09/03/2018 – 20/04/2018
Sabie river	S25,186237^0 E32,012945^0	Situated nest to the Sabie river, a permanent river system, few trees and rocky open areas, dominated by tall sedges (Cyperaceae) with patchy shurbs and grass	09/03/2018 – 20/04/2018
Shishangeni watering hole	S25,230926^0 E31,586215^0	Open animal grazing area with permanent water, open lowveld savannah surrounds a bare soil watering hole	09/03/2018 – 29/03/2018

The collection of tabanids was focused in the Sweet Lowveld Bushveld region (Sabie area) where the Lowveld, with its subtropical climate experiences generally hot summers and mild winters. The average annual rainfall of the area is between 300-600 mm increasing from northern to the southwest of the Lowveld. This bushveld is found mainly in flat plains from 170 to 250 m above sea level in a small strip located west of the Lebombo mountains. This type of bushveld

occurs from the Olifants River in the Kruger National Park southwards through Swaziland into the northern parts of KwaZulu Natal.

Figure 6: A map illustrating the collection sites in southern eastern Kruger National Park South Africa.

2.1.2 Trapping and collection of tabanids

A total of 14 different traps (four H traps, four Ngu traps and six Manitoba-style traps) were used in the study. Three different traps (one of each) were placed approximately 70-100 m apart at Sabie River, Sabie Matrix, Sabie drainage and Sabie reeds for trap comparisons (Figure 7). The remaining two Manitoba traps were placed at Shishangeni, Moamba river and Sabie bucket sites for opportunistic sampling during the first round of sampling. This was done to maximise catches for disease screening purposes. The traps were baited with 2 ml octenol sachets to increase the trapping efficiency of the traps. All traps were kept at each sampling site for 4 days whilst the flies were harvested daily. The trapping period was between March and April 2018.

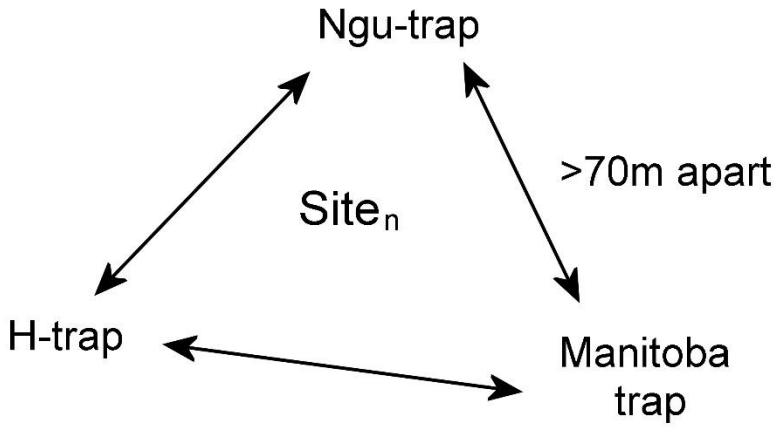


Figure 7: Showing trap placement in each of the focused sites in the study namely: Sabie river, Sabie drainage, Sabie matrix and Sabie reeds

2.1.3 Identification and characterisation of tabanid flies

Reference flies were pinned and placed in entomological boxes for morphological identification and labelled with area and date of collection. Entomological boxes are kept in the Biosystematic Support Unit (BSU) in the ecto-parasitology laboratory, University of Pretoria. The remaining flies were kept in a freezer for molecular work and others as reference flies for our collection. The Nikon AZ 100 M multi zoom microscope (Nikon Inc., Tokyo, Japan) was used to capture sequential photographs of different features of the flies. Subsequently, the flies were examined and identified to species level by discriminating unique characters that differentiated between species. The morphological keys used were those documented by Oldroyd (1952, 1954, 1957) with additions from Usher (1972) and Chainey (1993). Usher (1972) was used to confirm the distribution and occurrence of the tabanid flies in South Africa.

2.2 DNA extraction and analysis for identification

2.2.1 Molecular characterisation of flies

A leg was isolated from at least one fly of each species and placed in separate 2 ml tubes with 200 µl of >99 % ethanol each for DNA preservation. Fifty-two samples with the legs were then extracted for genomic DNA using the chelex extraction method with slight modifications. The fly legs were grinded with a pestel in a 2 ml Eppendorf tube containing 200 µl of 5 % chelex solution. This was followed by a 1 h incubation in the heating block at 56 ºC and 30 min denaturing at 95 ºC, after which they were stored at -20 ºC for further analysis. In a 20 µl volume containing 10 µl of 2x Phusion Flash Master Mix and 7 µl of double distilled water, conventional PCR targeting COI mitochondrial gene was conducted, 2 µl template DNA of the leg samples was added, using 0,5 µl of both primers LCOI1490: GGT CAA CAA ATC ATA AAG ATA TTG G and HCOI2198: TAA ACT TCA GGG TGA CCA AAA AAT CA as described by (Folmer *et al.* 1994). This was done for the amplification of an approximate 710 bp fragment of COI (cytochrome c oxidase I).

The PCR conditions of amplification were set as follows: were initial denaturation at 98 °C for 10 seconds, followed by 30 cycles entailing a 98 °C for 1 second of denaturation, annealing at 55 °C for 5 seconds, extension at 72 °C for 15 s, final extension at 70 °C for 1 min and holding temperature at 4 °C.

2.2.2 Phylogenetic analysis

All sequences were viewed, assembled and edited in CLC main workbench 8 (Hall, 1999) and exported as a FASTA file. To confirm sequences obtained from CO1 analysis, the nucleotide basic local alignment search tool (BLASTn) was used. Only gene sequences with 98 % to 100 % similarity match score were considered as significant. Gene sequences of species closely related to tabanid flies in the current study from the BLASTn search results were downloaded from NCBI database (https://www.ncbi.nlm.nih.gov/genbank/).

The CO1 gene sequences were aligned using the online version of MAFFT using standard parameters (https://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013). Thereafter, the aligned sequences were viewed, edited and truncated using Mega 7 (Kumar *et al.* 2016). The aligned matrix was used for all analysis and format changes to the dataset was conducted in format converter (https://www.hiv.lanl.gov/). jModelTest 2, via Cipres science gateway (www.phylo.org), was used for evolutionary model estimation.

Data-display networks (neighbour-net graphs) were constructed in SplitsTree 4.14.4 (Huson 1998), using all characters and uncorrected p-distances. Bootstrap support for the networks were calculated using 1000 replicates. Maximum likelihood analysis was executed using RAxML v. 8.2.4 (Vacher *et al.* 2016) on all codon positions, with a GTR+G+I model, invoking the autoMRE function for bootstrap calculation. For Bayesian inference, MrBayes v. 3.2.6 (Huelsenbeck & Ronquist, 2003) was employed using GTR+G+I as estimated model. Four cold MCM chains searched for 10 000 000 iterations saving every 500th sampled tree for analysis. 15% of sampled trees were discarded as burn-in with the remaining trees used for calculating posterior probabilities. Tracer v1.6 was employed for effective sample size determination, where ESS values of over 200 was deemed as sufficient.

2.3 Detection of the presence of *Babesia* and *Anaplasma* spp. from tabanid flies

DNA for disease screening was obtained by crushing the whole body of the selected tabanids samples. For a suitable sample size determination of tabanids for the detection of presence of parasites we used the formula:

$$n = (1 – (1 – P_1)^{1/d}) (N – d/2) + 1$$

Where:

N = Population size

d = number of affected animals

n = required sample size

P_1 = Probability of finding one case in the sample

The formula was only applied to a 60 % proportion of the strongly represented species of tabanid flies in our collection where in an infinite population size the probability of finding at least one case in the sample was 0,05 upper 5 % confidence limits of number of cases

2.3.1 Real time PCR assay for *Babesia bigemina*

The tabanid samples were analysed using the Applied Biosystems, Step One Plus Software v2.2.2, Real Time PCR System. The 20 µl volume contained 8 µl TaqMan universal PCR master mix, 0,5 µl of *B. bigemina*-specific primers Forward primer BIF and Reverse primer BIR, 0,5 µl Probe BIP (VIC-TAMRA), 8,5 water (Nuclease free) and 2 µl template DNA. PCR conditions were set as follows: 1 cycle of UNG incubation at 50 °C for 2 min, 1 cycle of AmpliTaq Gold pre-activation

at 95℃ for 10 minutes for, 45 cycles of denaturing were at 9 5℃ for 20 s and the annealing-extension step was at 57 ° C for 1 min.

2.3.2 Duplex real-time quantitative polymerase chain reaction for *Anaplasma marginale* and *A. centrale*

The tabanid samples were analysed using the duplex qPCR assay reported by Decaro *et al.* (2008) for simultaneous detection of *A. marginale* (detecting the msp1β gene) and *A. centrale* (detecting the groEL gene), with slight modifications of the *A. centrale* probe to adapt it for use in the Lightcycler realtime PCR system. The 20 µl reaction mixture contained 4 µl of FastStart Taqman mix (Roche Diagnostics), 0.5 µl UDG, 0.6 µM of *A. marginale*-specific primers AM-For and AM-Rev, 0.9 µM of *A. centrale*-specific primers AC-For and AC-Rev, 0.2 µM of probes AM-Pb and AC-Pb and 2.5 µl of template DNA (approximately 200 ng). DNA extracted from the *A. centrale* vaccine strain purchased from Onderstepoort Biological Products (OBP) and sample 9410 obtained from Dr Helena Steyn, Onderstepoort Veterinary Institute (OVI), Pretoria, South Africa, were used as positive controls for *A. centrale*. Samples C14 and F48 (originating from bovines in the Mnisi Community area) were used as positive controls for *A. marginale*. These samples were confirmed to contain *A. marginale* infections by amplification and sequence analysis of the msp1b gene. Nuclease-free water was used as a negative control. Thermal cycling was performed in a LightCycler v2 (Roche Diagnostics, Mannheim, Germany). Thermal cycling conditions were UDG activation at 40 °C for 10 min, preincubation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 1 min, annealing–extension at 60 °C for 1 min and a final cooling step at 40 °C for 30 s. The results were analysed using the Lightcycler Software version 4.0 (Roche Diagnostics, Mannheim, Germany).

Table 3: Oligonucleotide primers used in this study for the detection of *Anaplasma marginale* (AM) and *A. centrale* (AC)

Parasite	Target gene	Oligonucleotide name	Sequence	Amplicon size	Reference
AM	msp1β	AM-For	TTGGCAAGGCACAGCTT	95	Carelli *et al.* (2007)
		AM-Rev	TTCCGCGAGCATGTGCAT		Carelli *et al.* (2007)
		AM-Pb	6FAM - TCGGTCTAACATCTCCAGGCTTTCAT		Carelli *et al.* 2007
AC	groEL	AC-For	CTATACACGCTTGCATCTC	77	Decaro *et al.* (2008)
		AC-Rev	CGCTTTATGATGTTGATGC		Decaro *et al.* (2007)
		AC-Pb	LC610 – ATCATCATTCTTCCCCTTTACCTCGT – BHQ2		Decaro et al (2007)

2.4 *Trypanosoma* species identification

2.4.1 Detection of *Trypanosoma*

The PCR mixture was prepared using 2X Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, LTC Tech, South Africa). Reactions were performed in a 25 µl volume containing 12.5 µl of 2X Phusion Flash High-Fidelity PCR Master Mix, 20 µM of each primer (forward and reverse), nuclease-free water and 5 µl of DNA template. Forward primer 18ST nF2 (5'-CAACGATGACACCCATGAATTGGGGA-3') and reverse primer 18ST nR3 (5'-TGCGCGACCAATAATTGCAATAC -3') were used.

