Development of a toolkit for African horse sickness: identification of *Culicoides* vectors from Namibia and detection of African horse sickness virus

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"Wisdom is better than jewels" **Proverbs 8:11**

Preface

The research presented in this dissertation was conducted in the Unit of Environmental Sciences and Management, North-West University, Potchefstroom Campus, Potchefstroom, South Africa.

I hereby declare that this dissertation submitted, represents original work and has not previously been submitted for a degree at any other university. Where use was made of the work of other researchers, it was duly acknowledged in the text. The North-West University Harvard Referencing Guide was used as the referencing style in this dissertation.

Any opinion, findings and conclusions or recommendations expressed in this material are those of the author and therefore the National Research Foundation does not accept any liability in regard there to.

Summary

African horse sickness (AHS) is a non-contagious, viral, insect-borne disease of equids and this disease is caused by the African horse sickness virus (AHSV). The virus is part of the family Reoviridae of the genus *Orbivirus*. The virus has nine distinct serotypes. AHSV affects horses, mules, donkeys and zebras, resulting in severe animal health and welfare problems together with serious economic consequences. Main vectors of orbiviruses are haematophagous arthropods such as *Culicoides* Latreille midges, ticks, sand flies and mosquitoes. Female *Culicoides* biting midges are the primary vectors of AHSV. *Culicoides* midges (*C. imicola* Kieffer and *C. bolitinos* Meiswinkel) play a role in the abundance, prevalence and seasonal incidence of AHSV outbreaks.

The aim of this study was to establish DNA barcodes for *Culicoides* species collected in Namibia and to develop a simplified nucleic acid diagnostic toolkit for the detection of AHSV. The first objectives of the study were to extract DNA from morphologically identified *Culicoides* specimens, sequence the mitochondrial cytochrome oxidase subunit I gene for DNA barcoding and align amplicons with sequences from databases for phylogenetic identification. A phylogenetic tree of *Culicoides* species was drawn and 11 new sequences of morphologically identified species were obtained along with five previously sequenced species.

The second objective was to design specific primers for loop-mediated isothermal amplification (LAMP) assays of AHSV, optimise the reverse-transcription (RT)-LAMP method for AHSV detection and evaluate the assay with artificially infected *Culicoides* samples. Specific primers of the virus-protein-4 region of AHSV were designed for RT-LAMP assays. The RT-LAMP standard test was successful, with multiple band formation on an agarose gel indicating a positive result. The RT-LAMP method was optimised with virus RNA and evaluated through assays with artificially infected specimens to test sensitivity, but the primer set proved not to be sensitive enough. However, an RT-LAMP method can be used for AHSV detection in the near future, with additional research and better designed primers from different regions of the genome. A diagnostic toolkit will be helpful for the early detection of AHSV and will help manage and control epidemic outbreaks of AHS.

Keywords: African horse sickness, *Culicoides*, vectors, DNA barcodes, phylogenetic identification, RT-LAMP

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Acronyms and abbreviations

AHS: African horse sickness

AHSV: African horse sickness virus

ARC-OVI: Agriculture Research Council-Onderstepoort Veterinary Institute

B3: Backwards outer primer

BIP: Backwards inner primer

BLAST: Basic Local Alignment Search Tool

BLP: Backwards loop primer

BOLD: Barcode of Life Data system

bp: Base pair

BTV: Bluetongue virus

dH_2O: Distilled water

DNA: Deoxyribonucleic acid

EIP: Extrinsic incubation period

FIP: Forward inner primer

FLP: Forward loop primer

F3: Forward outer primer

LAMP: Loop-mediated isothermal amplification

MEGA: Molecular Evolutionary Genetics Analysis

MT-COI: Mitochondrial cytochrome oxidase subunit I

NCBI: National Center for Biotechnology Information

OIE: World Organisation for Animal Health

PCR: Polymerase chain reaction

qPCR: Real time polymerase chain reaction

RNA: Ribonucleic acid

RT-PCR: Reverse-transcription polymerase chain reaction

RT-qPCR: Real-time reverse-transcription polymerase chain reaction

RT-LAMP: Reverse-transcription loop-mediated isothermal amplification

TAE: Tris-acetate-EDTA

$TCID_{50}$: Tissue culture infectious dose

Tm: Melting temperature

UK: United Kingdom

USA: United States of America

UV: Ultraviolet

VP: Viral protein

VN: Virus neutralisation

w/v: Weight/volume ratio

CHAPTER 1: INTRODUCTION

1.1 African horse sickness

African horse sickness (AHS) is a non-contagious, infectious, insect-borne disease of equids (Boinas *et al*., 2009; Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004; Venter *et al*., 2000; Venter *et al*., 2010). It is a disease caused by a virus from the genus *Orbivirus* in the family Reoviridae (Wilson *et al*., 2008). African horse sickness virus (AHSV) is transmitted by adult female *Culicoides* biting midges (Diptera: Ceratopogonidae). The virus has nine antigenically distinct serotypes (Howell, 1962; Mellor & Hamblin, 2004). AHSV has similar morphological characteristics to other members of the *Orbivirus* genus, such as equine encephalosis virus, bluetongue virus (BTV), epizootic haemorrhagic disease.

1.1.1. AHS history

The earliest reference to AHS disease was in 1327 in Yemen (Moule, 1896). This disease is endemic to sub-Saharan Africa (Boinas *et al*., 2009; Guthrie *et al*., 2013; Mellor, 1993). AHS occasionally spreads northwards, with a few outbreaks outside the continent. Until the late 20th century it was believed that AHS was not able to survive outside of Africa for more than two years (Koekemoer & Van Dijk, 2004). However, Spain, Portugal, Cape Verde Islands and Middle Eastern countries have suffered considerable losses due to AHS (Boinas *et al*., 2009; Mellor, 1993; Mellor & Hamblin, 2004).

The disease was first recognised in southern Africa 60 years after the introduction of horses in 1657 (Mellor & Hamblin, 2004). The first major outbreak of AHS occurred in 1719, when over 1 700 horses died (Theiler, 1921, cited by Verwoerd, 2012; Henning, 1956). The largest outbreak recorded in South Africa was from 1854 to 1855, when over 70 000 horses died (Barnard, 1998; Coetzer & Erasmus, 1994; Venter *et al*., 2010; Bayley, 1856, cited by Verwoerd, 2012). AHSV was also detected in Nigeria, Ghana, Mali and Mauritania in 2007 (Wilson *et al*., 2009)

In 1908, two Namibian *Culicoides* species were described and this was the first research done on sub-Saharan *Culicoides* (Meiswinkel *et al*., 2004b). In 1943, *Culicoides* species were first studied in South Africa by Rene Du Toit and later, in 1951, O.G.H. Fiedler published the first identification key for South African *Culicoides* species which consisted of 22 species. The Imicola group consists of nine sibling

species, with seven out of the nine species occurring in sub-Saharan Africa (Meiswinkel *et al*., 2004a).

Significant research on AHS was conducted by Theiler at Onderstepoort, now known as the Agricultural Research Council – Onderstepoort Veterinary Institute. He focused on the incidence of AHS on the Onderstepoort farm. Later he discovered various serotypes (Howell, 1962) and developed the first effective vaccine against AHS and BTV (Verwoerd, 2012). The first AHSV propagation was in mouse brains by Alexander in 1935 and chicken embryos were used in 1938 (Alexander, 1935, 1938). In 1943, Du Toit identified the role of *Culicoides* species as vectors of the virus (Du Toit, 1944). The World Organisation for Animal Health (OIE) listed the disease as notifiable because of its rapid expansion and severity (Boinas *et al*., 2009; Mellor, 1993; Venter *et al*., 2010; Wilson *et al*., 2008).

1.1.2. Geographical distribution of AHS

The distribution of AHSV is endemic to sub-Saharan Africa (Hamblin *et al*., 1990; Wilson *et al*., 2008), with outbreaks particularly frequent and severe in southern Africa (Baylis *et al*., 1999). The distribution of virus stretches from Senegal to Ethiopia and Somalia and extends as far as South Africa (Mellor & Boorman, 1995). In Spain, the 1987 AHS outbreak was due to the importation of zebras from Namibia to a safari park in Madrid (Cullinane *et al*., 2013).

The natural reservoir of the virus is believed to be zebras, allowing circulation of the virus in areas with large zebra populations all year round (Lord *et al*., 2002). Most adult zebras have specific antibodies to all nine serotypes of the virus (Barnard, 1998). The spread of AHSV is prevented by the Sahara Desert that acts as an effective geographical barrier. Outbreaks outside Africa have occurred since AHSV is also endemic to Yemen (Arabian Peninsula) (Sailleau *et al*., 2000). Excluding Yemen, in 1959 to 1961 serotype 9 of AHSV expanded outside of Africa across to Syria, Lebanon, Iraq, Turkey, Cyprus, Saudi Arabia, Jordan, India, Pakistan and Afghanistan, with a death toll over 300 000 equids (Cullinane *et al*., 2013). In 1965, serotype 9 of AHSV once more spread outside its endemic borders in Africa to Morocco, Algeria and Tunisia, crossing over to Spain in 1966. This outbreak was quickly curbed following a vigorous vaccination and slaughter policy (Mellor & Hamblin, 2004). Numerous AHSV serotype-4 outbreaks followed in 1988, 1989 and 1990 in Spain, in 1989 in Portugal and in 1989, 1990 and 1991 in Morocco (Mellor, 1993).

No evidence of other causes of AHSV was documented within a radius of 2 000 km from Spain and Morocco during these outbreaks. AHSV outbreaks continued in these areas for five years and overwintered four times due to presence of efficient vector species (*Culicoides*) and suitably mild climatic conditions for adult activity (Mellor *et al*., 1994).

AHS is endemic in South Africa, with most appearances in the north-eastern parts of the country (Coetzer & Erasmus, 1994) throughout the 19th century and a few decades in the 20th century (Barnard, 1998). All nine of the AHSV serotypes are endemic in South Africa, but are not equally abundant throughout the country (Venter *et al*., 2010). The AHS OIE reference centre at the Agriculture Research Council-Onderstepoort Veterinary Institute (ARC-OVI), as described by Venter *et al*. (2010), found that during the period between 1981 and 2005, out of the 280 diagnostic samples, serotype 7 was diagnosed 32.9% and serotype 2, 22.9%. AHS is also endemic to Namibia with outbreaks mostly localised in the central and northern parts of the country (Liebenberg *et al*., 2015). In central Namibia, a few serotypes of AHSV have been isolated from horses (blood and organs). These samples were obtained from the Windhoek, Okahandja, Gobabis, Omitara and Mariental areas (Scacchia *et al*., 2009; Scacchia *et al*., 2015). In the Windhoek district, 72% (8 out of 10) of tested donkeys revealed the presence of antibodies against AHSV in a limited serological study (Venter *et al*., 1999).

1.1.3. Aetiology of the AHSV

There are nine distinct serotypes of AHSV (Howell, 1962; Mellor & Hamblin, 2004). All of the serotypes occur in eastern and southern Africa and only serotype 9, 4 and 2 in northern Africa (OIE, 2013). Serotype 9 is the most widespread serotype in Africa and is also responsible for most of the epidemics outside of Africa, with serotype 4 being an outlier responsible for the outbreaks in Spain and Portugal (Mellor & Hamblin, 2004); this was the first time that AHSV serotype 4 was recorded outside of Africa (Cullinane *et al*., 2013; Mellor & Hamblin, 2004).