The PCR conditions were as follows: initial denaturation at 98 °C for 10 s and then 35 cycles each of denaturation at 98 °C for 1 sec, annealing at 58 °C for 5 sec and elongation at 72 °C for 15 sec, and a final extension step at 72 °C for 1 min.

A semi-nested PCR was then performed with the same forward primer, but a different reverse primer: 18ST nR2 (5'-GTGTCTTGTTCTCACTGACATTGTAGTG-3').The PCR was performed as mentioned above with slight modications which included: 2.5 µl of the PCR product

- from the first PCR reaction – was used as template and the PCR was run for only 25 cycles, not 35.

A negative- and positive control should be included in each PCR amplification. The negative control consists out of the PCR mixture without DNA template and the positive control is of a known *Trypanosoma* DNA sample. The amplification products were examined for the presence of trypanosome DNA on a 2 % agarose gel. A 100 bp ready to use DNA ladder (Thermo Scientific, Inqaba Biotec, South Africa) was included in every gel for fragment size determination. The samples were run for 35 min at 120 V in 1x Tris/Acetic acid/EDTA (TAE) buffer, stained with ethidium bromide and photographed under UV illumination using the ChemiDoc™ XRS system (Bio-Rad Laboratories, South Africa). Detection of *Trypanosoma* DNA was regarded as a positive infection (amplicons between 600 and 720 bp were obtained – this is in accordance with Geysen et al (2003).

Restriction Fragment Length Polymorphism (RFLP): A double-digest reaction was performed on the positive PCR amplicons in 15 µl reactions using the restriction enzymes Msp1 (New England BioLabs, Inqaba Biotec, South Africa) and Eco571 (New England BioLabs, Inqaba Biotec, South Africa) in the CutSmart® Buffer according to the manufacturer's instructions. Four microlitres of the restricted PCR product was mixed and loaded with 2 µl of loading dye buffer onto a 10 % Tris/Boric acid/EDTA (TBE) polyacrylamide gel (PAGE) and the fragments were thereafter separated by gel-electrophoresis in 1x TBE buffer at 100 V for 95 min.

A 100 bp DNA ladder (Thermo Scientific, Inqaba Biotec, South Africa) for fragment size determination was also included in each TBE-PAGE gel. The gel was then stained in 1x TBE buffer with SYBR Green I gel stain (Roche, South Africa) for 30 min and photographed using the ChemiDoc™ XRS system (BioRad, South Africa). The Enzymes used was MspI (Cat numbers: R0106 AcuI & R0641 (This enzyme is the same as Eco571).

2.5 Ethical Considerations

The animal ethics committee for animal experimentation at the University of Pretoria, South Africa, reviewed and approved the research protocol. Tabanid samples were collected by the MSc student or researcher, (Mr. X. Mazibuko) in accordance with the guidelines on research and testing of animals. The study was conducted with the assistance of the State Veterinarians and support from South African National Parks (SANparks).

Chapter 3

3.1 Sampling results

A total of 273 tabanid flies were collected from the sampled sites in south-eastern Kruger National Park South Africa. Morphological identification revealed a total of five genera collected from the sampled areas namely: *Atylotus, Philoliche, Haematopota, Tabanus* and *Chrysops*. A total of 13 different species were collected whereby, the dominant species from the sampled sites were *Tabanus minuscularius* 147 (55 %) and *Philoliche (Buplex) suavis* 38 (14%). The least species caught were *Haematopota vittata* 1 (0,4 %), *Haematopota daveyi* 1 (0.4 %), *Tabanus chevaleiri* 1 (0,4 %), *Tabanus sericiventris* 1 (0,4 %) (Table 4, 5).

Table 4: Captured horseflies from south-eastern Kruger National Park South Africa.

Tabanid Species	Sabie River	Sabie Matrix	Sabie Drainage	Sabie Reeds
Tabanus minuscularius	10	24	101	4
Tabanus chevalieri	0	1	0	0
Tabanus gratus	9	8	4	4
Tabanus atrimanus	5	1	0	0
Tabanus sericiventris	0	0	0	1
Atylotus agrestis	10	3	5	2
Chrysops obliquefasciata	5	10	0	0
Philoliche (Buplex) suavis	1	3	29	0
Haematopota vittata	0	0	1	0
Haematopota decora	0	1	0	0
Heamotopota daveyi	0	0	1	0
Unknown	0	1	7	0
Total	40	52	148	11

From the 13 species captured, 11 species were caught in the main four sampling sites focused in the study (Table 4) namely: *Tabanus minuscularius, Tabanus chevalieri, Tabanus gratus, Tabanus atrimanus, Tabanus cf. sericiventris, Atylotus agrestis, Chrysops obliquefasciata, Philoliche (Buplex) suavis, Haematopota vittata, Haematopota decora* and *Haemotopota daveyi* .

Table 5 Captured tabanids in opportunistic sites in south-eastern Kruger National Park

Tabanid Species	Bucket site	Shishangeni watering hole	Moamba river
Tabanus minuscularius	1	1	6
Tabanus gratus	1	0	1
Tabanus par	0	0	1
Tabanus taeniola	1	0	0
Atylotus agrestis	1	0	0
Philoliche (Buplex) suavis	0	2	2
Haematopota decora	1	0	1
Unknown	0	1	1
Total	5	6	11

One species that was only accounted for in the opportunistic sites was, *Tabanus par* (Table 5). *Tabanus minuscularius* was also dominant in opportunistic catches with (n=8/22) 36,4 % , *Phloliche Buplex suavis* (n=4/22)18.2 %, *Tabanus gratus* (n=2/22) 9,1 %, *Haematopota decora* (n=2/22) 9,1 %, *Tabanus taeniola* (n=1/22) 4,5 % and *Atylotus agrestis* (n=1/22) 4,5 %

3.1.1 Trap comparisons

The H trap captured the most average number of tabanid species per day across all sites (Figure 8). The H trap was followed closely by the Manitoba trap with 1,7 catches per trap per day and

lastly the Ngu trap with 0.4 catches per trap per day.

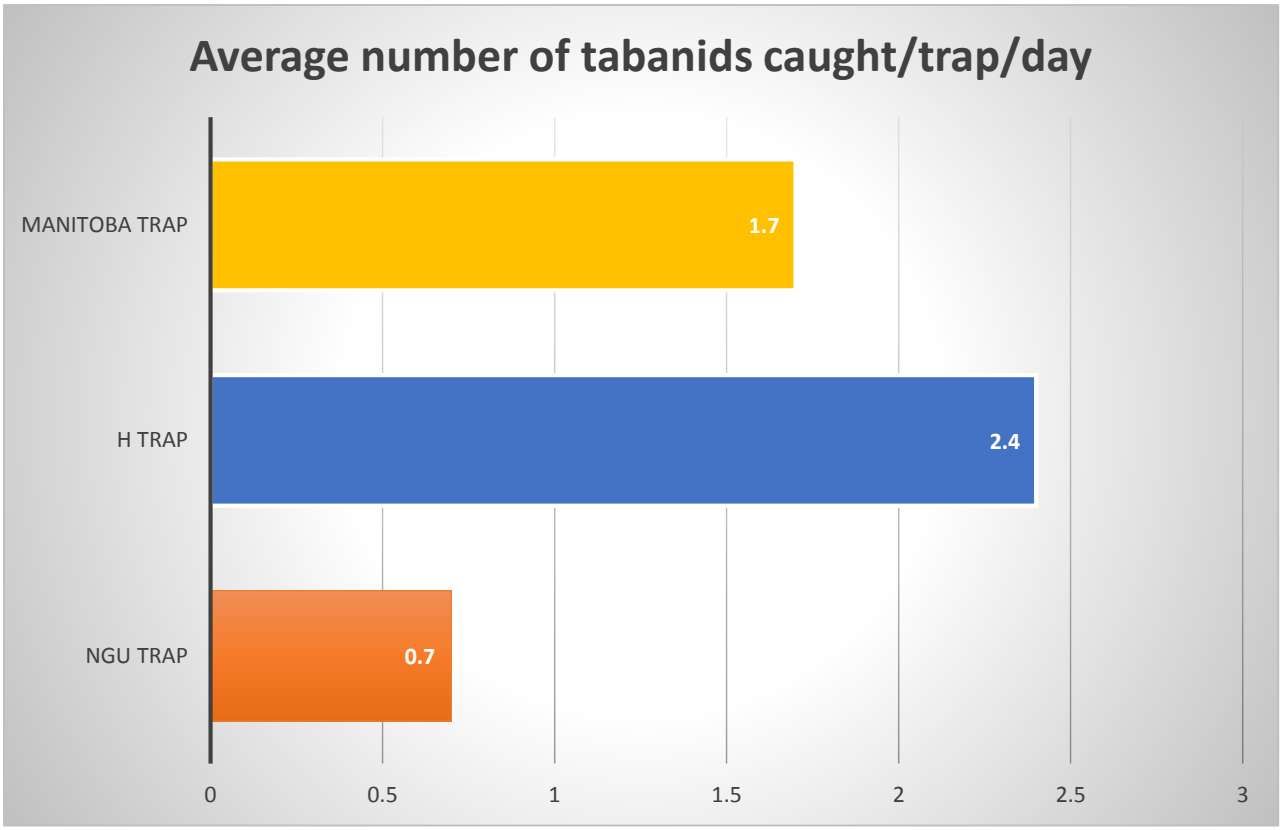


Figure 8: Bar chart showing the average number of catches per trap per day across all sites. Ngu trap is represented by orange, H-trap by blue and Manitoba trap by yellow

3.1.2 Site comparisons

The Sabie drainage site saw the greatest number of catches when compared with the other four sites (Table 4) in south eastern region of the Park. Composing of (n=148) 59 % (Figure 9) in the collection of tabanids, the Sabie drainage represents more than half of the entire collection of tabanids in the study with *Tabanus minuscularius* (n=101/251) 40% and *Philoliche (Buplex) suavis* (n=29/251) 12 % as the dominant species caught respectively (Table 4).

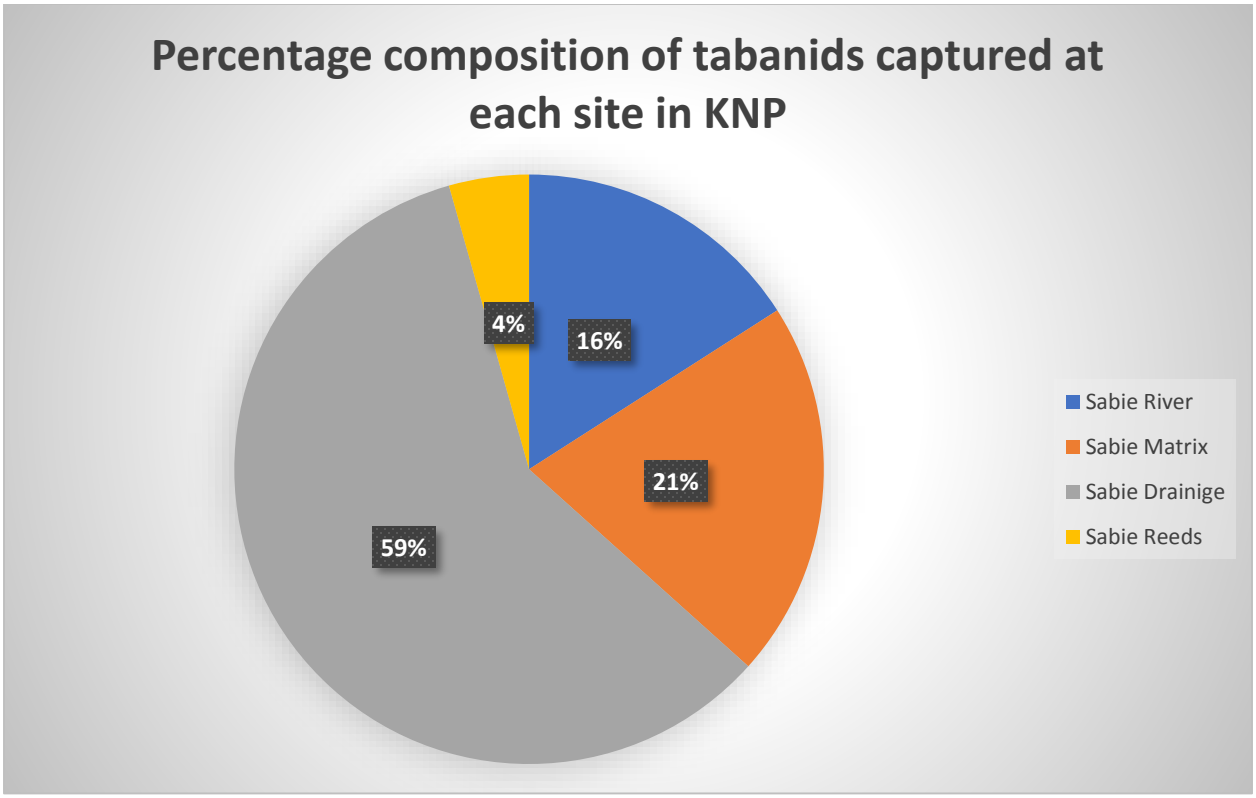


Figure 9: Pie chart showing the percentage composition of tabanids captured at the different sites: Sabie River represented by blue, Sabie Matrix by orange, Sabie Drainage by grey and Sabie Reeds by yellow

3.2 Morphological identification

Identification of horsefly species was based on discerning unique characteristics as proposed by the monograph series by Oldroyd (1952, 1954, 1957) supplemented by Usher (1972) and Chainey (1993): Only 5 species from our collection was described as identifications of other species were doubtful.

Tabanus par Walker, 1858

The specimen was identified on characteristics described by Oldroyd (1952, 1954,1957). *Tabanus par* is easily recognised by the complete yellow appearance of the species, only shared by *Tabanus donaldsonii* (18-19 mm). *Tabanus donaldsonii* is, however, quite larger than *T. par* and known to occur more in the northern regions of Africa like the Gold Coast as compared to *T. par* which is mostly found in the southern areas of Africa. They also differ in the colour of the legs where in *T. par* the rest of the legs are yellow to a slight brown (Figure 10) whereas for *T. donaldsonii* the rest

of the legs are red. *T. par* is small with a body length of this species between 10-12 mm. The eyes are dark green, lacking bands on preserved specimens (Figure 4). The calli are yellowish to a brown, united into an elongated shape. The frons tomentum is golden yellow including scattered, small black hairs. The face, subcallus and parafacials are yellowish with yellow hairs. The palpi are dark yellow with black hairs basally and pale white hairs toward the apex. The proboscis is dark yellow to orange. The antennae segments are light yellow to bright orange. The thorax lacks patterns, the scutellum is black in ground colour and grey in non-ground colour. The abdomen is yellowish basally and darker towards the apex with no patterns and covered with fine black and golden yellow hairs. The wings are clear with golden yellow veins and stigma.

Figure 10: Sequential images of *Tabanus par*, from top left clockwise, habitus, venter, facial and lateral views.

Tabanus minuscularius Austen (1912)

The species was identified on characteristics described by (Oldroyd 1954; Usher 1972). The species can be easily recognised by its small size and abdominal patterns with a head structure and abdomen patterns that resembles *T. sufis* and *T. taeniatus* belonging in the Sufis group. These

species though can be easily distinguished as *T. minuscularius* apart from its small size it has bare eyes (Figure 11) compared to the hairy eyes seen in *T. taeniatus*. The average body size of the species is 9-10 mm. The eyes are microscopically emerald green with three faint crimson bands. Calli is black, bare and shining (Figure 11). The frons with grey tomentum, including black, brown and yellowish hairs. Face and parafacials white with short white hairs. The palpi dark yellow with a uniform distribution of white and black hairs. Proboscis dark brown. The antennae basally brown to brown-orange with black hairs, apex of flagellum black and had terminal segments with a tinge of brown dorsally. Abdomen is a black to brown colour with grey pattern of two longitudinal stripes, median stripes is very faint and scarcely indicated. Sublateral stripes are well developed with roundish grey spots on all segments. Wings are clear with yellowish veins on the basal half, the stigma is a yellow to brown colour with no appendix on R4.

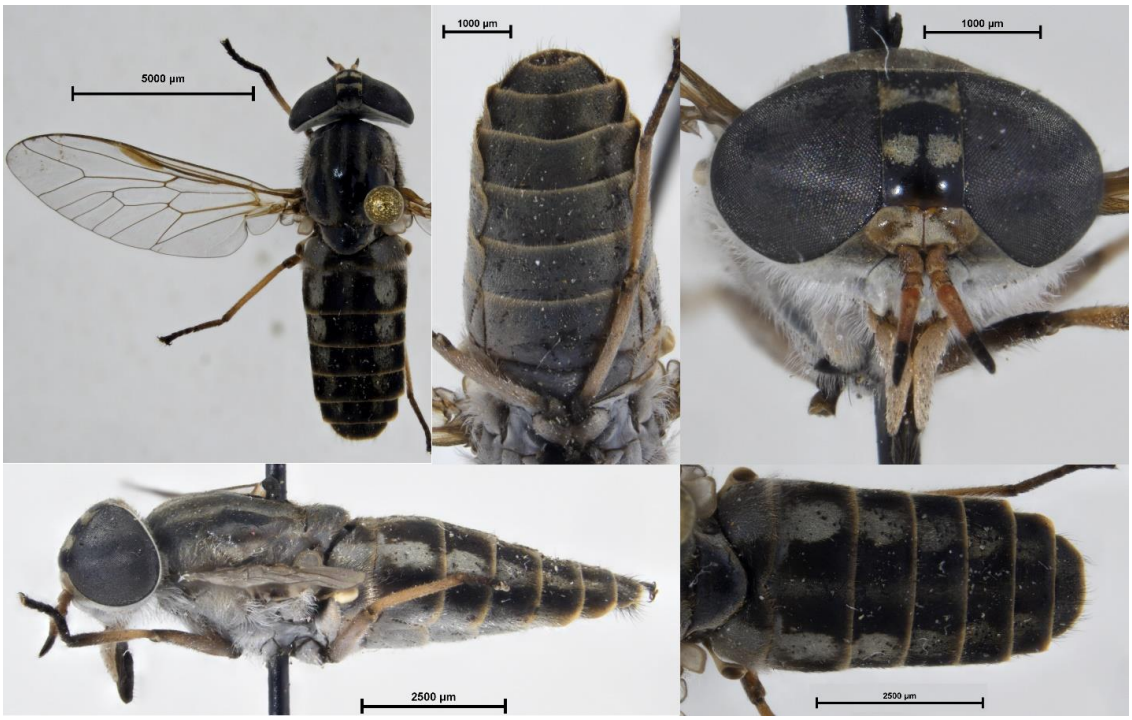


Figure 11: Sequential images of *Tabanus minuscularius*, starting from top left clockwise, habitus, venter, facial, dorsal abdomen and lateral views.

Tabanus atrimanus Loew (1858)

The species was identified based on characteristics described by Oldroyd (1954). The average body length of the species is 10-13 mm. The eyes are microscopically black with a very faint purple band that is clearly seen in life and slowly fade in preserved specimens (Figure 12). The frons with white tomentum and large brown patch on each side of upper callus. Calli had a light brown to a strong brown colour. The subcallus, face and parafacials are snow white with bright white hairs. Palpi white with mostly white hairs and few fine black hairs towards the apex. Proboscis with a shining black colour. Antennae whitish in the first two segments with a mixture of small white and black hairs, the rest of the antennae is light black. Thorax. Mesonontum black and brown with a reddish colour on the sides especially along the lines of sublateral stripes. Abdomen is a black and brown colour, with a white coloured pattern built up of median spots. Wings are clear with yellowish stigma.

Figure 12: Sequential images of *Tabanus atrimanus* from top left going clockwise habitus, venter, facial and lateral views.

Chrysops obliquefasciata Macquart (1838)

The species was identified based on characteristics described by Oldroyd (1957). *Chrysops obliquefasciata* is easily recognised by its overall black appearance. The average body length of the species is 8-10 mm, uncharacteristically small for a tabanid in Africa (Figure 7). The thorax has two unique yellow sublateral spots (Figure 7). The eyes are black and microscopically hairy. Frons are black with ash-grey tomentum. Thorax. Mesonontum and scutellum black, shining through thin black and strong yellow tomentum. Abdomen is predominantly black with yellow hairs and small black hairs. The wings are patterned with a black colour with two yellow spots across the stigma

Figure 13: Sequential images of *Chrysops obliquefasciata* from top left clockwise; habitus, frontal facial, lateral facial and lateral views.

Philoliche (Buplex) suavis Loew (1858)

The species was identified based on characteristics described by Oldroyd (1957). The species is usually recognised with its bee-like appearance with quite a large and wide abdomen (Figure 14). The eyes are black and lacks bands. The average body length of this species is 15-16 mm. Palpi are light brown to a partly yellow with whitish hairs. Proboscis is long and a shining black colour.

Thorax: mesonotum is black with bold yellow hairs. Abdomen is with a mixture of black and strong yellow colour built up by yellow hairs. Wings have yellow veins with membranes stained yellowish with a unique black colour on the hyaline apex only for *Philoliche (Buplex)* species, vein R4 specifically has a very long appendix (Figure 14) top left.

Figure 14 Sequential images of *Philoliche (Buplex) suavis* from top left going clockwise habitus, facial, venter ad lateral views

3.3 Phylogeny of horseflies from south-eastern Kruger National Park South Africa

Phylogeny of southern African tabanid flies using CO1 gene sequences had a high identity score when compared with species from the NCBI database (Table 6). The first sequence matrix resulted in 91 ingroup CO1 sequences with a single outgroup sequence comprising 656 nucleotides. A GTR+G+I model was estimated by jModeltest using both AIC and BIC criteria. The matrix resulted in similar topologies across all analyses. The second, Tabanini specific, dataset had dimensions of 62 taxa with a length of 611 which managed to resolve some lineages that was not resolved by the first. Details of all specimens can be found in the (Appendix I).

Table 6: Table with species support over different analyses (DDN bs: bootstrap support from data-display network, ML bs: bootstrap support from Maximum Likelihood analysis, BI pp: posterior probabilities from Bayesian inference, Tabanini DDN bs: bootstrap support form DDN using the Tabanini dataset)

Species	DDN bs	ML bs	BI pp	Tabanini DDN bs
P. (B.) sauvis	99	100	1	N\A
C. obliquefasciatus	87	94	0.95	N\A
H. daveyi	100	99	1	100
T. atrimanus	N/A	99	1	100
T. taeniola	N\A	99	0.99	100
T. gratus	83	62	0.99	93
T. par	74	100	1	100
T. minuscularius	N\A	98	1	100
H. decora	N\A	99	1	100
T. sericiventris	N\A	99	1	100
A. agrestis	N\A	100	1	N\A
T. chevaleiri	N\A	99	1	100

Haematopini, Philolichini and Chrysopsini where well supported across all methods. Within Philolichini, *Buplex* formed a well-supported group (Figure 9). Tabaninae formed a well-supported outgroup in the DDN (BS: 87) (Figure 15) and formed an unsupported monophyletic group in both the ML ab BI analyses (Figure 16, 17). Species generally clustered into monophyletic groups across all analyses, except for various *Tabanus* species which formed an unresolved *Tabanus* cluster.