The capsid of the virus is 70 nm in width and an unenveloped particle (Figure 1.1). The virion consists of a double-layered icosahedral capsid and has 32 capsomeres (Coetzer & Erasmus, 1994). The virus genome comprises 10 linear double-stranded ribonucleic acid (RNA) segments (Firth, 2008; Maan *et al*., 2011; Roy *et al*., 1994). There are four non-structural and seven structural proteins. The seven structural

proteins are viral proteins (VPs) 1–7 and the non-structural proteins are NS1, NS2, NS3/NS3a and NS4 (Wilson *et al*., 2008). The core particle that enfolds the genome consists of two major proteins, VP3 and VP7 and three minor proteins, VP1, VP4 and VP6. Throughout the nine serotypes, VP3 and VP7 are conserved (Roy *et al*., 1991). The core particle is surrounded by an outer capsid that consists of two proteins, namely VP2 encoded by genome segment 2 and VP5 by segments 6 (Figures 1.1 & 1.2) (Maan *et al*., 2011; Roy *et al*., 1994). Two proteins are primarily involved in cell penetration and attachment during the early stages of infection (Maan *et al*., 2011). VP2 is considered as the protein responsible for antigenic variation (Martinez-Torrecuadrad & Casal, 1995) and determines the range of host type cells, thus influencing the virus replication site and tissue specificity.

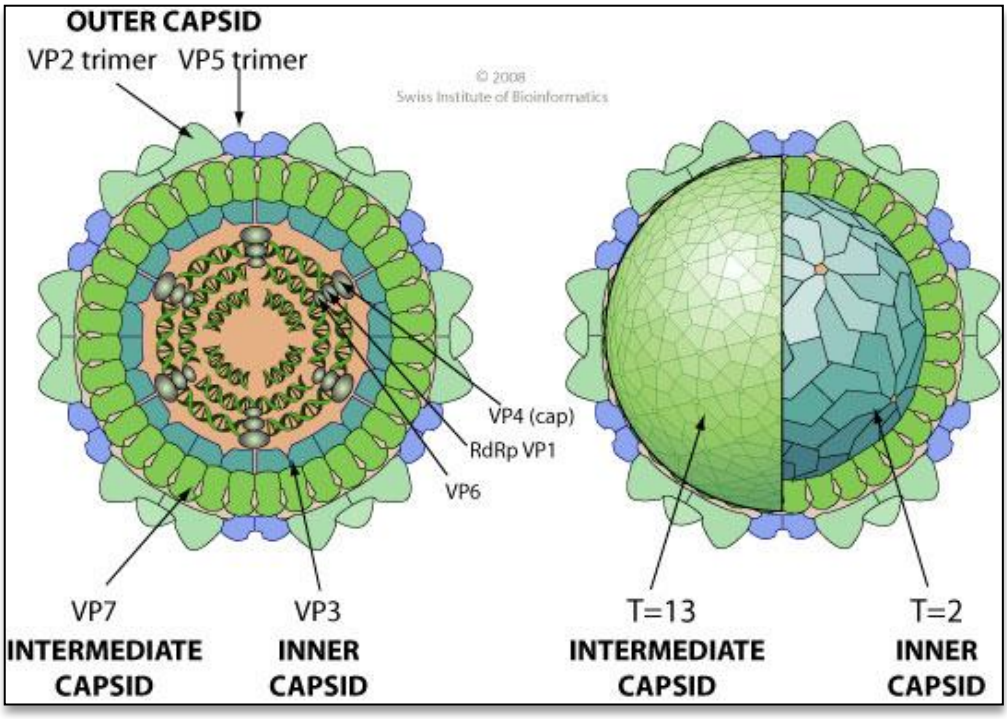


Figure 1.1: *Orbivirus* virion with the outer capsid, intermediate capsid and the inner capsid (Source: SIB, 2016).

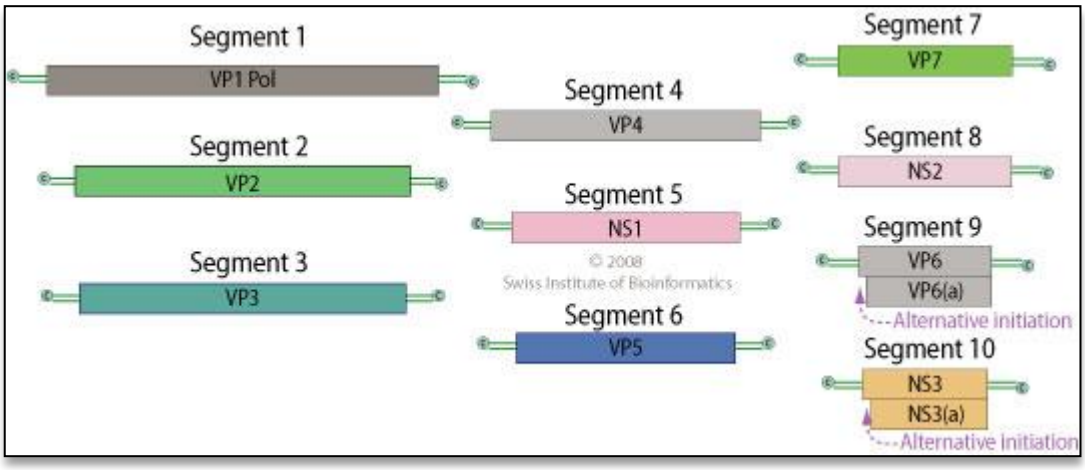


Figure 1.2: *Orbivirus* genome with all the segments and viral proteins (Source: SIB, 2016).

The physico-chemical characteristics of AHSV are distinctive. The virus survives in environments with a pH between 6 and 12. It can be inactivated at a pH below 6.0, which shows that it is acid-sensitive, but remains stable at a more alkaline pH of 7.0–8.5 (OIE, 2013). AHSV is relatively heat resistant with an ideal temperature of between 27ºC and 45ºC, but it has little activity below 12ºC (Wilson *et al*., 2009). Nonetheless, the infectivity of the virus is stable at 4ºC. Viral RNA synthesis and replication is largely controlled by ambient temperature and AHSV is particularly stable in the presence of stabilisers, for instance serum (Mellor & Hamblin, 2004). When the virus is stored between at –20ºC and –30ºC it is labile, but has a minimal loss of titre when it is lyophilised or frozen at –70°C with Parker Davis Medium (Coetzer & Erasmus, 1994).

1.1.4. Pathogenesis of the disease

AHSV cyclic hosts includes equids such as horses, mules, donkeys and zebras, but zebras have long been considered the natural vertebrate and amplifying host of AHSV (Centre for Food Security and Public Health, 2006; Mellor & Hamblin, 2004; OIE, 2009). It is believed that the persistence of the virus in Africa is related to zebra distribution and these equids rarely show clinical signs of infection (Mellor & Hamblin, 2004).

When an equid becomes infected with AHSV, the virus multiplies in the lymph nodes and spreads to the pulmonary microvascular endothelial cells (Wilson *et al*., 2009; Coetzer & Guthrie, 2004). From there it spreads by means of the bloodstream (primary viraemia), infecting secondary organs. AHSV is found in most of the organs (Mellor & Hamblin, 2004) and while replicating in these organs, viraemia is observed. Virus titre

and viraemia are determined by the host species (Wilson *et al*., 2009; Coetzer & Guthrie, 2004).

The incubation period of this virus is normally 7–14 days but can be as short as 2 days or as long as 21 days (OIE, 2013). Normally the incubation period of secondary viraemia is less than 9 days (Mellor & Hamblin, 2004). High-titre – up to 105.0 tissue culture infectious dose ($TCID_{50}$) of virus/ml – viraemia is typically demonstrated for 4–8 days in horses and 28 days for lower viraemia (<103.0 $TCID_{50}$/ml) in donkeys, mules and zebras (Coetzer & Erasmus, 1994).

There are four forms of AHS that can be classified according to the extent and severity of the disease, namely horse sickness fever, cardiac/subacute (dikkop), pulmonary/acute (dunkop) and mixed form. The severity of the strain of the virus and the horse's immunity influence the clinical form of the disease. Pulmonary, cardiac and mixed forms are located in the cardiovascular and lymphatic systems, while the horse sickness fever form is located mostly in the spleen (Wilson *et al*., 2009).

The horse sickness fever is the form usually observed in donkeys and zebras. It occurs when the host is infected with a less virulent strain or when some form of immunity is present in the host (Mellor & Hamblin, 2004). Following the infection, the host only shows a mild fever of 40–40.5ºC (OIE, 2013). Other signs can be seen, including mild anorexia or depression, congested mucous membranes and increased heart rate; some horses may show partial loss of appetite, congestion of the conjunctivae and slightly laboured breathing, but these signs are transient. This form of the disease is rarely fatal (OIE, 2013).

The most common form of AHS is the mixed form, which is a combination of cardiac and pulmonary forms. This form has a mortality rate of 70%, with death occurring within 3–6 days after a fever has begun. Symptoms of affected horses include respiratory distress followed by oedematous swellings or oedematous swellings before the onset of respiratory distress (Coetzer & Erasmus, 1994).

The cardiac form (dikkop) begins with a fever that lasts for 3–6 days and can occur for several weeks. Mortality rates of this form may exceed 50% (Coetzer & Guthrie, 2004). Just before the fever begins to drop, swelling appears in the head, neck, eyes, chest and supraorbital fossae (Figures 1.3 & 1.4). This swelling can also spread to the lips, cheeks, tongue, intermandibular space and shoulders.

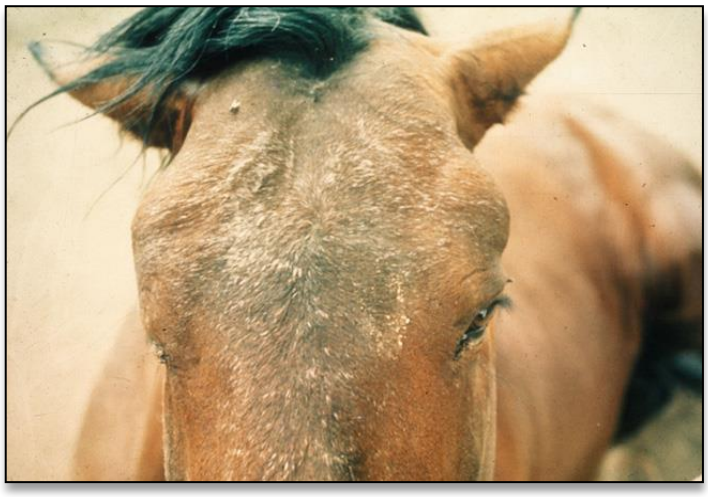


Figure 1.3: Facial swelling and oedema of the supraorbital fossae of a horse showing symptoms of the dikkop form of African horse sickness (Source: Anon, 2016b).

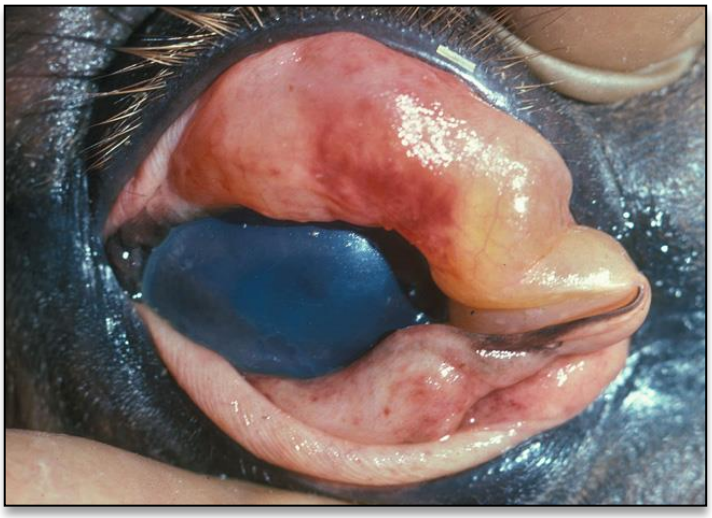


Figure 1.4: Severe oedema of the eyelids in a horse suffering from African horse sickness (Source: Anon, 2016b).

The pulmonary form (dunkop) of AHS develops rapidly without the horse appearing ill or showing any symptoms (Figures 1.5 & 1.6). The mortality rate of this form is about 95%. A fever of 39–41ºC occurs, followed by respiratory distress and severe dyspnoea (Mellor & Hamblin, 2004). Clinical signs in infected horses include severe sweating, head and neck extension and coughing spasms. Great amounts of frothy fluid is possibly discharged from areas of the body like the nose (Coetzer & Erasmus, 1994). This is also the form usually observed in dogs after feeding on infected equid carcases (Coetzer & Erasmus, 1994).

Figure 1.5: Abundant froth draining from the nostrils reflects severe pulmonary oedema in the pulmonary form of African horse sickness (Source: Anon, 2016a).

Figure 1.6: Froth and serofibrinous fluid that may be gelatinous in the trachea of a horse that died of the pulmonary form of African horse sickness (Source: Anon, 2016b).

The skin of the equid is a critical organ in the transmission cycle between the vector and host due to its direct involvement in infection. The evolutionary fitness of a viral strain and clinical form influences the ability of that strain to infect endothelial cells (Wilson *et al*., 2009; Coetzer & Guthrie, 2004).

Studies have shown that other animals besides equids can be infected with the disease. Camels have been infected and antibodies were found, but no details of

viraemia are available and their role in epidemiology is unlikely to be significant. This is also true for African elephants and black and white rhinoceros. After ingestion of infected horsemeat, dogs can be fatally infected. Although dogs are vulnerable to experimental infection they are not a preferred host by *Culicoides* spp. and are unlikely to play any role in the transmission of the virus (MacLachlan & Guthrie, 2010).

1.2. Vector genus: *Culicoides*

Culicoides midges can serve as biological vectors for several protozoa, filarial nematodes and viruses, ultimately affecting humans, birds and other animals. This blood-feeding species can be an annoyance to humans, but at the same time harmful to animals due to it being a vector of veterinary arbovirus diseases (Venter *et al*., 2012). Globally, 1 387 species of *Culicoides* Latreille have been described, with 1 343 being extant and 44 extinct (Borkent, 2014a), making it the largest genus of the Ceratopogonidae (Harrup *et al*., 2015). Thirty of these species are believed to be competent vectors. There are no records of *Culicoides* occurring in Antarctica, Hawaii and New Zealand (Meiswinkel *et al*., 2004b; Bellis, 2013; Nolli *et al*., 2014; Mellor *et al*., 2000; Borkent, 2005). Recently, *Culicoides* was found in Iceland (Arabrsdóttir, 2015). An estimated 120 species are found in southern Africa and 105 have been recorded in South Africa since 1990 (Meiswinkel, 1996). From the estimated 120 *Culicoides* species recorded from southern Africa, 31 were described from six southern African countries (Labuschagne, 2016).

Culicoides imicola Kieffer is not the only vector for orbiviruses in South Africa as a result of the somewhat irregular pattern of appearances in both warm and cold areas (Venter *et al*., 2010). *Culicoides bolitinos* Meiswinkel is considered to be a vector of AHSV after the virus was isolated from field-collected specimens of *C. bolitinos* during an outbreak of AHS in the high-lying eastern Free State province in 1998 (Meiswinkel & Paweska, 2003). This species is common in this area and in other cooler highland areas of South Africa. Morphologically, these two species are similar, but *C. bolitinos* readily enters stables, while other *Culicoides* species do not. Virus transmission can be significantly reduced and control led to several regulatory measures. These controlling measures include screening stables with mesh, stabling horses during the night, vaccination and vector control through the use of insecticides or repellents (Meiswinkel *et al*., 2004b; Carpenter *et al*., 2008).

Several surveys have been conducted, showing that the most widespread and abundant species in southern Africa that have the greatest potential as arbovirus vectors (Meiswinkel *et al*., 2004b) are *C. imicola*, Schultzei group, *C. zuluensis* de Meillon, *C. pycnostictus* Ingram and Macfie, *C. leucostictus* Kieffer, *C. bedfordi* Ingram and Macfie, *C. magnus* Colaco, *C. ravus* de Meillon, *C. gulbenkiani* Caeiro, *C. similis* Carter, Ingram and Macfie and *C. bolitinos*. Other abundant and widespread species have a more limited host preference, which leads to a smaller chance of them being potential vectors for AHSV.

Worldwide, approximately 75 arboviruses have been isolated from different *Culicoides* species, with the most recent being the Schmallenberg virus (Elbers *et al*., 2013). Most of the arboviruses belong to the Reoviridae, Bunyaviridae and Rhabdoviridae families (Meiswinkel *et al*., 2004a). Twenty-three of the 75 arboviruses have been isolated from the Imicola group of the subgenus *Avaritia* Fox 1955 (Nevill, 2007). In South Africa, AHSV has been isolated from *C. bolitinos*, *C. imicola*, *C. nivosus* and *C. leucostictus* (Goffredo *et al*., 2015; Scheffer *et al*., 2012; Venter *et al*., 2006).

1.2.1. Life cycle, feeding and habitat of *Culicoides* species

The life cycle of *Culicoides* consists of four stages, namely eggs, larval, pupal and imago (adult midge) stages. Thus, the life cycle of *Culicoides* can be referred to as a holometabolous life cycle. Nearly all *Culicoides* females need a blood meal for the purpose of developing eggs and there are four main types of larval habitats: (i) soil and surface water interface, (ii) large mammal manure pats, (iii) hollows of plants, rocks and trees and (iv) rotting fruits and plants (Meiswinkel *et al*., 2004a).

The first stage of the *Culicoides* life cycled involves *Culicoides* females laying white cylindrical eggs that change into a darker colour over time (Borkent, 2005). The eggs are laid in large batches varying in size from 30 to 450 worldwide (Liebenberg, 2012 UF). Normally, eggs are 0.5 mm in size and hatch within 2–7 days (Noli *et al*., 2014). Unfavourable environmental conditions can cause the eggs to enter diapause, where delayed development over a long period (7–8 months) can occur (Kettle, 1995). The second stage involves larvae being released when eggs hatch, after which the four larval stages begin. The development stage can stretch over a period of four days up to several weeks (Noli *et al*., 2014).

Temperature is critical because the development of larvae depends on it. The development of the larvae can range from 11 to 16 days (Veronesi *et al*., 2009). Under

unfavourable conditions, larvae can overwinter. The third stage involves larvae developing into pupae. The pupae stage can be described as a non-feeding stage (Kettle, 1995) and only lasts for 2–3 days (Noli *et al*., 2014). The fourth and final stage of the *Culicoides* life cycle is the imago stage, where pupae develop into young, winged adults. The life span of *Culicoides* varies between 15 and 21 days (Mellor *et al*., 2000), depending on environmental conditions, but research showed that the life span can vary from up to 63–90 days (Mellor *et al*., 2000).

There is a broad spectrum of hosts on which female *Culicoides* midges feed, e.g. reptiles, mammals, birds, humans and blood-engorged mosquitoes (Meiswinkel *et al*., 2004b). Southern African *Culicoides* species have a preference to feed on animals, in contrast to some European *Culicoides* species that feed on humans (Carpenter *et al*., 2013). After years of studies, *C. imicola* was shown to be the most abundant livestock-associated *Culicoides* species, especially in the summer rainfall and frost-free areas of South Africa (Meiswinkel *et al*., 2004b). This species breeds in moist, organically-enriched, clayey soils that are either bare or covered by short grass only (Meiswinkel & Linton, 2003; Meiswinkel *et al*., 2004a; Nevill *et al*., 2007, 2009).

1.2.2. Morphology of *Culicoides* species

Ander *et al*. (2013) described *Culicoides* midges as being a highly diverse group. This vector is one of the smallest haemophagous flies described, only 1–3 mm in body length (Labuschagne, 2016). Their colour varies from yellow-brown to black. Their legs are small and antennas are prominent (Figure 1.7a & b), with both males and females having antennas that typically comprise 6–13 flagellomeres (Labuschagne, 2016). Male antennas are feathery (plumose), while those of females are like small hair (pilos). Normally, seven types of sensilla are found on an antenna (Meiswinkel, 1995), with the antenna having 13 segments of flagellomeres (eight short and five long).

The mouth of the midge is vertically suspended, the labrum is sharp and adapted for piercing. *Culicoides* midges also have mandibles and paired maxillae, where serrated mandibles in females are present (Borkent, 2005). The hypopharynx of the midges carries a salivary duct and delivers anticoagulants to the host tissue. The number, shape and size of the spermathecae have been examined and *Culicoides* female may have one, two or three fully developed (functional) spermathecae. In species with two functional spermathecae, a rudimentary (undeveloped) third spermatheca is often observed and a sclerotised ring may be present at the junction of the spermathecal

ducts (Wirth & Hubert, 1989). The wings of *Culicoides* midges are 0.4–7 mm in length with 1–3 radial veins (Labuschagne, 2016). Patterned wings are visible on some midges but other species do not have any patterns at all (Labuschagne, 2016).

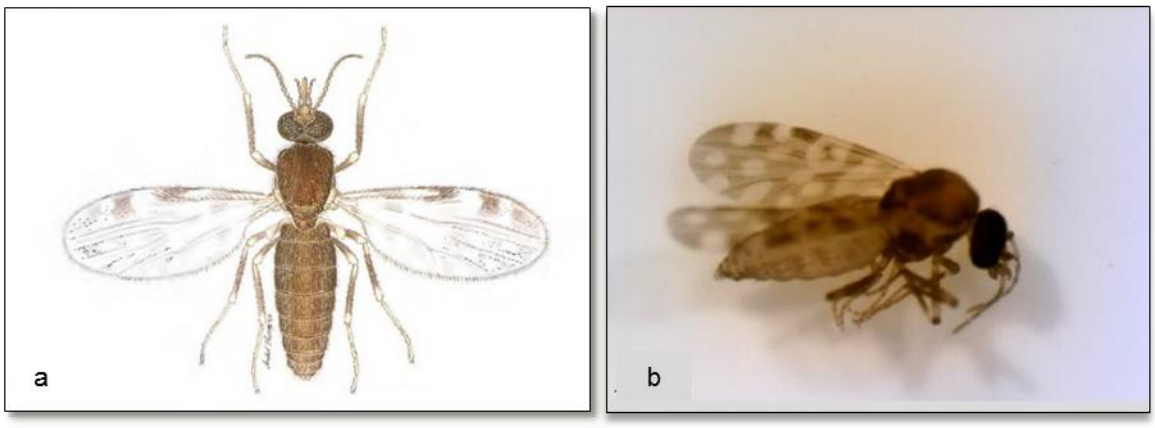


Figure 1.7: a) Sketch of *Culicoides zuluensis* female (Source: Meiswinkel, 1993). b) Microscopic image of *Culicoides sp*.: lateral view (Source: BOLD, 2013).

Three characteristics are used to distinguish the *Culicoides* genus from other midge genera, namely that the thorax has two humeral pits, the claws are equal in length after tarsomere 5 and flagellomere 13 are rounded can be observed (Labuschagne, 2016).

1.2.3 Transmission of AHS

One million midges can be collected in a single trap when an outbreak occurs (Meiswinkel, 1998). In certain - of summer and winter rainfall areas, *C. imicola* represents more than 90% of all species in one catch due to its wide distribution and rich livestock association (Venter *et al*., 1996, 2006, 2010). Midges like *C. imicola* are more exophilic and AHSV transmission can be reduced if horses are stabled in adequetly screened stables (Barnard, 1997; Meiswinkel *et al*., 2000). In hot and low-lying areas of South Africa, like Mpumalanga, an estimate of 500 000 *Culicoides* can be found at horse stables at night (Meiswinkel, 1997).

Thus, it is essential to screen stables. Based on geographical distribution, vector status, host preference and abundance, *C. imicola* is the main vector involved in the transmission of AHSV to susceptible equids (Scheffer, 2011). Virus infection is decreased when *Culicoides* populations are reduced because of seasonal changes or reduction of susceptible hosts. In countries like Namibia where drought is common, it leads to a wide-ranging variance in rainfall. AHSV outbreaks in Namibia are driven by

rainfall and humidity (Liebenberg *et al*., 2015). A total of 70% from Namibian collections of *Culicoides* consisted of *C. imicola*.