P. (B.) sauvis, A. agrestis, T. taeniola, T. gratus and *T. minuscularius,* was supported across all analyses where reference sequences were available from the COI sequence analysis (Table 6, Figures 15-18).

Well-supported monophyletic clusters across all methods without reference sequences were formed for *C. obliquefasciatus, H. decora, H. daveyi* and *T. atrimanus* (Figures 15-18). *T. par* grouped with reference species

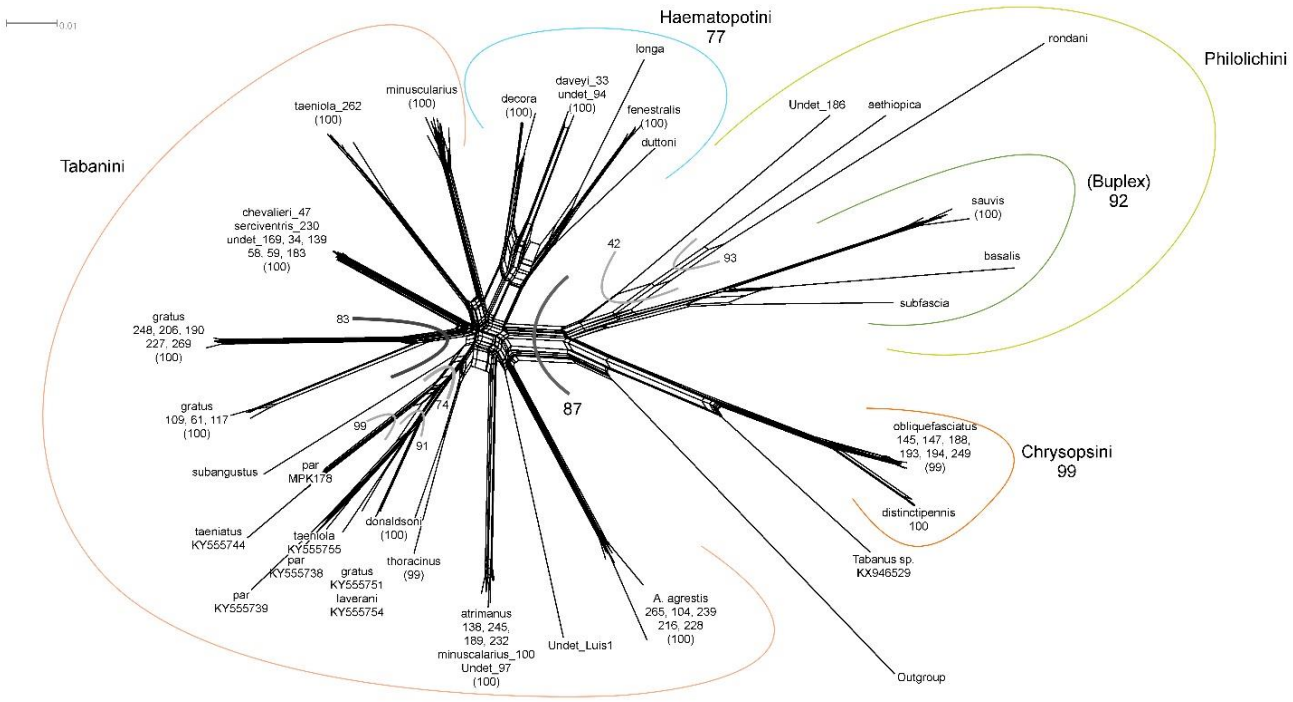


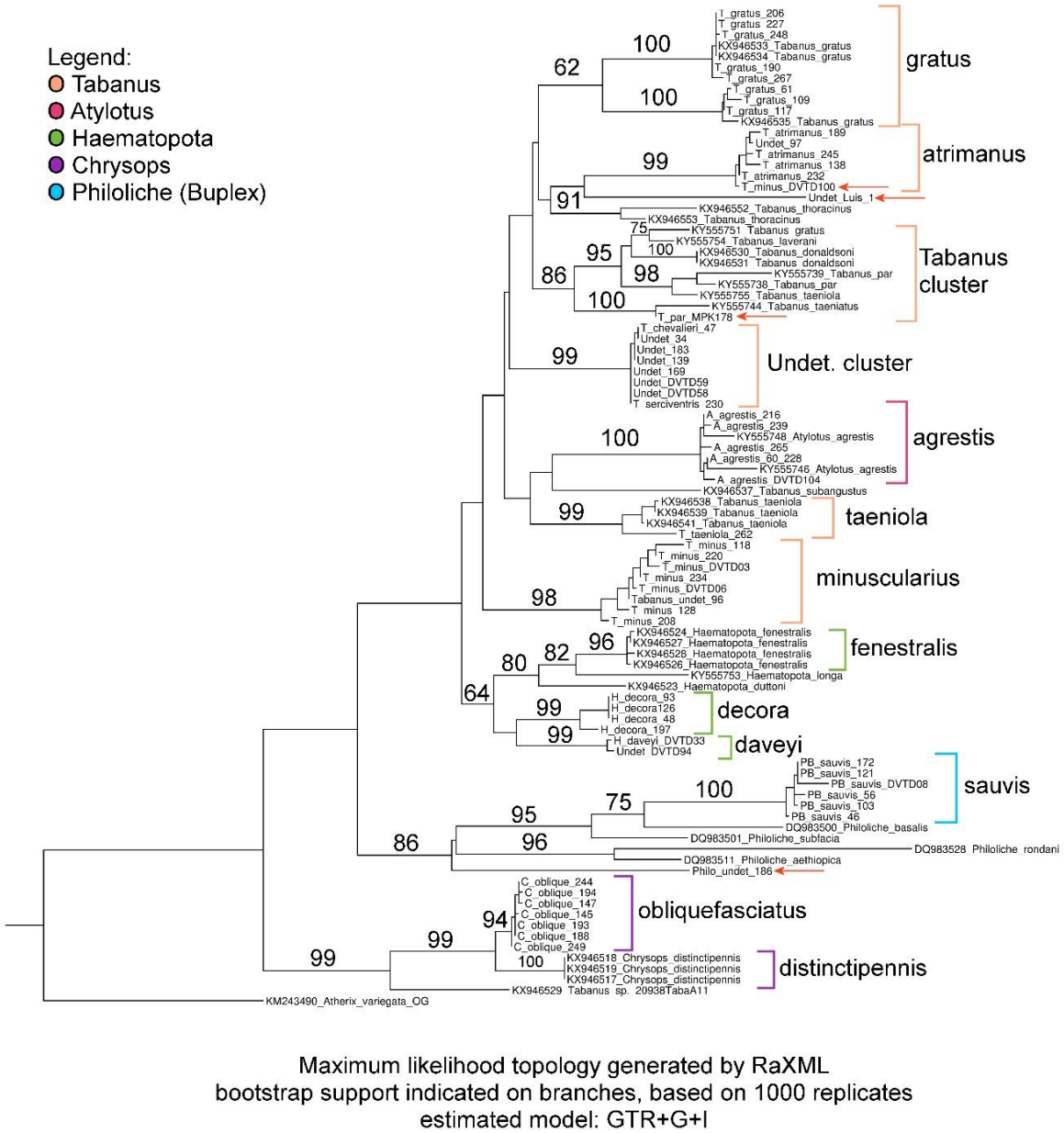
Figure 15: Data display network generated by Splitstree on the full dataset, all tribes are indicated by coloured brazier curves. Bootstrap branch support indicated and calculated from 1000 replicates.

Tabanini was analysed separately (Figure 18). Within the grouping, a well separated *T. taeniatus* sequence was recovered. Several unidentified specimens grouped well with two specimens identified as *T. chevaleiri* and *T. sericiventris* respectively (Figures 15-18).

T. gratus formed a well-supported sister grouping within the species, each clade included specimens from this study as well as reference sequences from GenBank (Figures 15-18).

Two specimens, a *Philoliche* sp. (Undet_186) and a *Tabanus* sp. (Undet_Luis_1), could not be identified morphologically and since reference sequences for the species were lacking, it could also not be identified molecularly (Figures 15-18).

A *T. minuscularius* erroneously identified by morphology, grouped with *T. artrimanus*. An unresolved but well supported Tabanus cluster contained several reference sequences from downloaded from GenBank (Figures 15-18).



Maximum likelihood topology generated by RaXML
bootstrap support indicated on branches, based on 1000 replicates
estimated model: GTR+G+I

Figure 16: Maximum likelihood topology generated from RAxML analysis with bootstrap support calculated using the autoMRE function is indicated on the branches. GTR+I+G was employed as evolutionary model as estimated by jModelTest. Red arrows indicate specimens that could not confidently be identified by CO1.

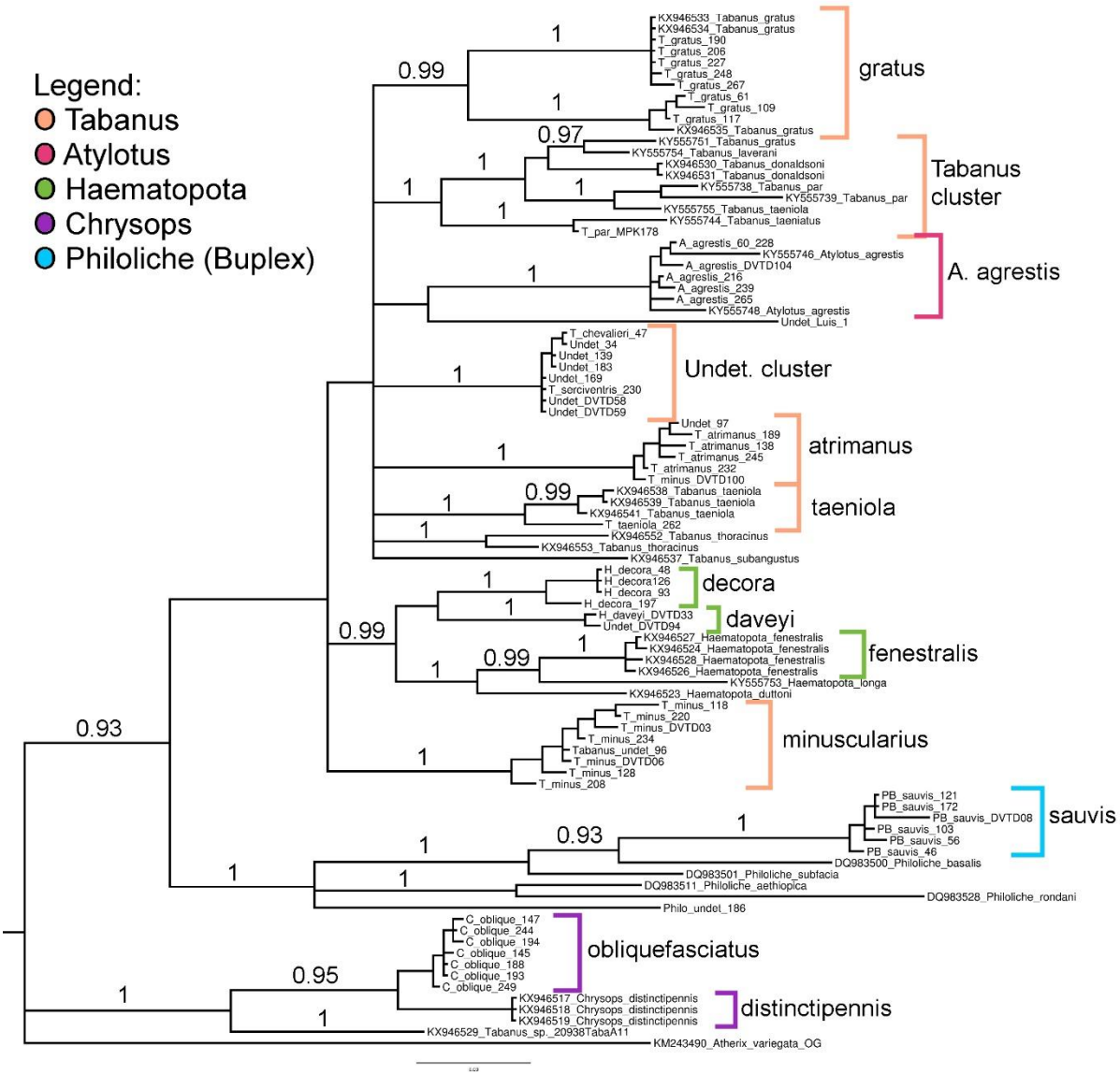


Figure 17: Topology generated by MrBayes with posterior probabilities indicated on the branches (four MCMC, 10 000 000 iterations, every 500th tree saved, 15% burn-in, GTR+G+I model, ESS>>200):