Climatic parameters impact transmission of vector-borne viruses. Across southern Africa there is considerable variation in climate. Changes in climate will affect the viral epidemiology largely on the vector population size (Lord *et al*., 2002). Previous field studies have shown that soil moisture and temperature are the main factors of determining AHS prevalence (Lo Lacono *et al*., 2014; Venter *et al*., 2000). The activity of *Culicoides* is also affected by humidity and wind speed (Sinclair, 2007). Wind has been implicated in the dispersal of infected *Culicoides* in some epidemics and can move the midges over long distances of up to 700 km over water and 150 km over land (Sellers *et al*., 1977). Outbreaks occurred as a result of this type of dispersal method in the Cape Verde Islands, Spain and Cyprus (MacLachlan & Guthrie, 2010; Sellers *et al*., 1977).

AHSV transmission is only possible during the summer/late summer, beginning of autumn or during the winter and in cooler areas (Coetzer & Gurthrie, 2004; Monaco *et al*., 2011; Gordon *et al*., 2013). After the first frost, AHS outbreaks decrease despite the continuing presence of the vertebrate host. Both cyclic and seasonal incidences (Scacchia *et al*., 2009) are observed in AHSV and epidemics occur in cyclic intervals related to drought followed by heavy rain. It has been observed that epidemics are linked with the timing of AHSV outbreaks and the warm (El Niño) phase in South Africa (Venter *et al*., 2010; Baylis *et al*., 1999). The link between these two factors is due to the combination of heavy rain and drought that the El Niño/Southern Oscillation brings to South Africa (Brown & Torres, 2008; Baylis *et al*., 1999).

Transmission of AHSV to vulnerable equine by *Culicoides* biting midges is possible after *Culicoides* midges have been infected after a blood meal (Venter *et al*., 2010; Venter *et al*., 2000) (Figure 1.8). Favourable conditions are necessary for the virus to survive in the vector. The virus must survive long enough in the gut of the vector to penetrate the gut wall to infect the cells (Mellor & Hamblin, 2004). For a vector to be effective the virus must be able to replicate and avoid pathogenesis during the extrinsic incubation period (EIP); EIP is the time between ingestion and transmission of the virus. After infecting the cells, it spreads to the salivary glands, which makes it possible for the vector to transmit the virus back to the host. EIP depends on the temperature experienced by the vector (Wilson *et al*., 2009).

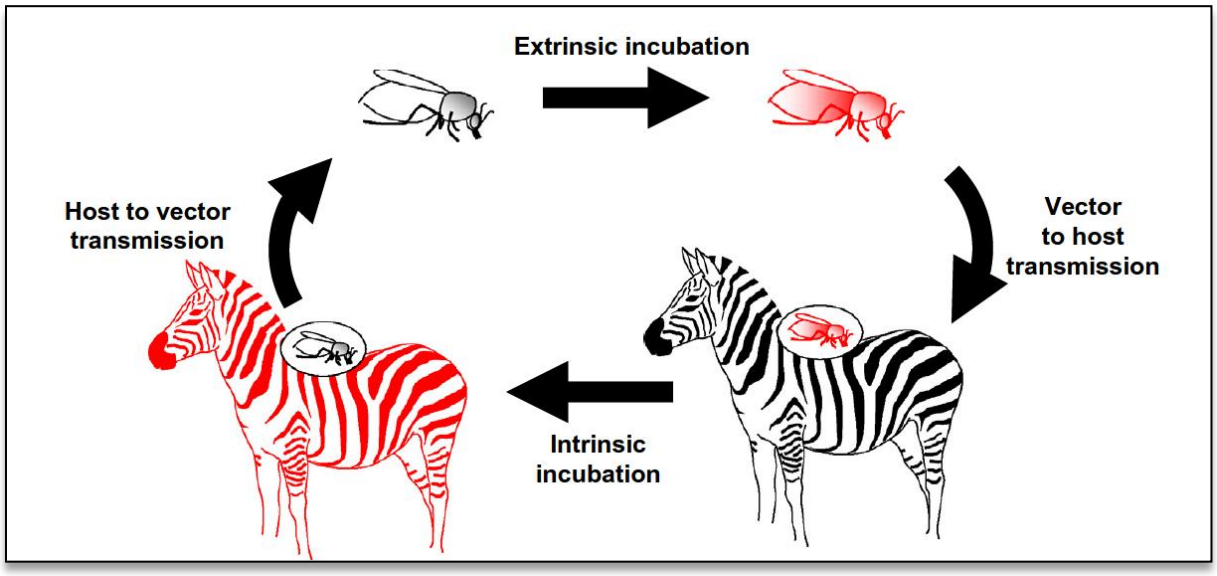


Figure 1.8: The African horse sickness transmission cycle (Source: Wilson e*t al*., 2009).

Temperature is the most important extrinsic variable affecting the rate of replication of the virus within the insect vector (Wilson *et al*., 2009). Vector replication and production rates of the virus increases in high temperatures (Baylis *et al*., 1999; Sinclair, 2007; Gordon *et al*., 2013; Welby *et al*., 1996). Replication of AHSV within the vector is possible for up to 12 days with incubation at 26ºC, but not at temperatures lower than 15ºC. Thus, when temperatures drop below this level, infection rate decreases.

The insect vector has an effect on the activity of viral RNA polymerase and the ability to modulate viral replication within its cells (Wilson *et al*., 2009). In the case of increasing temperature, infection of *Culicoides* increases along with a decrease in their survival rates, which leads to faster virogenesis (production) and transmission of the virus (Mullens *et al*., 1995; Wellby *et al*., 1996).

1.3 Perspective and outline of the study

1.3.1 Problem statement

AHS is an important intercontinental disease, which is listed by the OIE as a notifiable disease (Maan *et al*., 2011; Becker *et al*., 2012; Manole *et al*., 2012; Venter *et al*., 2010). This vector-borne disease is known to be transmitted via bites of haematophagous arthropods such as female *Culicoides*, which are the primary vectors of AHSV.

Culicoides species classified according to their morphological features and are placed accordingly into subgenera (Borkent, 2014b). However, the subgeneric classification of these species and their placement in molecular trees can lead to phylogenetic confusion as they group differently in the subgenera than in the trees. Currently morphological identification is used to identify *Culicoides* species, but this method is labour intensive, requires high-precision instruments and can only be done by a specialist in the field.

Identification of vectors is crucial for the epidemiology of vector-borne diseases (Rawlings, 1996). By identifying *Culicoides* vectors, a clear representation of the distribution between the vector and host of AHS can be given. Molecular identification of *Culicoides* species has been done in other parts of the world (Diarra *et al*., 2014). In southern Africa, *Culicoides* species have been broadly studied, but very little phylogenetic data are available. Species groups within subgeneras have very similar morphological characteristics, especially wing patterns, which are the primary identification tool, thus making classification of the genus difficult and in some cases unreliable. Sequence data on *Culicoides* species, particularly from Namibia, is lacking. Presently, the majority of identification is done through morphology tools. For a more effective identification tool, molecular methods must be approached to gain and improve phylogenetic data (Borkent, 2014b). Thus, it will help support morphological identification.

Establishing deoxyribonucleic acid (DNA) sequence barcodes of *Culicoides* species will be helpful for identifying species using a molecular method and not only by phenotypical characteristics. If standard molecular methods including PCR and Sanger sequencing are recognised for identification of *Culiciodes*, unknown specimens can be sequenced and compared to existing databases. Moreover, *Culicoides* species transmitting AHSV can be identified more efficiently.

By using bioinformatics systems, sequence data and morphological data of specimens can be compared. Through these approaches, a clear overview of the different species, the relationship between species and their preferred environment can be obtained. This study aimed to explain the phylogenetic of *Culicoides* classification and the development of a molecular detection tool for the identification of species.

AHSV is detected through molecular techniques (Staggemeier *et al*., 2012) that usually consist of PCR-based methods. Several diagnostic techniques for AHSV are recognised by the OIE. Serological tests of AHSV are done by enzyme-linked immunosorbent assay, using soluble AHSV or a recombinant protein VP7 to detect anti-AHSV group-reactive antibodies (OIE, 2016). A virus neutralisation test is also done to detect serotype-specific antibodies. Cell culture and inoculation of new-born mice are used to isolate the virus. On nucleic acid level, PCR tests are done, consisting of reverse-transcription qPCR (RT-qPCR) with viral RNA extraction using commercial kits.

Previous studies used PCR and real-time PCR (qPCR) to detect AHSV specifically in tissue samples and cell cultures (Aradaib *et al*., 2006; Guthrie *et al*., 2013; Quan *et al*., 2010; Saileau *et al*., 1997). Scheffer *et al*. (2011) used qPCR to detect AHSV in vector midges and proposed that both dissected and whole midges could be used with the RT-qPCR protocol. The latter was recently used to detect AHSV in *C. imicola* (De Waal *et al*., 2016). All of these methods are carried out in the laboratory with the results available within several days, up to several weeks. Shortcomings still appear with these methods, sophisticated instruments are needed, specificity of the target sequence needs to be detected through elaborate methods (Notomi *et al*., 2000) and amplifications efficiency are relatively low (Parida, 2008). These methods are also time-consuming, complex and costly.

An AHSV reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method has recently been developed for diagnostic purposes by Fowler *et al*. (2016). No in-field testing technique has been documented at present for the detection of AHSV within *Culicoides*. Thus, a loop-mediated isothermal amplification (LAMP) combined with a RT-LAMP (Notomi *et al*., 2000) can be developed for the detection of AHSV in field samples for instant identification. This will help to detect an outbreak of AHSV within a specific area or region.

This simple, rapid, specific and cost-effective nucleic acid amplification method (Notomi *et al*., 2000) will aid the equestrian industry in detecting the virus early on (Mulholland *et al*., 2014), minimising fatalities and economic impacts. Accurate identification of the AHSV vector and the presence of the virus are vital in the early detection of the disease. The development of a diagnostic toolkit will therefore be particularly helpful to take preventative actions such as manage and control epidemic outbreaks of this disease.

1.3.2 Aim and objectives

The aim of the study was to develop a toolkit for the identification of *Culicoides* from Namibia for AHS and for the detection of AHSV in *Culicoides*.

Therefore, the objectives of this study were:

- To establish DNA barcodes by mitochondrial cytochrome oxidase subunit I (MT-COI) gene sequencing for *Culicoides* species collected from Namibia.
- To develop a LAMP assay for the detection of AHSV in *Culicoides* as a simplified diagnostic tool.

1.3.3 Outline of dissertation

Chapter 1 is the introduction to the study, including the outlook and outline of the dissertation. In this chapter the history of AHS, geographical distribution, aetiology, pathogenesis, factors influencing the transmission of AHSV and the genus *Culicoides* are discussed.

Chapter 2 gives a brief description of *Culicoides* in Namibia, focusing on the methodology for establishing DNA barcodes for correct classification and identification of species. The results of sequence data of different species are discussed together with phylogenetic analyses.

Chapter 3 includes a description of the use of LAMP for detection of various viruses in previous studies. Primer design and optimisation are described along with the results of sensitivity and novel methodology.

Chapter 4 provides a conclusion and all the stated objectives.

CHAPTER 2: CLASSIFICATION AND IDENTIFICATION OF *CULICOIDES* SPECIES

2.1 Introduction

Two key components of determining the epidemiology of disease transmission are phenotypic and genetic characteristics of the vector species (Harrup *et al*., 2015). Miniscule differences concerning the ecology and biology of closely affiliated species can have substantial effects on transmission. Most important is the capability of the vector to become competent, infected with and transmit the virus to a specific host. Thus, correct identification of vector species is vital in the comprehension of epidemiological disease transmission (Harrup *et al*., 2015). However, the evolution of vector capability within the genus of *Culicoides*-borne viruses cannot be formally concluded due to the lack of competence data (Harrup *et al*., 2015).

2.1.1 *Culicoides* in Namibia

Comprehensive molecular information regarding *Culicoides* insects in Namibia is lacking. Research on AHSV serotyping, detection of AHSV in *Culicoides* (Goffredo *et al*., 2015; De Waal, 2016) and occurrence of *Culicoides* (Becker *et al*., 2012, 2013; Liebenberg *et al.*, 2016) in Namibia has been done. However, more research is needed. Studies have been done on the morphological and phylogenetic characterisation of different *Culicoides* species, but not in Namibia in recent times. Thus, only a few *Culicoides* species have been identified and classified through the use of molecular methods and phylogenetics. *Culicoides imicola* is one of the most abundant and widespread species in Africa, Europe and the East (Mellor *et al*., 2009; Venter *et al*., 2010). It was also found to be the most abundant and widespread species in Namibia (Goffredo *et al*., 2015; Liebenberg *et al*., 2016).

In 2009 and 2010 Becker *et al*. (2012) studied the presence of *Culicoides* in Namibia (south-western Khomas and Windhoek region). From July to September 2009 (Table 2.1) 34 collections, 9 091 *Culicoides* specimens were collected comprising of 25 species. Between February and October 2010 (Table 2.2), Becker *et al*. (2013) made 20 collections, 10 178 *Culicoides* specimens were collected comprising of 30 species. Research by Liebenberg *et al.* (2016), a multidisciplinary assessment of the distribution of AHS in Namibia was done, with one of the objectives to look at the occurrence of *Culicoides* species in the Karas (Aus), Khomas (Windhoek) and the Otjozondjupa (Okahandja) regions, where 48 different species were collected (Table

2.3) out of the 295 collections. A study on *Orbivirus* detection from *Culicoides* collected during AHS outbreaks in Namibia in the Khomas (Windhoek and Steinhausen), Erongo (Karibib and Omaruru), Otjozondjupa (Okahandja) and Omaheke (Gobabis) regions was conducted in 2011 by Goffredo *et al.* (2015) (Table 2.4). Eight collections were made, 194 211 *Culicoides* specimens comprising of 6 species.

Table 2.1: The different *Culicoides* species collected in Khomas region, Namibia, in 2009 (Becker *et al.*, 2012), where the presence and absence of species are indicated by +/-.

Species	Avis	Neu Heusis	Hureb Süd	Isabis	Corona
***C.* sp. #89**	-	-	-	-	+
***C.* sp. #90**	-	+	-	-	-
***C.* sp. #94**	-	+	-	-	+
Accraensis group	-	+	-	-	+
C. bedfordi	-	-	+	-	+
C. brucei	+	+	-	-	+
C. cornutus	-	-	+	-	-
C. exspectator	+	-	+	-	+
C. herero	+	+	+	-	+
C. imicola	+	+	+	+	+
C. kanagai	-	+	-	-	-
C. leucostictus	+	+	+	+	+
C. magnus	-	-	-	-	+
C. macintoshi	-	+	+	+	+
C. nivosus	+	+	+	-	-
C. olyslageri	-	-	-	-	+
C. pretoriensis	-	-	+	-	+
C. pycnostictus	+	+	+	+	+
C. ravus	+	+	+	+	+
C. remerki	-	-	+	-	-
C. schultzei	+	-	+	+	+
C. similis	-	-	-	-	+
C. subschultzei	+	+	+	+	+

Table 2.1 (cont.): The different *Culicoides* species collected in Khomas region, Namibia, in 2009 (Becker *et al.*, 2012).

C. trifasciellus	**-**	**-**	**-**	**-**	**-**
C. tropicalis	+	+	+	+	+
C. tuttifrutti	-	-	-	+	+

Table 2.2: *Culicoides* species collected in Khomas region, Namibia, in 2010 (Becker *et al.*, 2013), where the presence and absence of species are indicated by +/-.

Species	Neu Heusis	Hureb Süd	Isabis	Corona
***C.* sp. #33**	-	-	-	+
***C.* sp. #50**	-	+	-	-
***C.* sp. #61**	+	-	-	-
***C.* sp. #89**	-	+	-	+
***C.* sp. #94**	-	-	-	+
Accraensis group	+	+	-	+
***C.* nr. albopunctatus**	+	-	-	-
C. bedfordi	+	+	-	+
C. bolitinos	+	+	-	-
C. brucei	+	-	-	+
C. cornutus	+	-	-	-
C. exspectator	+	+	+	-
C. herero	+	+	-	+
C. imicola	+	+	+	+
C. leucostictus	+	+	+	+
C. macintoshi	+	+	+	-
C. neavei	-	+	-	+
Nigripennis group	+	-	-	-
C. nivosus	+	+	+	-
C. olyslageri	+	-	-	+
C. pretoriensis	+	+	+	+
C. punctithorax	+	+	-	-
C. pycnostictus	+	+	+	+

Table 2.2 (cont.): *Culicoides* species collected in Khomas region, Namibia, in 2010 (Becker *et al.*, 2013).

C. ravus	**+**	**+**	**+**	**+**
C. schultzei	+	+	-	-
C. similis	+	-	+	-
C. subschultzei	+	+	+	+
C. trifasciellus	-	+	-	-
C. tropicalis	+	+	+	+
C. tuttifrutti	+	+	+	+

Table 2.3: *Culicoides* species collected in the Karas, Khomas and Otjozondjupa regions, Namibia, in 2013 and 2014 (Liebenberg *et al*., 2016), where the presence and absence of species are indicated by +/-.

Species	**Windhoek**	**Okahandja**	**Aus**
***C.* sp. #107**	-	+	-
***C.* sp. #33**	+	-	+
***C.* sp. #50**	+	+	-
C.* sp. #54 (d/f)	+	+	-
C.* sp. #54 (p/f)*	-	+	-
***C.* sp. #61**	+	+	-
***C.* sp. #62**	+	-	-
***C.* sp. #69**	-	+	-
***C.* sp. #89**	+	+	+
***C.* sp. #94**	-	+	+
Accraensis group	+	+	-
C. albopunctatus	+	+	-
C. bedfordi	+	+	+
C. bolitinos	-	+	-
C. brucei	+	+	+
C. coarctatus	+	+	-
C. cornutus	+	+	-
C. distinctipennis	-	+	-
C. dekeyseri	-	-	+

Table 2.3 (cont.): *Culicoides* species collected in the Karas, Khomas and Otjozondjupa regions, Namibia, in 2013 and 2014 (Liebenberg *et al*., 2016).

C. enderleini	**+**	**+**	**+**
C. eriodendroni	+	+	-
C. exspectator	+	+	+
C. glabripennis	+	-	-
C. herero	+	+	+
C. imicola	+	+	+
C. kanagai	-	+	-
C. leucostictus	+	+	+
C. loxodontis	-	+	-
C. macintoshi	-	-	+
C. miombo	+	+	-
C. neavei	+	+	-
C. nevilli	-	+	-
Nigripennis group	+	+	-
C. nivosus	+	+	+
C. olyslageri	-	+	-
C. ovalis	-	+	-
C. pretoriensis	+	+	+
C. punctithorax	+	+	+
C. pycnostictus	+	+	+
C. ravus	+	+	+
C. rhizophorensis	-	+	-
C. schultzei	+	+	+
C. similis	+	+	+
C. subschultzei	+	+	-
C. tororoensis	+	-	-
C. trifasciellus	+	+	-
C. tropicalis	+	+	+
C. tuttifrutti	+	+	+

Table 2.4: Six *Culicoides* species collected in Khomas, Erongo, Otjozondjupa and Omaheke regions, Namibia (Goffredo *et al*., 2015), where the presence and absence of species are indicated by +/-.

Species	Stein-hausen	Windhoek	Karibib	Omaruru	Okahandja	Gobabis
C. imicola	+	+	+	+	+	+
C. leucostictus	-	+	-	-	-	+
C. nivosus	-	+	-	-	+	+
C. pycnostictus	+	+	+	-	+	+
Schultzei complex	-	+	+	+	+	+
C. tropicalis	-	+	-	+	-	-

2.2 Classification and identification of *Culicoides*

2.2.1 Classification of *Culicoides*

Borkent (2014a) divided the genus *Culicoides* into 31 subgenera, 38 groups of species not placed into any subgenus and approximately 13% of the known species not placed in any group or subgenus. Some groups like the subgenus *Avaritia* have a larger number of vector species, although economically important species are placed into a wide variety of subgeneric groups (Meiswinkel *et al*., 2004a; Wirth & Dyce, 1985, cited by Harrup *et al*., 2015).

In South Africa, 105 species of *Culicoides* have been recorded at present. Of these, 73 species have been named and described (morphologically). The subgeneric classification of these species is as follows: nine are unplaced, 44 are placed into nine subgenera and 20 into five species groups (Borkent, 2014b). Only 26 of the 73 species' immature stages are described. Thus, up to date, the descriptions of 26 pupae, 14 larvae, 70 females and 68 males are available (Labuschagne, 2016).

It is believed that some of the subgenera or species groups are monophyletic. This is based on unpublished synapomorphies (OIE, 2016). Specific area evaluations were done in the past to classify *Culicoides* subgenera, with few attempts to justify groupings with those from other areas (Fox, 1948, 1955; Khalaf, 1954; Root & Hoffman, 1937, cited by Harrup *et al*., 2015). Synapomorphies of the genus as a whole was discussed by Borkent (2014b) and Shults *et al*. (2016). Both Gomulski *et al*. (2006) and Schwenkenbecher *et al*. (2009) suggested that current subgenera are polyphyletic

and descended from one or more common ancestors (Perrin *et al*., 2006). Subgeneric classification has also been based on adult specimens and only a small percentage of studies included immature stages of *Culicoides*, making the classification almost completely phenetic (Nevill & Dyce, 1994; Nevill *et al*., 2009).

Previous studies of *Culicoides* subgeneric classification have never been effective enough (Borkent, 2012). Although numerous species were placed in subgenera, there are various species that are still not described (Table 2.5). Various separate species groups are even placed in uncertain affiliation, since single specimens are collected every so often, contributing to the lacking of character variation when describing species (Liebenberg, 2016).

Table 2.5: Classification of *Culicoides* species relevant to this study (Meiswinkel, 1996).

SUBGENUS	GROUP	SPECIES
Remmia Glukhova	Schultzei	*C. enderleini*, *C. schultzei*, *C. subschultzei*
Beltranmyia Vargas	Unspecified	*C. nivosus*, *C. pycnostictus*
Meijerehelea Wirth and Hubert	Unspecified	*C. leucostictus*
Synhelea Kieffer	Unspecified	*C. tropicalis*
Unspecified	Similis	*C. exspectator*, *C. herero*, *C. pretoriensis*, *C. ravus, C. similis*
Unspecified	Unspecified	*C.* sp. #61, *C. eriodendroni*, *C. punctithorax*

2.2.2 Morphological identification

The Imicola group, the most widespread complex in South Africa, consists of 13 species. Four species of the Imicola group have yet to be described (Nevill *et al.*,

2007). Nine of the 13 species can be identified through the use of a key, where the adult stage has been described (Meiswinkel *et al*., 1995). Although the wing patterns of these species are very similar, they can be distinguished with practice or by experts in the field. The wing patterns of all *Culicoides* are composed of grey and white spots and are unique to each species (Meiswinkel *et al*., 2004a), but some species do not have spots on their wings. Most *Culicoides* species have a distinguishable wing pattern, but within closely related species groups and complexes, the patterns become more similar. Thus, misidentification can occur if the researchers are inexperienced.

Globally, only a small number of taxonomic experts are studying midges on a daily basis. Identification keys for specific regions, countries or groups are available. Several keys have been published to identify African *Culicoides* species with Khamala and Kettle (1971), Boorman and Dipeolu (1979) and Glick (1990) being used regularly. Only East African, Nigerian and Kenyan *Culicoides* species are dealt with in these keys and they do not enclose all the species occurring in the Afrotropical region. Other countries also have their own identification keys, such as Angola (Caeiro, 1961), Congo (Itoua & Cornet, 1986) and South Africa (Fiedler, 1951; Meiswinkel, 1996).

Meiswinkel (1996) developed a wing picture atlas, a key for southern African *Culicoides*, to make identification easier. This key describes each species' wing pattern, distribution and habitat. Numbers are typically given to identify *Culicoides* species that have not been officially named and described. The numbering system devised by Rudy Meiswinkel is still used in South Africa for undescribed species, e.g. *C.* sp. #33 (Labuschagne, 2016).