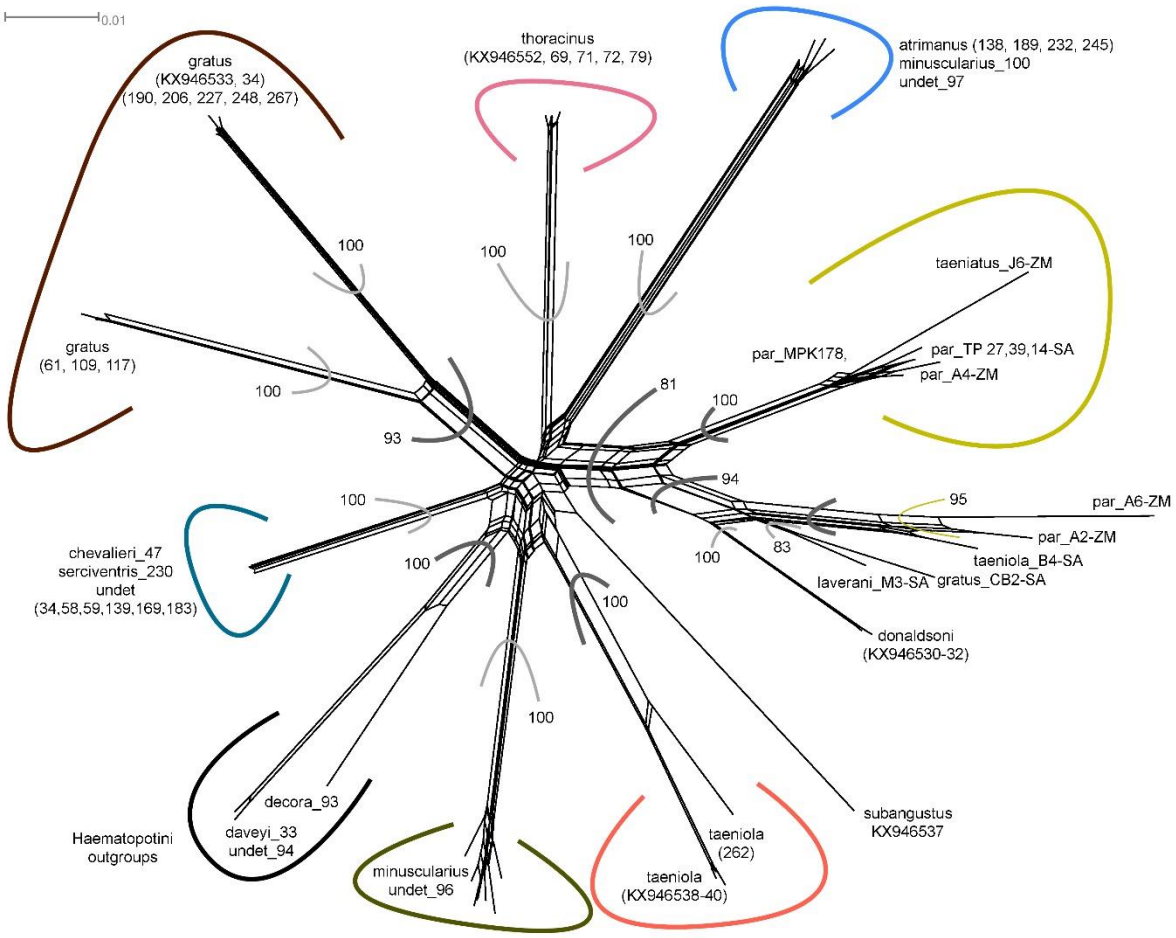


Figure 18: Data display network generated using Splitstree showing the different groupings of Tabanini species

3.4 Molecular detection of pathogens from sampled flies

3.4.1 Molecular detection *Anaplasma* species

A 60% proportion of the well represented species of tabanids in our collection was calculated using the formula n = (1 – (1 – P1)1/d) (N – d/2) + 1, to determine the presence of disease. Out of 60 samples tested for *Anaplasma marginale* and *A. centrale*, none tested positive for either of the species (Figure 19).

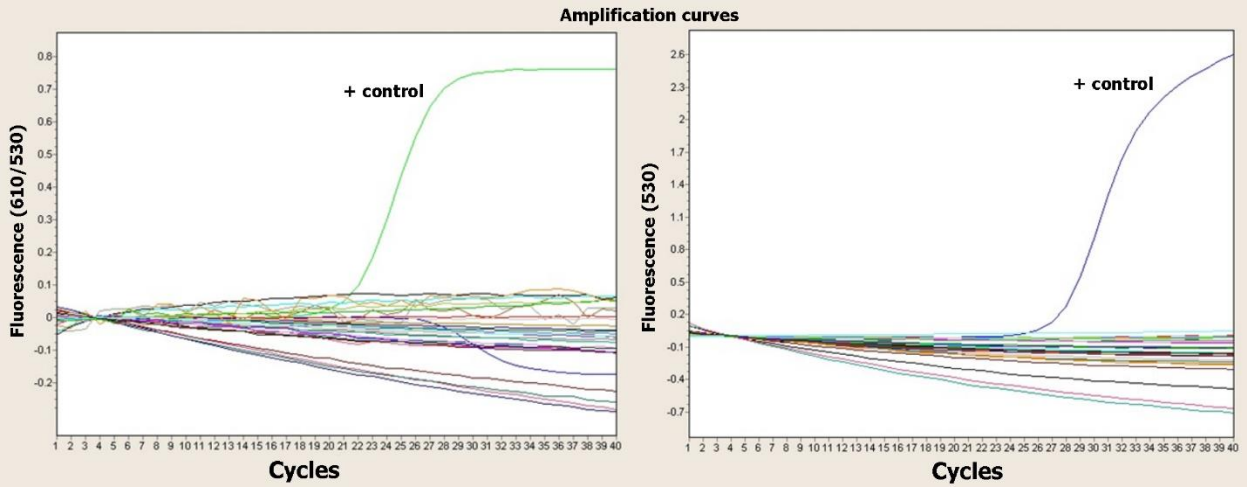


Figure 19: Amplification curve showing a Duplex PCR assay of *Anaplasma marginale* (left) and *A. centrale* (right)

3.4.2 Molecular detection of *Babesia bigemina*

A 60 % proportion of the well represented species of tabanids in our collection was calculated using the formula n = (1 – (1 – P1)1/d) (N – d/2) + 1, to determine the presence of disease out of the 60 samples that were chosen based on the proportions of the well represented species in the study. All samples tested negative for *Babesia bigemina* (Figure 20)

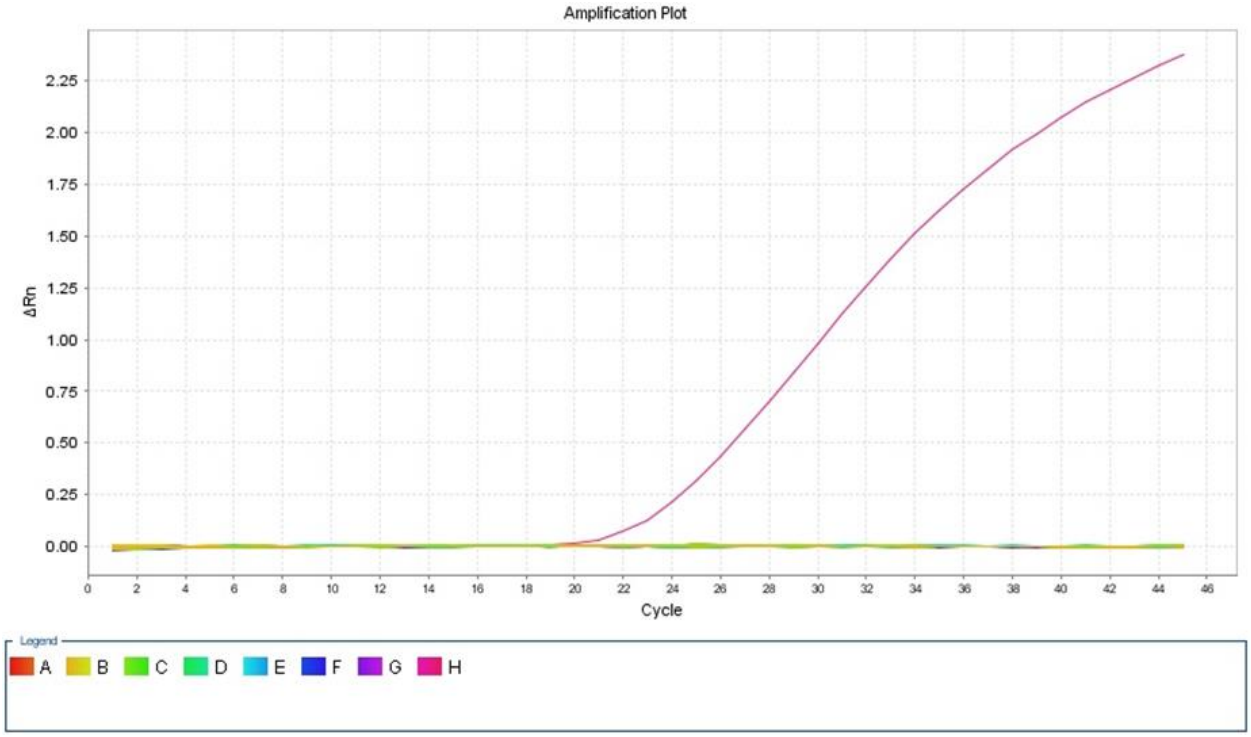


Figure 20: Amplification plot showing qPCR of *Babesia bigemina*

3.4.3 Molecular detection of Trypanosoma parasites

A 60 % proportion of the well represented species of tabanids in our collection was calculated using the formula n = (1 – (1 – P1)1/d) (N – d/2) + 1, to determine the presence of disease. Out of the 60 samples chosen on proportions of the well represented species in the study. Only one sample from collected tabanids tested positive for *Trypanosoma theileri* (1/60) 1.6 % (Figure 21) Detection of *Trypanosoma theleiri* was regarded as positive when amplicon size between (600 and 700bp) were obtained. Sample 109 (*Tabanus gratus*) shows tabanid positive for *Trypanosoma theileri*. Sample 228 (*Atylotus agrestis*) show tabanid negative for *Trypanosoma theileri*