In plain-wing species, species groups or complexes dissection and mounting of specimens may be required, as wing pattern alone may not provide a conclusive identification (Labuschagne, 2016). Mounted evidence on microscope slides can display other characteristics of species, which include sensilla on the antennae, size and shape of the third segment of the maxillary palpus and the genitalia (Labuschagne, 2016). Additional characters can also be examined, including spermathecae shape, size and number (Meiswinkel *et al*., 1993), the intraocular space between the eyes (Meiswinkel *et al*., 2004b; Borkent, 2005) and whether the chitinous area between the ocelli are decked with hair or not (Meiswinkel *et al*., 1993).

Morphological analyses are dependent on the number of taxonomic studies (Harrup *et al*., 2015). Thus, the availability of taxonomic expertise and infrastructure is

significant for entomology and epidemiology studies. As described by Tautz *et al*. (2003) and Harrup *et al*. (2015), the start of molecular classification has provided an alternative shift in standard taxonomy expertise, with increased significance regarding the concept of phylogenetic characterisation. Due to record appearances of arboviruses in novel regions, the concept has been fast-tracked to answer epidemiological questions (Carpenter *et al*., 2009). Morphological comparison with outgroups has been used for identification of taxa with shared synapomorphies (Borkent, 2000a, 2000b). Within the *Culicoides* genus, only a few studies testing subgeneric groupings have been done by utilising genetic and phylogenetic data (Bellis *et al*., 2014; Pagès *et al*., 2009), with Bellis *et al*. (2014) expanding on morphology of *Culicoides*.

2.2.3 Molecular and phenotypic identification

Generally, DNA barcoding is described as the amplification of a uniform region of the mitochondrial gene that is sequenced, analysed and compared to a database (Keele *et al*., 2014). In this study, DNA barcoding refers to the identification of species through the sequencing of the 5'-region (Rebijith *et al*., 2012) of the MT-COI gene (Ander *et al*., 2013). Mitochondrial DNA is considerably smaller than nuclear genome, thus amplification and subsequent sequencing is more successful (Archana *et al*., 2015). DNA barcoding can have several advantages, e.g. reduction in uncertainty, more specific identification, corrections of field misidentifications, expansion of taxonomic expertise (Stoeckle *et al*., 2004; Keele *et al*., 2014) and it is reliable and cost-effective (Hebert *et al*., 2003). Thus, MT-COI DNA barcoding can be the solution for problematic species identification.

Over the last decade, DNA barcoding has become a fast-developing tool for species identification (Keele *et al*., 2014) and is commonly used in research comprising identification and biodiversity of species (Kim *et al*., 2012). A number of studies have disclosed that once morphological differentiation between *Culicoides* species becomes difficult, the DNA barcoding of the MT-COI gene delivers useful results (Sebastiani *et al*., 2001; Pagès & Sarto i Monteys, 2005; Nolan *et al*., 2007; Pagès *et al*., 2009; Monaco *et al*., 2010). By using this gene, useful sequences for phylogenetic analyses at species and other taxonomic levels can be produced (Folmer *et al*., 1994). Numerous studies developed assays using other genes than MT-COI, e.g. the ribosomal RNA genes internal transcribed spacer 1 or 2 or the nuclear carbomoyl phosphate synthetase genes (Raich *et al*., 1993; Linton *et al*., 2002; Dallas *et al*., 2003;

Pagès & Sarto i Monteys, 2005; Nolan *et al*., 2007; Pagès *et al*., 2009; Monaco *et al*., 2010; Bellis *et al*., 2013).

These studies not only encompassed universal primers but also genus-specific primers. Folmer *et al*. (1994) developed universal primers by comparing a wide range of different species' DNA sequences and used a number of highly conserved regions of these genes. The sequences of 11 taxa, including humans, nematodes, a fin whale and a cow, were involved in the development of the universal MT-COI primers (Folmer *et al*., 1994). In 2003, Dallas *et al*. (2003) designed genus-specific primers for their study on *C. imicola*. A partial sequence of the MT-COI gene was used from five species of the Imicola complex, which contain at least 10 species (Meiswinkel, 1995).

The use of bioinformatics tools is critical in phylogenetic studies. There are several databases freely accessible worldwide (Harrup *et al*., 2015). DNA analysis programmes such as BioEdit (biological sequence alignment editor) (http://www.mbio.ncsu.edu/bioedit/bioedit.html), FinchTv (http://www.geospiza.com/ftvdlinfo.html) and Molecular Evolutionary Genetics Analysis (MEGA, http://www.megasoftware.net/) are used to edit or view DNA sequences (Treves, 2010). Other programmes are used to compare sequence data of not only the animal kingdom but all living organisms. Some of these databases are The Barcode of Life Data system (BOLD) (Etzler *et al*., 2014; Ratnasingham & Herbert, 2007), Consortium for the Barcode of Life (Etzler *et al*., 2014; Ratnasingham & Herbert, 2007), International Nucleotide Sequence Database Consortium and GenBank (Harrup *et al*., 2015; Ratnasingham & Herbert, 2007). Records of *Culicoides* species DNA barcode sequences are accessible in GenBank.

The aim of this chapter is to showcase the establishment of DNA barcodes for *Culicoides* species collected in Namibia.

The specific objectives included the following:

- To extract DNA from morphologically determined *Culicoides* specimens.
- To sequence the MT-COI gene of *Culicoides* species for DNA barcoding.
- To align amplicon sequences generated in this study to known databases for phylogenetic comparisons.

2.3. Materials and methods

2.3.1 Sample collection

2.3.1.1. Site selection and description

Taxonomic and molecular studies can be done on *Culicoides* collected with light traps (Meiswinkel *et al*., 2004a). *Culicoides* specimens were collected for identification in Namibia from 2 to 7 April 2016. Namibia is known as a dry country where drought usually occurs, with rainfall between November and March (Sweet & Burke, 2000). Winter rainfall can occur in some parts of the country (Sweet & Burke, 2000). Two sites where chosen: Windhoek (Khomas region) and Okahandja (Otjozondjupa region) (Table 2.6). The collection sites were chosen according to the results of Liebenberg *et al*. (2016). A total of 48 different species – 36 in Windhoek and 41 in Okahandja – were collected and identified in the present study.

Table 2.6: *Culicoides* collection sites in Windhoek (W) and Okahandja (O) districts, Namibia, during April 2016.

Site	Trap identification	Global positioning system coordinates	Number of collections
Windhoek	W1	22°26'38.8"S 17°35'37.7"E	5
	W2	22°26'37.7"S 17°35'36.7"E	5
Okahandja	O1	21°58'21.4"S 16°55'21.3"E	5
	O2	21°58'21.8"S 16°55'20.0"E	5
	O3	21°58'21.1"S 16°55'20.8"E	4

2.3.1.2. Collection method of Culicoides midges

Culicoides midges were collected using the OVI 220 V suction ultraviolet (UV)-light trap described by Venter *et al*. (1998). The OVI trap is the most sensitive for collecting widespread diversity and large numbers of *Culicoides* species (Venter *et al*., 2009). These traps consist of the following: UV light that attracts insects, a gauze, fan and collection beaker (Figure 2.1).

Biting midges are most active from sunset to sunrise (Meiswinkel *et al*., 2004a; Mullen & Durden, 2009) and therefore collections were made every day from 19:15 to 22:15 (sunset) at night and from 03:30 to 06:30 (sunrise) in the morning local time. *Culicoides* and other insects were collected in a 500 ml beaker with 250 ml phosphate buffered

saline buffer. After every collection, the catch was roughly cleaned and preserved in 70% ethanol at room temperature for further analysis.

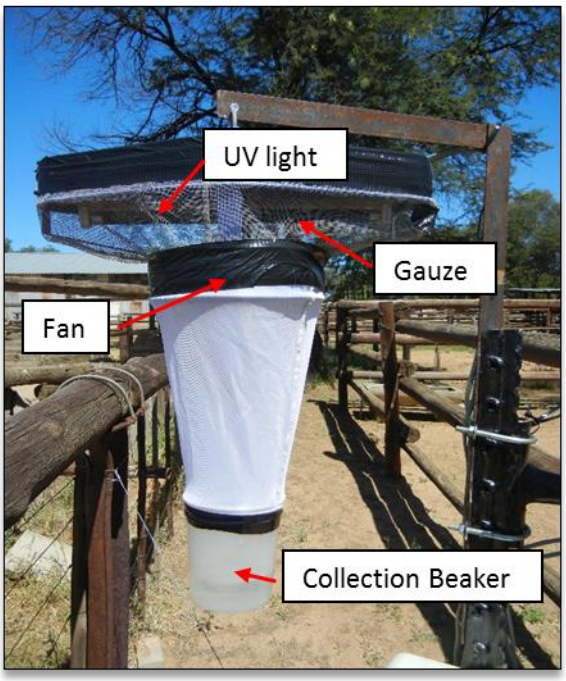


Figure 2.1: The Onderstepoort Veterinary Institute 220 V suction ultraviolet-light trap used for insect collection, particularly *Culicoides*, in this study (Source: Van Zyl, 2016).

2.3.1.3. Khomas region – Windhoek

The warmblood horse stud farm used for *Culicoides* collection is located approximate 60 km outside Windhoek in the Khomas region. Traps W1 and W2 were set up close to the open paddocks on the farm (Figure 2.2). While collection was being done, horses were roaming in the open paddocks or nearby during the day and night. Together with the stabled horses, free-roaming horses could also be found on the farm. Both traps were used for morphological and molecular analysis.

Figure 2.2: Windhoek collection sites of *Culicoides* midges. Traps W1 and W2 were used for morphological identification and DNA barcoding.

2.3.1.4. Otjozondjupa region – Okahandja

The second site chosen for *Culicoides* collection is a horse farm situated on the border of Okahandja town in the Otjozondjupa region. Three sampling areas were chosen and marked O1, O2 and O3 (Figure 2.3). Traps O1 and O2 were set up close to the semi-open horse stables and trap O3 was set up inside the horse stables, where sheep were kept during the night. Along with the stabled horses, zebras, sheep, cattle, goats, camels, oryx and honey badgers could also be found on the farm. All three traps were used for morphological and molecular analysis.

Figure 2.3: Okahandja collection sites of *Culicoides* midges. Traps O1, O2 and O3 were used for morphological identification and DNA barcoding.

2.3.2 *Culicoides* identification

Morphological identification of *Culicoides* species was carried out by the use of the wing picture atlas of Afrotropical *Culicoides* (Meiswinkel, 1996) at ARC-Onderstepoort with the help of Dr K Labuschagne, an expert in the field of *Culicoides* taxonomy. *Culicoides* wing patterns were used for primary identification of species. With the specimens used for DNA extraction the head, one wing and in males the genitalia were mounted on a microscope slide (Meiswinkel, 1995) for future reference if needed.

2.3.3 DNA extraction

DNA was extracted from the remaining parts of the midges using a modified procedure of the High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) for isolation of nucleic acids from mammalian tissue, as used by Grobler *et al*. (2011). Grobler *et al*. (2011) applied this approach to extract DNA from invertebrate genera *Palirhoeus* Kuschel, *Bothrometopus* Jeannel and *Ectemnorhinus* GR Waterhouse (all from weevil family) and delivered good DNA that was viable for sequencing. Similarly, this methodology was applied in the present study and delivered good quality DNA. However, a modification was made to the elution step, reducing the volume from 200 µl to 25 µl, because of the low concentration values of DNA obtained with a higher

volume. Each individual *Culicoides* midge was placed into a 1.5 ml Eppendorf tube with 200 µl phosphate buffered saline and homogenised with a 3-mm stainless steel ball for 2 minutes at 50 rpm. The concentration (ng/µl) of extracted nucleic acid was determined with the use of the ND-1000 Spectrophotometer (NanoDrop Technologies. Inc., United States of America (USA))

2.3.4 MT-COI DNA amplification

A Phire Tissue Direct PCR Master Mix (Thermo Scientific, Inc., USA) kit was used for PCR amplification. Universal primers LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and HC02198: 5'-taaacttcagggtgaccaaaaaatca-3' were used (Folmer *et al*., 1994), amplifying a 710-base pair (bp) fragment of the MT-COI gene.

An Alpha Cycler 1 PCRmax thermal cycler (PCRmax, United Kingdom (UK)) was used to amplify target sequences. Each reaction comprised a total volume of 25 µl: 1 µl of each MT-COI primer (0.4µM), 12.5 µl 2x Master Mix, 1 µl (10–20 ng/µl) DNA extracted from *Culicoides* specimens and distilled water (dH_2O). A three-step protocol for cycling conditions was used according to the manufacturer's instructions, which included the following steps: (i) initial denaturation step of 98ºC for 5 min, (ii) 35 cycles, comprising a denaturation step of 98ºC for 5 s, annealing step at 46ºC for 5 s and extension step at 72ºC for 20 s and (iii) final extension at 72ºC for 1 min, with a hold at 4ºC.

Amplified DNA product was confirmed using 1.0% (w/v) agarose gel electrophoresis. Then, 3 µl DNA was mixed with 2 µl of loading dye (6X Orange Loading Dye, Fermentas, USA) containing GelRed (1000x) (Biotium, USA) and loaded onto the 1.0% agarose gel. The buffer used was 1x tris-acetate-EDTA (TAE) (20 mM acetic acid, 100 mM EDTA, 40 mM Tris at pH 8.0) gel electrophoresis buffer. A 1 kb molecular weight marker (O'GeneRuler Express DNA Ladder, Thermo Scientific, Inc., USA) was used to analyse the PCR product. Conditions for gel electrophoresis were set at 80 V for 45 min in a Mini Sub-cell GT and Power-Pac (Bio-Rad, USA). The Bio-Rad Chemidoc MP (Bio-Rad, USA) was used to capture images of gels.

2.3.5 Cycle sequencing of amplicons

The amplified PCR product was cleaned by using a NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The concentration (ng/µl) of purified nucleic acid was determined with the use of the ND-1000 Spectrophotometer (NanoDrop Technologies. Inc., USA). Cycle sequencing was done with the use of the Cycle Sequencing BigDye Terminator Kit v3.1 (Applied

Biosystems by Thermo Fisher Scientific, USA). Each reaction contained the following: 4 µl 1:10 dilution Ready Reaction Premix (2.5x), 2 µl BigDye Sequencing Buffer (5x), 3.2 pmole of the specific forward or reverse primer (Inqaba Biotech, South Africa), template DNA (20 ng) and nuclease-free water (Fermentas Life Sciences, USA) with a final volume of 20 µl. An Alpha Cycler 1 PCRmax, (PCRmax, UK) was used for thermal cycling. The following cycling conditions were used: an initial denaturation step at 96ºC for 1 min for one cycle, followed by 25 cycles including a denaturation step at 96ºC for 10 s and an annealing step at 50ºC for 5 s. A final elongation step at 60ºC for 4 min followed, with a hold at 4ºC.

The labelled products were purified using a ZR DNA-Sequencing Cleanup kit (Zymo Research, USA) according to the manufacturer's instructions. Five µl of clean sequencing reaction product was mixed with 8 µl of Hi-Di formamide (Applied Biosystems by Thermo Fisher Scientific, USA) and analysed with an ABI 3130 Genetic Analyser (Applied Biosystems, UK) under standard conditions to obtain electropherograms of sequencing reactions.

2.3.6 Bioinformatic tools for data analyses

After sequencing amplicons, the generated DNA sequence data were available for further analysis. Forward and reverse fragments of MT-COI were sequenced, creating chromatograms of the 5'-3' and 3'-5' ends of the fragment. In this study, the basic local alignment search tool (BLAST) function of BOLD and the National Center for Biotechnology Information (NCBI) were used to obtain an identity match and rule out any misidentification or contaminated specimens. Sequences obtained were then used to draw a phylogenetic tree.

The NCBI assembled and disseminated GenBank (Benson *et al*., 2005; Clark *et al*., 2015) (www.ncbi.nlm.nih.gov/genbank/) is a free database of nucleotide sequences consisting of over 340 000 species (Clark *et al*., 2015). In the past, DNA sequences were submitted to the database, but new developments allow 16S and ribosomal RNA sequences to be submitted. Files uploaded onto GenBank have been divided into separate divisions for convenience purposes. A total of 17 divisions (including bacteria, viruses, primates and high-throughput complementary DNA) can be seen on GenBank and some larger divisions are divided into multiple files (Benson *et al*., 2005). The BOLD system was also used, by comparing MT-COI gene sequences using the barcode identification engine in BOLD v3 (http://www.barcodinglife.org/).

2.3.6.1 Sequence editing

Chromatograms obtained by sequencing were edited with the use of biological sequence alignment editor of Windows 95/98/NT/2000/XP/7 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html0). A complementary sequence was used for the species where only a reverse sequence was obtained. Sequences could be aligned and edited with the use of BioEdit software (Hall, 1999). This programme is extensively used in molecular biological studies. Over recent years, the programme has integrated numerous other features and function tools that include manual alignment, restriction mapping and annotation (Hall, 2011).

2.3.6.2 Sequence identification with GenBank

The chromatograms obtained through BioEdit were subjected to a BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) to identify *Culicoides* species. BLAST is referred to as an algorithm to search for DNA and protein sequence similarities (Altschul *et al*., 1997). This tool is maintained by the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) (Ye *et al*., 2006). NCBI is a division of the National Library of Medicine, situated in Bethesda, Maryland, USA (Benson *et al.*, 2005; Clark *et al*., 2015).

All combinations of nucleotide or protein queries with nucleotide or protein databases were compared through BLAST to variants as described by Ye *et al*. (2006). These tools provided statistics about alignment (false-positive rate, expected value) and could create alignments from hot spots through finding short matches between two sequences (Ye *et al*., 2006). DNA sequences were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/).

2.3.6.3 Phylogenetic tree construction

MEGA 7 software (http://www.megasoftware.net/) was used for phylogenetic analysis. The programme is able to construct a phylogenetic tree from a set of sequences. It consists of various sophisticated tools and methods (Kumar *et al*., 2016). In addition to drawing phylogenetic trees, this programme is also able to align DNA sequences, consider molecular clocks, infer ancestral sequences and compute pairwise distances. Bootstrap values are given on the branches of the tree. Information of the stability of the tree branching order (topology) can be derived from the bootstrap percentage. If

the bootstrap values are >90%, the node data are strongly supported, 70–90% means that the data are well supported, 50–70% means that they are weakly supported and <50% means that they are not supported.

Sequences form BioEditwere exported to MEGA 7 and aligned. Both the phylogenetic trees of *Culicoides* sequences were constructed by MEGA 7. The Neighbour-Joining method was used and the tree was drawn to scale. All sequences were manually aligned and sequences that were too long were deleted at the 5' or 3' ends. The quality check was done through the bootstrap method (1 000 bootstraps) and the evolutionary distance was computed using the p-distance method with complete deletion. All the gaps and missing data were eliminated.

2.4 RESULTS AND DISCUSSION

The aim of this chapter is to establish DNA barcodes by MT-COI gene sequencing for *Culicoides* species collected from Namibia. *Culicoides* was first identified morphologically and DNA was then extracted from these *Culicoides* specimens, followed by sequencing of the MT-COI gene for DNA barcoding. Lastly, alignment of sequences generated during this study as well as from databases was done for phylogenetic identification.

2.4.1. Morphologically identified *Culicoides* of Namibia

Morphological identification of specimens was done to identify each specimen. As previously described in Section 2.4.2, *Culicoides* species can be identified through morphological characteristics. Wings from the different species found in the collections from Namibia are illustrated below. A total of 163 specimens were classified based on their wing patterns and 19 different species were identified. All 19 species were used for DNA extraction and phylogenetic study. As can be seen in Figure 2.4 species from Figure 2.4a to Figure 2.4p was successful and Figure 2.4q to Figure 2.4s were unsuccessful (lower concentrations).

All of the 19 species identified in this study were formerly found in and identified from various regions in Namibia during occurrence and distribution studies (Becker *et al*., 2012, 2013; Goffredo *et al*., 2015; Liebenberg *et al*., 2016). In association to the sites of this study, *C. schultzei*, *C. enderleini*, *C. punctithorax*, *C. eriodendroni* and *C. pretoriensis* were only found at Okahandja, whereas *C. tropicalis*, *C. tuttifrutti*, *C. bolitinos* and *C.* sp. #89 were only found at Windhoek. The remaining species were found at both Okahandja and Windhoek. In reference to Liebenberg's (2015) work, all 19 species collected in this study were previously found at Windhoek and Okahandja with the exception of *C. bolitinos* only being caught in Okahandja. Goffredo *et al*. (2015) collected *C. leucostictus* and *C. tropicalis* at Windhoek and *C. nivosus*, *C. schultzei*, *C. subschultzei*, *C. enderleini*, *C. imicola* and *C. pycnostictus* in Okahandja. The Schultzei and Imicola groups are generally distributed in higher quantities in Okahandja, with *C. tropicalis* being limited to Windhoek. This could be helpful for future catchments to identify sites that will deliver species with inadequate sequences.