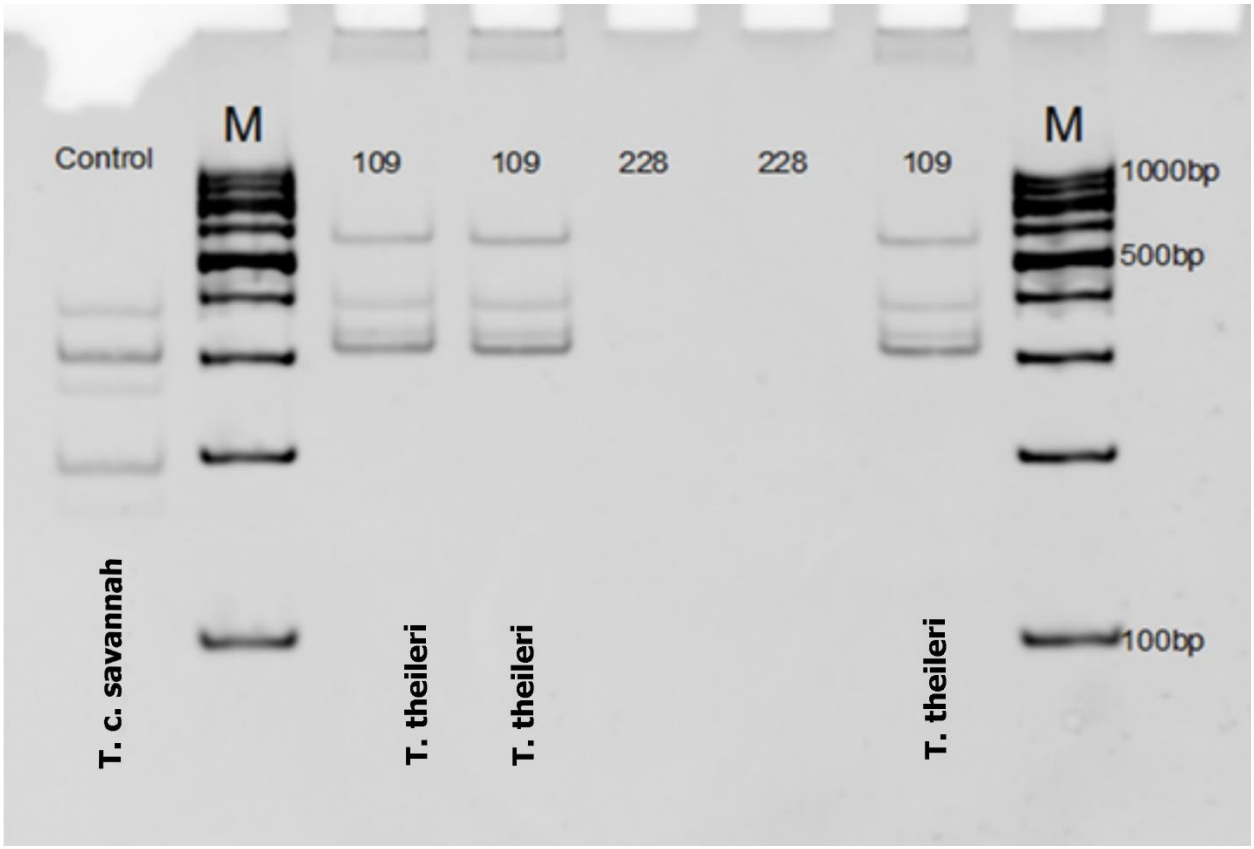


Figure 21: Gel image representing PCR amplification of *Trypanosoma* parasite DNA. Sample 109, an extraction from a *Tabanus gratus* specimen, conforms to *T. theileri*.

Chapter 4

4.1 Discussion

The lowveld biome of South Africa is home to at least 29 species of Tabanidae of which approximately half belong to Tabanini (Usher 1972). This corresponds with studies on horsefly abundance and distribution worldwide as the genus Tabanus alone has approximately 1300 species from the 4400 reported worldwide (Baldacchino *et al.* 2014a; Taioe *et al.* 2017). In this study, the overall number of members from the genus *Tabanus* was greater than all other genera combined with multiple species identified belonging to several species groups which include, the sufis-group in which the upper and lower calli are separated but not reduced or circular as is found in in *Atylotus*, (1) the insignis-group which is moderately sized (ranging from 10 mm to 17 mm) and is composed of black and white species with a bold thoracic and abdominal pattern, (2) the plain group with no patterns on the thorax, abdomen and wings. Lastly (3) the patterned group which are composed of small to moderately sized species with segmented patterns on the abdomen whereas some have no patterns but indefinite markings towards the tip of the abdomen (Oldroyd 1954).

The relatively rich diversity of tabanids in the Lowveld region as described by Usher (1972) is emulated by the results of this study. Unfortunately, few similar studies are available for adequate comparison, with Esterhuizen (2006) and Taioe *et al.* (2017) being the only authors that conducted studies on tabanids in the lowveld, albeit it be in the northern regions of Kwa-Zulu Natal. In the current study the most common species collected in south-eastern Kruger National Park which is a region nearing the border of Mozambique was *Tabanus minuscularius*. This agrees with reports from Usher (1972) whom described the species as a common species in open savannah present in eastern RSA extending into Mozambique. Esterhuizen (2006) recorded only two species from the genus *Chrysops* from four years of sampling, similar to the one *Chrysops* species recorded in this study. The review by Usher (1972) indicated a relatively low diversity of *Chrysops* in southern Africa with only *C. obliqufasciatus* (as *C. obliquefasciata*) in the KNP. Apart from the uncommonness of the species shown in previous studies, the scarcity of *Chrysops* in this study may also be attributed to the evident shortness of the period of sampling. To add to that, Baldacchino *et al.* (2014a) reported that, a seasonal succession of tabanid species is often observed

and the seasonally high density of tabanids does not often last beyond three months. This is indicative of the importance of studies focusing on seasonal abundance, targeting uncommon species such as the genus *Chrysops* in other under-sampled regions. Apart from the reports that *Chrysops* may be scarce species in southern Africa, they have also been implicated with the mechanical transmission of *Anaplasma marginale* in many areas around the world including Africa (Baldacchino *et al.* 2014a), highlighting the importance of species of this genus.

The research studies conducted on the seasonal abundance of tabanid flies mostly concluded that high numbers of flies are usually observed during the summer months (November-March) or rainy season (Barros 2001; Ahmed *et al.* 2005; Esterhuizen, 2006; Itina *et al.* 2013; Baldacchino *et al.* 2014a). The small sample size observed in the study may therefore be largely attributed to the time of capture (March-April) and can consequently be regarded as after the seasonal peak of tabanid activity. Museum data compiled and reported by Snyman *et al.* (unpublished) indicates an activity peak during November and December, or early to mid- summer with only a slight second peak before winter (Figure 22).

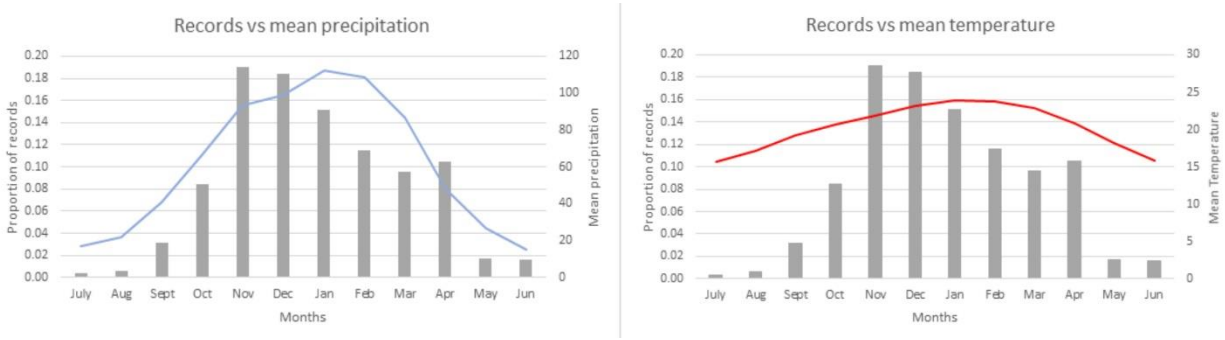


Figure 22: A clustered column and line chart showing proportion of museum records of tabanids in lowveld savannah versus mean precipitation (left) and mean temperature (right) across a year period.

The Kruger Park provides a different and wide range of habitats which in turn form the basis for the distribution of most animal populations including tabanids (Venter & Gertenbach 1986). The flora of the KNP is known to consist of over 1968 plant species. The botanical diversity of the Kruger National Park is not only manifested with a large number of species, but also in a large variety of structural features ranging from dense forests to open, shrubby grasslands (Venter & Gertenbach 1986). The Lowveld are generally open savanna characterised by marula (*Sclerocarya birrea*), knob thorn (*Senegalia nigrescens*), leadwood (*Combretum imberbe*) and

sicklebush (*Dichrostachys cinerea*). Furthermore, the Lowveld provides adequate ecological factors for tabanid occurrence. These characteristics might also have an effect on the abundance and composition of tabanid flies as it was noted by (Usher 1972). In the current study, the Sabie drainage site (Figure 9) saw the greatest number of fly catches as compared to other sites. The possible reason for this may be that collections of tabanid flies from this specific site provide favourable vegetative or environmental characteristics for the occurrence of tabanids which seasonal water, open savannah, ample flowering plants and wet muddy areas (Table 2). This might have lured more tabanids to the area.

Overall the H trap slightly outcompeted the Manitoba trap as the most efficient, with the Ngu trap catching the least. This is despite it being refined for capturing *G. brevipalpis* and *G. austeni* (Kappmeier & Nevill 1999). The Manitoba trap had very similar number of catches and is the only specialised tabanid trap used in this study. The result of it also following close with the catches corresponds with the report as to why it is regarded as one of the efficient horsefly traps to date (Thorsteinson 1958; Lempereur *et al.* 2018). Interestingly, unpublished data from earlier in the season in similar vegetation pointed to the Ngu-trap as the most effective trap which might indicate seasonal effects on the efficacy of traps.

CO1 has shown promise as a molecular barcode for the identification of Tabanidae (Simon *et al.* 1994). However, only a low number of studies on the performance of COI as a barcode has been published are therefore still worth investigating (Barnerjee *et al.* 2015). All morphologically identified species from the genus *Haematopota, Philoliche* and *Chrysops* were well supported across all analyses indicating good performance of the marker (Mugasa *et al.* 2015). However, a low diversity of species sequences from these genera can result in phylograms with strong support, but as the number of species increase, the performance of the marker can decrease (Cywinska et al 2010). Interestingly, *Tabanus gratus* formed two well supported clusters, with both groups representing specimens from this study and previous studies (Taioe *et al.* 2017). This point to well separated sympatric populations and the status of *T. gratus* as a single species should be investigated using more specimens, additional gene regions and/or other methodologies (Banerjee *et al.* 2015). Other species like *Tabanus par, Tabanus chevaleiri* and *Tabanus minuscularius* (Figure 11) clustered with other Tabanus species. For instance, *T. par*_10 grouped together with *T. donaldsonii* (KX4569895) and both species share similar physical characteristics which is the yellow coloured body appearance as described by (Oldroyd 1954). This might be due to

misidentification of species from both Genbank and in our study. As the use of variable physical characters of flies like body appearance, wing colour and eyes as described by (Oldroyd 1954) can be misleading especially in closely related species. Another possible reason is the evident limited reference of COI barcodes of tabanid species available on GenBank (Banerjee et al 2015; Taioe *et al.* 2017). *Tabanus chevaleiri* and *Tabanus sericiventris* clustered together with several unidentified species which might indicate that the morphological separation of *Tabanus chevaleiri* and *Tabanus sericiventris* is difficult and erroneous identifications were made in this study. However, with the well supported cluster, the species is probably a single species that could not be identified morphologically. Cywinska *et al.* (2010), stated that low fly activity leading to small sample size of tabanids capture may result in underestimations of variability amongst the different tabanid species, which may also lead to over estimations of diversity among species. Therefore, the use of CO1 as a barcode for comparing all known species of horseflies needs to be evaluated in South Africa. This will provide the possibility of a classification tool for unknown tabanid species. All of this will be made much easier once there is a tabanid reference library constructed. This library should include reference barcodes, including high-resolution photographs of relevant characteristics for all known horsefly species and their known morphological differences (diagnostic description) from wide sampled areas in compared to just a small region in the Kruger Park. Furthermore, molecular data on other molecular markers e.g. ITS 2 and CAD (Moulton & Wiegmann 2004) combined with morphological characters may be useful in providing a much clearer understanding of the phylogenetic relationships of Tabanidae species from the region.

The absence of *Babesia* and *Anaplasma* does not suggests that tabanids are not mechanical vectors of these parasites (Foil 1989; Baldacchino *et al.* 2014a). The occurrence of these parasites is well documented in southern Africa (Taioe *et al.* 2017). There are many factors often involved with the mechanical transmission of these parasites by tabanids (Nevill *et al.* 1994). Firstly, for the mechanical transmission of pathogen, the titre of pathogen bodies in the host should be high enough to contaminate the mouthparts of the vector when feeding. Secondly, the physical characteristics of the vector, such as the size of the mouthparts may play an important role. It has been shown that larger mouthparts are more effective in mechanical transmission of parasites and that mouthpart size differs between species (Sumba *et al.* 1998; Baldacchino *et al.* 2013; Taioe *et al.* 2017). The other is the abundance of susceptible hosts (which was not observed in our study) which results in interrupted feeding of tabanids thus more target host for disease transmission. This

may have an impact in making horse-flies possible vectors for these two pathogens that are known to be tick-borne (Azmi *et al.* 2016). The time of year, vector density, species of vectors, density of hosts and titre of pathogens are thus all factors that might have resulted in the absence of *Anaplasma* and *Babesia*. Apart from the above-mentioned factors encountered in the field, there are other factors which should be looked at in future when studying tabanids as vectors of disease transmission. The survival time of the pathogen outside the host seems to play a major role as parasite survival on the mouthparts is known to be shorter (24 h) compared to the inside of the gut (5–7 d) (Foil 1989). This would allow delayed transmission as the flies can regurgitate infected blood into the host (Baldacchino *et al.* 2013; Taioe *et al.* 2017). It has to be mentioned that these are preliminary results, and this calls for further studies that look at other regions in the Kruger National Park, factors like highest and lowest tick activity (Howell *et al.* 1941) which might limit the efficiency of tabanid flies as mechanical vectors. This might suggest a possible re-evaluation of current vector control strategies as to whether tabanid flies should be monitored and included in arthropod vector control approaches.

It is well documented that tabanids are vectors of many pathogens including viruses, bacteria and protozoan parasites (Table 1). However, the efficacy of transmission is often put to question (Foil 1989). In the current study only one protozoan parasite has been detected by PCR. The well-known parasite, *Trypanosoma theileri*, is described as a widespread species of low pathogenicity in specifically cattle and wild animals throughout the world (Foil 1989). This parasite develops within the midgut and hindgut of tabanid flies (Krinsky & Pechuman, 1975; Foil 1989). Previous studies have shown several species of tabanids infected with *T. theileri* or *T. theileri*-like species (Krinsky & Pechuman 1975). In South Africa this is the first report of *Trypanosoma theileri* detected in *Tabanus gratus* from south-eastern Kruger National Park elevating the status of *T. gratus* to a possible vector of *Trypanasoma* and possible biological host to *T. theileri*. Other tabanid species reported to have *T. theleiri* include *Tabanus par* and *Tabanus taeniola* as reported by Taioe *et al.* (2017). The limited knowledge present in southern Africa for tabanid species harbouring *T. theileri*, suggests that further confirmation of *Trypanosoma theileri* detection is still required to elucidate the vector-pathogen relationship in this cycle.

Previous studies on *Trypanasoma* conducted in South Africa focused on either tsetse flies as vectors or susceptible hosts (Van den Bossche 2001; Mamabolo *et al.* 2009; Gillingwater *et al.* 2010; Mwandiringana *et al.* 2012; Motloang *et al.* 2014). Moreover, in all studies conducted, the

infections are mostly associated with tsetse flies as vectors. Neglecting all the other possible mechanical vectors like tabanids. Tabanids are thus deprived of attention and subsequent financial support. The possibility of prevalence of trypanosome parasites in regions where tsetse flies are absent is evident (Desquesnes *et al.* 2009). This suggests that other haematophagous flies such as tabanid and *Stomoxys* flies might be responsible for the transmission of these pathogens (Desquesnes *et al.* 2013). Lastly, this also suggests the need for further studies and attention on not only tabanids, but other Diptera as mechanical transmitters of trypanosomes.

4.2 Conclusion

Tabanids have evidently fallen short in research studies with a focus on insect vectors (Foil 1989; Baldacchino *et al.* 2014a; Taioe *et al.* 2017). The data generated in this study is useful in distinguishing the different species in comparisons with the voluminous works of (Oldroyd 1952, 1954, 1957) and those from the Genbank database. There are difficulties with other species such as *Tabanus chevaleiri* and *Tabanus sericiventris* which have no barcodes on the database of which has been an ongoing challenge in the identification system of horseflies in South Africa. Hence, more data from other under-sampled areas in other wide geographical regions in South Africa would be of immense importance for evaluating species status, and more comparable results in trapping efficacy and generation of molecular markers for identification of tabanid species. The generation of a DNA barcode library will sure make it easier for taxonomists and biologists to connect variability of genes with taxonomy and management of vectors. Incorporating this with sampling from much wider areas will produce a platform for a much viable system of identification. This is the first report of *Trypanosoma theileri* detection in *Tabanus gratus* by PCR. As this is a preliminary study that was focused on the small south-eastern region of the Kruger National Park, further confirmation of these parasites is still required as well as studies that look at other regions in the Park. The control of vectors such as ticks and *Glossina* spp. reduces the prevalence of tick-borne diseases and trypanosomosis, respectively, but these diseases will not be completely eradicated if tabanid flies are not attended to as mechanical vectors pathogens.

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Appendix I

Table A1: Showing all the Tabanidae specimens to be submitted to GenBank and those downloaded from GenBank for comparisons in the study (data matirx 1)

Subfamily	**Tribe**	**Genus**	**Species**	**Acc. Num.**	**Country of Origin**	**ID status**
NA	NA	*Atherix*	*variegata*	KM243490		NA
Chrysopsinae	Chrysopsini	*Chrysops*	*brucei*	KX946516	Uganda	NA
Chrysopsinae	Chrysopsini	*Chrysops*	*distinctipennis*	KX946517	Uganda	NA
Chrysopsinae	Chrysopsini	*Chrysops*	*distinctipennis*	KX946518	Uganda	NA
Chrysopsinae	Chrysopsini	*Chrysops*	*distinctipennis*	KX946519	Uganda	NA
Chrysopsinae	Chrysopsini	*Chrysops*	*obliquefasciatus*	*DVTD268	RSA	Confirmed
Chrysopsinae	Chrysopsini	*Chrysops*	*obliquefasciatus*	*DVTD145	RSA	Confirmed
Chrysopsinae	Chrysopsini	*Chrysops*	*obliquefasciatus*	*DVTD147	RSA	Confirmed
Chrysopsinae	Chrysopsini	*Chrysops*	*obliquefasciatus*	*DVTD188	RSA	Confirmed
Chrysopsinae	Chrysopsini	*Chrysops*	*obliquefasciatus*	*DVTD193	RSA	Confirmed
Chrysopsinae	Chrysopsini	*Chrysops*	*obliquefasciatus*	*DVTD244	RSA	Confirmed
Chrysopsinae	Chrysopsini	*Chrysops*	*obliquefasciatus*	*DVTD194	RSA	Confirmed
Chrysopsinae	Chrysopsini	*Chrysops*	*obliquefasciatus*	*DVTD249	RSA	Confirmed
?	?	*Tabanus*	sp. (20938TabaA11)	KX946529	Uganda	Grouped with *Chrysops*
Tabaninae	Tabanini	*Atylotus*	*agrestis*	*DVTD104	RSA	Confirmed
Tabaninae	Tabanini	*Atylotus*	*agrestis*	KM111665	India	NA
Tabaninae	Tabanini	*Atylotus*	*agrestis*	KY555746	Zambia	NA

Tabaninae	Tabanini	*Atylotus*	*agrestis*	KY555748	RSA	NA
Tabaninae	Tabanini	*Atylotus*	*agrestis*	MG587915	Bangladesh	NA
Tabaninae	Tabanini	*Atylotus*	*agrestis*	*DVTD216	RSA	Confirmed
Tabaninae	Tabanini	*Atylotus*	*agrestis*	*DVTD239	RSA	Confirmed
Tabaninae	Tabanini	*Atylotus*	*agrestis*	*DVTD265	RSA	Confirmed
Tabaninae	Tabanini	*Atylotus*	*agrestis*	*DVTD60_228	RSA	Confirmed
Tabaninae	Haematopotini	*Haematopota*	*daveyi*	*DVTD33	RSA	Confirmed
Tabaninae	Haematopotini	*Haematopota*	*undet*	*DVTD94	RSA	*H. daveyi*
Tabaninae	Haematopotini	*Haematopota*	*decora*	*DVTD197	RSA	Confirmed
Tabaninae	Haematopotini	*Haematopota*	*decora*	*DVTD48	RSA	Confirmed
Tabaninae	Haematopotini	*Haematopota*	*decora*	*DVTD126	RSA	Confirmed
Tabaninae	Haematopotini	*Haematopota*	*decora*	*DVTD93	RSA	Confirmed
Tabaninae	Haematopotini	*Haematopota*	*duttoni*	KX946523	Kenya	NA
Tabaninae	Haematopotini	*Haematopota*	*fenestralis*	KX946524	Kenya	NA
Tabaninae	Haematopotini	*Haematopota*	*fenestralis*	KX946526	Kenya	NA
Tabaninae	Haematopotini	*Haematopota*	*fenestralis*	KX946527	Kenya	NA
Tabaninae	Haematopotini	*Haematopota*	*fenestralis*	KX946528	Kenya	NA
Tabaninae	Haematopotini	*Haematopota*	*longa*	KY555753	Zambia	NA
Pangoniinae	Philolichini	*Philoliche (Buplex)*	*basalis*	DQ983500	Unknown	NA
Pangoniinae	Philolichini	*Philoliche (Buplex)*	*subfascia*	DQ983501	Unknown	NA
Pangoniinae	Philolichini	*Philoliche (Buplex)*	*sauvis*	*DVTD08	RSA	Confirmed
Pangoniinae	Philolichini	*Philoliche (Buplex)*	*sauvis*	*DVTD23	RSA	Confirmed

Pangoniinae	Philolichini	*Philoliche (Buplex)*	*sauvis*	*DVTD103	RSA	Confirmed
Pangoniinae	Philolichini	*Philoliche (Buplex)*	*sauvis*	*DVTD121	RSA	Confirmed
Pangoniinae	Philolichini	*Philoliche (Buplex)*	*sauvis*	*DVTD172	RSA	Confirmed
Pangoniinae	Philolichini	*Philoliche (Buplex)*	*sauvis*	*DVTD46	RSA	Confirmed
Pangoniinae	Philolichini	*Philoliche (Buplex)*	*sauvis*	*DVTD56	RSA	Confirmed
Pangoniinae	Philolichini	*Philoliche*	*undet*	*DVTD186	RSA	Unconfirmed
Pangoniinae	Philolichini	*P. (Philoliche)*	*aethiopica*	DQ983511	Afrotropical	NA
Pangoniinae	Philolichini	*P. (Philoliche)*	*rondani*	DQ983525	Afrotropical	NA
Pangoniinae	Philolichini	*P. (Philoliche)*	*rondani*	DQ983528	Afrotropical	NA
Pangoniinae	Philolichini	*P. (Philoliche)*	*aethiopica*	DQ983535	Afrotropical	NA
Tabaninae	Tabanini	*Tabanus*	*atrimanus*	*DVTD138	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*atrimanus*	*DVTD245	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*atrimanus*	*DVTD189	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*atrimanus*	*DVTD232	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*chevalieri*	*DVTD47	RSA	*chevalieri/serciventris*
Tabaninae	Tabanini	*Tabanus*	*donaldsoni*	KX946530	Kenya	NA
Tabaninae	Tabanini	*Tabanus*	*donaldsoni*	KX946531	Kenya	NA
Tabaninae	Tabanini	*Tabanus*	*gratus*	KX946533	Tanzania	NA
Tabaninae	Tabanini	*Tabanus*	*gratus*	KX946534	Tanzania	NA
Tabaninae	Tabanini	*Tabanus*	*gratus*	KX946535	Kenya	NA
Tabaninae	Tabanini	*Tabanus*	*gratus*	KY555751	RSA	NA
Tabaninae	Tabanini	*Tabanus*	*gratus*	*DVTD190	RSA	Confirmed

Tabaninae	Tabanini	*Tabanus*	*gratus*	*DVTD206	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*gratus*	*DVTD227	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*gratus*	*DVTD248	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*gratus*	*DVTD267	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*gratus*	*DVTD117	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*gratus*	*DVTD61	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*gratus*	*DVTD109	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*laverani*	KY555754	RSA	NA
Tabaninae	Tabanini	*Tabanus*	*minuscularius*	*DVTD03	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*minuscularius*	*DVTD06	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*minuscularius*	*DVTD100	RSA	as *T. atrimanus*
Tabaninae	Tabanini	*Tabanus*	*minuscularius*	*DVTD118	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*minuscularius*	*DVTD220	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*minuscularius*	*DVTD234	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*minuscularius*	*DVTD128	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*minuscularius*	*DVTD208	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*par*	KY555737	Zambia	NA
Tabaninae	Tabanini	*Tabanus*	*par*	KY555738	Zambia	NA
Tabaninae	Tabanini	*Tabanus*	*par*	KY555739	Zambia	NA
Tabaninae	Tabanini	*Tabanus*	*par*	*MPK178	RSA	Unconfirmed
Tabaninae	Tabanini	*Tabanus*	*serciventris*	*DVTD230	RSA	Unconfirmed
Tabaninae	Tabanini	*Tabanus*	*subangustus*	KX946537	Kenya	NA

Tabaninae	Tabanini	*Tabanus*	*taeniatus*	KY555744	Zambia	NA
Tabaninae	Tabanini	*Tabanus*	*taeniola*	KX946538	Tanzania	NA
Tabaninae	Tabanini	*Tabanus*	*taeniola*	KX946539	Tanzania	NA
Tabaninae	Tabanini	*Tabanus*	*taeniola*	KX946541	Tanzania	NA
Tabaninae	Tabanini	*Tabanus*	*taeniola*	KY555755	RSA	NA
Tabaninae	Tabanini	*Tabanus*	*taeniola*	*DVTD262	RSA	Confirmed
Tabaninae	Tabanini	*Tabanus*	*thoracinus*	KX946552	Uganda	NA
Tabaninae	Tabanini	*Tabanus*	*thoracinus*	KX946553	Uganda	NA
Tabaninae	Tabanini	*Tabanus*	undet	*DVTD96	RSA	as *T. minuscularius*
Undet	Undet	Undet	undet	*DVTD58	RSA	*chevalieri/serciventris*
Undet	Undet	Undet	undet	*DVTD59	RSA	*chevalieri/serciventris*
Undet	Undet	Undet	undet	*Luis_1	Mozambique	Unconfirmed
Undet	Undet	Undet	undet	*DVTD34	RSA	*chevalieri/serciventris*
Undet	Undet	Undet	undet	*DVTD139	RSA	*chevalieri/serciventris*
Undet	Undet	Undet	undet	*DVTD169	RSA	*chevalieri/serciventris*
Undet	Undet	Undet	undet	*DVTD183	RSA	*chevalieri/serciventris*
Undet	Undet	Undet	undet	*DVTD97	RSA	*as T. atrimanus*

Table A2: Showing Tabanini specimens to be submitted on GenBank and those downloaded from GenBank for comparisons in the study (Tabanini data matrix)

Tribe	Genus	Epithet	Acc. Num.	Country of Origin	ID status
Haematopotini	*Haematopota*	*daveyi*	*DVTD33	RSA	Outgroup
Haematopotini	*Haematopota*	*daveyi*	*DVTD94	RSA	Outgroup
Haematopotini	*Haematopota*	*decora*	*DVTD93	RSA	Outgroup
Tabanini	*Tabanus*	*atrimanus*	*DVTD138	RSA	Confirmed
Tabanini	*Tabanus*	*atrimanus*	*DVTD245	RSA	Confirmed
Tabanini	*Tabanus*	*atrimanus*	*DVTD189	RSA	Confirmed
Tabanini	*Tabanus*	*atrimanus*	*DVTD232	RSA	Confirmed
Tabanini	*Tabanus*	*minus*	*DVTD100	RSA	as *T. atrimanus*
Tabanini	*Tabanus*	undet.	*DVTD97	RSA	as *T. atrimanus*
Tabanini	*Tabanus*	*chevalieri*	*DVTD47	RSA	*chevalieri/serciventris*
Tabanini	*Tabanus*	*serciventris*	*DVTD230	RSA	*chevalieri/serciventris*
Tabanini	*Tabanus*	undet.	*DVTD34	RSA	*chevalieri/serciventris*
Tabanini	*Tabanus*	undet.	*DVTD139	RSA	*chevalieri/serciventris*
Tabanini	*Tabanus*	undet.	*DVTD183	RSA	*chevalieri/serciventris*
Tabanini	*Tabanus*	undet.	*DVTD169	RSA	*chevalieri/serciventris*
Tabanini	*Tabanus*	undet.	*DVTD58	RSA	*chevalieri/serciventris*
Tabanini	*Tabanus*	undet.	*DVTD59	RSA	*chevalieri/serciventris*
Tabanini	*Tabanus*	*donaldsoni*	KX946530	Kenya	Confirmed
Tabanini	*Tabanus*	*donaldsoni*	KX946531	Kenya	Confirmed
Tabanini	*Tabanus*	*donaldsoni*	KX946532	Kenya	Confirmed

Tabanini	*Tabanus*	*gratus*	*DVTD190	RSA	Confirmed (L1)
Tabanini	*Tabanus*	*gratus*	*DVTD206	RSA	Confirmed (L1)
Tabanini	*Tabanus*	*gratus*	*DVTD227	RSA	Confirmed (L1)
Tabanini	*Tabanus*	*gratus*	*DVTD248	RSA	Confirmed (L1)
Tabanini	*Tabanus*	*gratus*	*DVTD267	RSA	Confirmed (L1)
Tabanini	*Tabanus*	*gratus*	*DVTD117	RSA	Confirmed (L2)
Tabanini	*Tabanus*	*gratus*	*DVTD61	RSA	Confirmed (L2)
Tabanini	*Tabanus*	*gratus*	*DVTD109	RSA	Confirmed (L2)
Tabanini	*Tabanus*	*gratus*	KY555751	RSA	Doubtful
Tabanini	*Tabanus*	*gratus*	KX946533	Tanzania	Confirmed (L1)
Tabanini	*Tabanus*	*gratus*	KX946534	Tanzania	Confirmed (L1)
Tabanini	*Tabanus*	*laverani*	KY555754	RSA	Unconfirmed
Tabanini	*Tabanus*	*minuscularius*	*DVTD118	RSA	Confirmed
Tabanini	*Tabanus*	*minuscularius*	*DVTD220	RSA	Confirmed
Tabanini	*Tabanus*	*minuscularius*	*DVTD03	RSA	Confirmed
Tabanini	*Tabanus*	*minuscularius*	*DVTD234	RSA	Confirmed
Tabanini	*Tabanus*	*minuscularius*	*DVTD06	RSA	Confirmed
Tabanini	*Tabanus*	*minuscularius*	*DVTD128	RSA	Confirmed
Tabanini	*Tabanus*	*minuscularius*	*DVTD208	RSA	Confirmed
Tabanini	*Tabanus*	*undet*	*DVTD96	RSA	as *T. minuscularius*
Tabanini	*Tabanus*	*par*	KY555737	Zambia	Confirmed
Tabanini	*Tabanus*	*par*	KY555743	RSA	Confirmed

Tabanini	*Tabanus*	*par*	KY555741	Zambia	Confirmed
Tabanini	*Tabanus*	*par*	*MPK178	RSA	Confirmed
Tabanini	*Tabanus*	*par*	KY555740	RSA	Confirmed
Tabanini	*Tabanus*	*par*	KY555742	RSA	Confirmed
Tabanini	*Tabanus*	*par*	KY555738	Zambia	Doubtful
Tabanini	*Tabanus*	*par*	KY555739	Zambia	Doubtful
Tabanini	*Tabanus*	*subangustus*	KX946537	Kenya	Unconfirmed
Tabanini	*Tabanus*	*taeniatus*	KY555744	Zambia	Unconfirmed
Tabanini	*Tabanus*	*taeniola*	KY555755	RSA	Doubtful
Tabanini	*Tabanus*	*taeniola*	KX946538	Tanzania	Confirmed
Tabanini	*Tabanus*	*taeniola*	KX946539	Tanzania	Confirmed
Tabanini	*Tabanus*	*taeniola*	KX946540	Kenya	Confirmed
Tabanini	*Tabanus*	*taeniola*	*DVTD262	RSA	Confirmed
Tabanini	*Tabanus*	*thoracinus*	KX946552	Uganda	Confirmed
Tabanini	*Tabanus*	*thoracinus*	KX946569	Uganda	Confirmed
Tabanini	*Tabanus*	*thoracinus*	KX946579	Uganda	Confirmed
Tabanini	*Tabanus*	*thoracinus*	KX946571	Uganda	Confirmed
Tabanini	*Tabanus*	*thoracinus*	KX946572	Uganda	Confirmed

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

Ref: V114-17

30 November 2017

Prof. L Neves
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
(eric.etter@cirad.fr)

Dear Prof Neves

Project V114-17
Investigating the occurrence of haematophagous flies and their potential role in haemoparasite transmission in the Kruger National Park (South Africa) (X Mazibuko)

The committee has evaluated your project. No ethical approval is required since the study does not involve vertebrates or higher invertebrates.

Yours sincerely