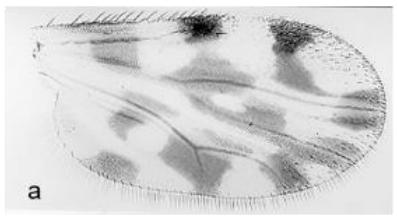
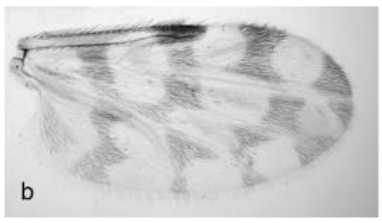
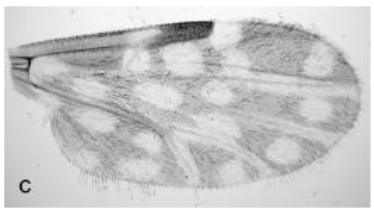
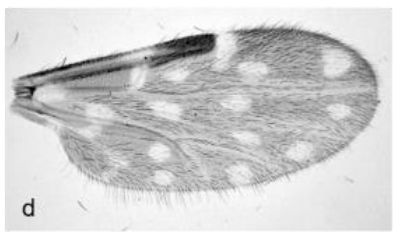
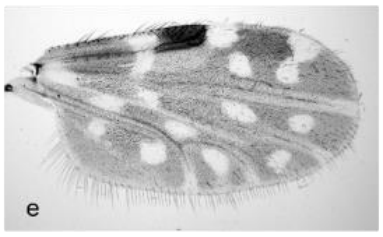
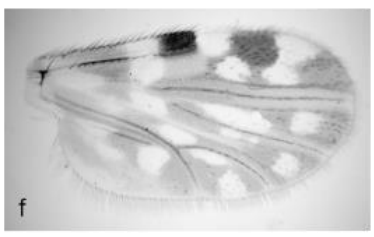
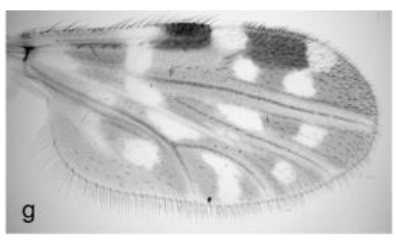
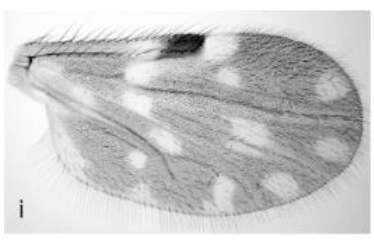
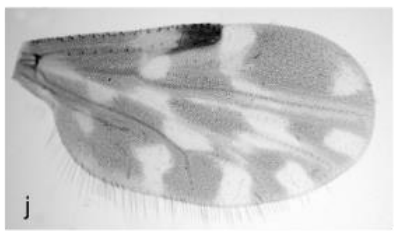
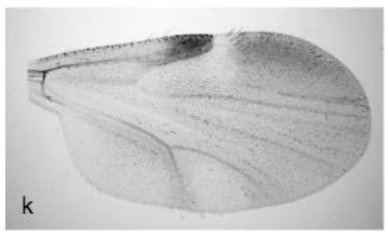
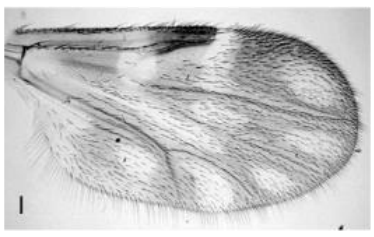
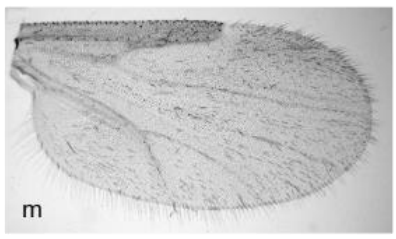
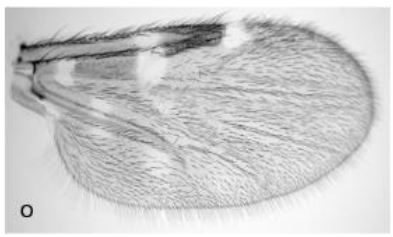
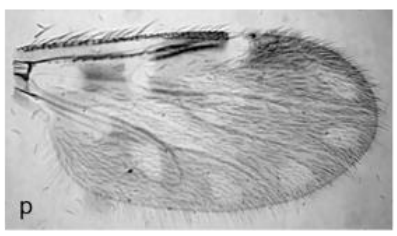
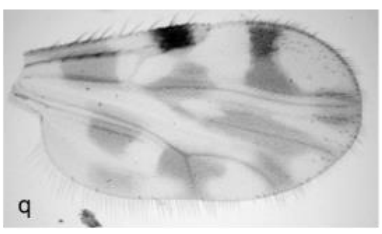
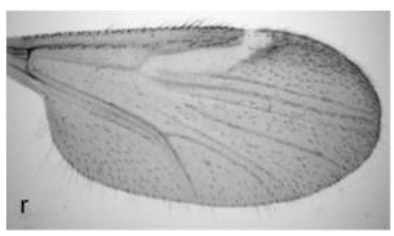
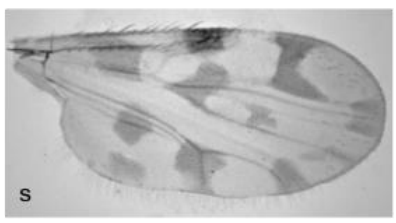
Culicoides imicola **Kieffer**  a	***Culicoides nivosus*** **De Meillon**  b	***Culicoides pycnostictus*** **Ingram and Macfie**  c
Culicoides leucostictus **Kieffer**  d	***Culicoides enderleini*** **Cornet and Brunhes**  e	***Culicoides schultzei*** **(Enderlein)**  f
Culicoides subschultzei **Cornet and Brunhes**  g	***Culicoides tropicalis*** **Kieffer**  h	***Culicoides similis*** **Carter, Ingram and Macfie**  i
Culicoides exspectator **Clastrier**  j	***Culicoides herero*** **(Enderlein)**  k	***Culicoides pretoriensis*** **Kremer and Nevill**  l
Culicoides ravus **de Meillon**  m	***Culicoides eriodendroni*** **Carter, Ingram and Macfie**  n	***Culicoides punctithorax*** **Carter, Ingram and Macfie**  o
C. sp. # 61 (undescribed)  p	***Culicoides bolitinos*** **Meiswinkel**  q	***Culicoides gerdesae*** **sp. nov. (C. sp. # 89)**  r
Culicoides tuttifrutti **Meiswinkel, Cornet and Dyce**  s		

Figure 2.4: Digital photographs of wing patterns of the different *Culicoides* species identified from specimens collected in Namibia. Where **a** to **p** indicates species from which DNA extraction and amplification were successful, and **q** to **s** indicates species with low-concentration DNA yield and unsuccessful amplification reactions (Source: Labuschagne, 2016).

A major problem in identifying *Culicoides* species is the lack of identification keys for specific countires or areas. This may lead to misidentification by inexperienced researchers. Molecular sequencing have indicated the possibility of closely related species i.e species that may have been relegated to synonomy. These species need further study to determine if these are separate species or variation within the species. Therefore, this highlights the importance of this study.

2.4.2. MT-COI DNA amplification of *Culicoides* species

DNA extraction was done, followed by MT-COI specific PCR that resulted in 750 bp amplicon for all species. Figure 2.5 is an illustration of the amplified PCR products from 16 different *Culicoides* species whose DNA extractions were successful. Gel electrophoresis of PCR products is the standard method for analysing reaction quality and yield. Visible bands in the gel were strong and the correct size was amplified with no primer-dimers detected below 100 bp. Amplification of the desired fragment size of the MT-COI target gene was shown, indicating accurate primer set concentrations and cyclic conditions of PCR. As can be seen, some lanes do not have any bands or very light bands. These amplified DNA were not used for sequencing, due to the quality of the bands formed in Figure 2.5. Only the numbered lanes in the figure are used for further studies.

For three of the 19 species, namely *C. bolitinos*, *C.* sp. #89 and *C. tuttifrutti*, MT-COI DNA amplification was unsuccessful. Only one specimen of *C. bolitinos* and *C. tuttifrutti*, respectively, were collected in Namibia and several *C.* sp. #89 were collected. Lighter bands were also detected (Figure 2.5). Low DNA yield could be the main reason for unsuccessful amplification and practical errors before and after amplification could have occurred such as pipetting or contamination. Therefore, only 16 of the 19 species were sequenced. For the majority of species, DNA concentrations ranged from 1.11 ng/µl to 55.94 ng/µl. Even though some of the midge DNA concentrations were <10 µg/ul, the amplification technique still produced valuable data.

Midges identified morphologically were subjected to specific PCR using the universal MT-COI primers (Folmer *et al*., 1994). These markers are frequently used as an identification tool to clarify relationships between species and identify different *Culicoides* species (Harrup *et al*., 2015). The results of this study showed that the MT-COI gene can be used as a diagnostic tool for the identification of *Culicoides* species.

The molecular analysis done in this study confirmed the morphological identification, with parts of the Culicoides midges slide-mounted for future reference purposes.

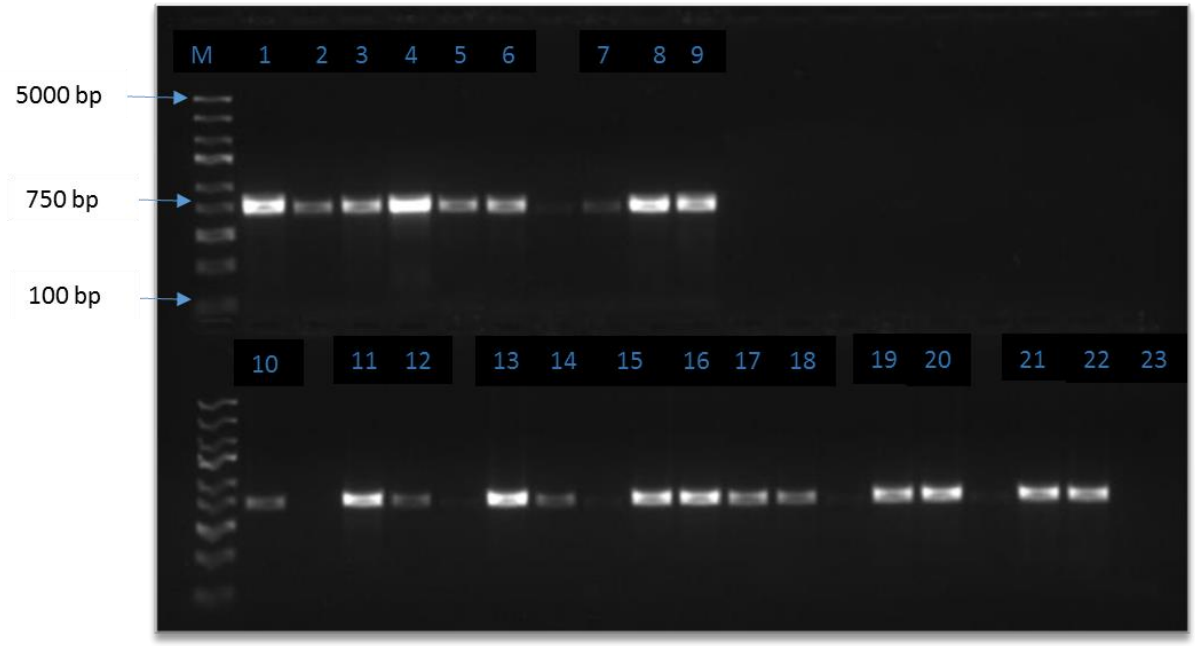


Figure 2.5: Agarose gel of PCR products from 16 different *Culicoides* species. Mitochondrial cytochrome oxidase subunit I gene amplification resulted in 750 base pair fragments. These species were collected in Namibia at different sampling sites. Lane M, molecular weight marker. Lane 1: *C*. sp. #61; Lane 2: *C. eriodendroni*; Lane 3: *C. enderleini*; Lane 4: *C. exspectator*; Lanes 5–6: *C. pycnostictus*; Lane 7: *C. herero*; Lane 8: *C. leucostictus*; Lane 9, *C. tropicalis*; Lane 10: *C. pretoriensis*; Lane 11–12: *C. punctithorax*; Lane 13: *C. schultzei*; Lane 14: *C. similis*; Lane 15–16: *C. imicola*; Lane 17–18: *C. ravus*; Lane 19–20: *C. nivosus*; Lane 21–22: *C. subschultzei*; Lane 23: non-template control PCR reaction with all components except template DNA. Sequence results obtained from these samples were used for phylogenetic analysis.

A novel DNA extraction method was described by Bellis *et al*. (2013), where midges are only partially destroyed or not at all. This is known as a non-destructive DNA extraction method; as a result, the whole midge can be mounted for more detailed taxonomic studies. This method described in Bellis *et al*., (2013) was among the methods tested before establishing a suitable methodology to extract DNA from midges in this study. Undesirable results were obtained with the use of the non-destruvtive method. Establishing a standard methodology for studies of vector competence in *Culicoides* species with reference to a non-destructive method would be helpful. If the whole specimen can be slide-mounted, more detailed studies on

species and species within specific areas worldwide can be done. Comparison of detailed taxonomic characteristics can showcase morphological differences with the same species collected from different areas.

2.4.3. DNA barcoding of Namibian *Culicoides* through sequencing of MT-COI gene

A total of 51 sequences were obtained from 16 different *Culicoides* species, with n indicating the number of specimens; *C. imicola* (n=5), *C. leucostictus* (n=5), *C. ravus* (n=5), *C. subschultzei* (n=5), *C. nivosus* (n=4), *C. schultzei* (n=4), *C*. sp. #61 (n=3), *C. eriodendroni* (n=3), *C. exspectator* (n=3), *C. pycnostictus* (n=3), *C. tropicalis* (n=3), *C. enderleini* (n=2), *C. similis* (n=2),*C. herero*. (n=1), *C. pretoriensis* (n=1), *C. punctithorax* (n=1). Of these 16 species, *C. enderleini*, *C. schultzei*, *C. similis*, *C. subschultzei* and *C. imicola* have been previously sequenced. The barcodes of these five species along with over 180 other different species can be found on the GenBank and BOLD system databases. Thus, the remaining 11 species (*C*. sp. #61, *C. eriodendroni*, *C. exspectator*, *C. herero*, *C. leucostictus*, *C. nivosus, C. punctithorax*, *C. pretoriensis*, *C. pycnostictus*, *C. ravus* and *C. tropicalis*) sequences are new to the taxonomy and phylogenetics of *Culicoides*. As these 11 Namibian *Culicoides* sequences have never been published and can be described as novel sequences, they will be submitted to GenBank. The BLAST search results of *Culicoides* species collected in Namibia can be found in Table 2.7. All the Namibian collected *Culicoides* sequences including the 11 novel sequences obtained from Namibian species can be found in Appendix A along with complete BLAST results of sequences.

The obtained sequences of *C. imicola* (highlighted in light grey) from Namibia had an identity match from 93% and greater, with the following GenBank accession numbers: KT945263.1, KT339721.1, KT339716.1 and KT339720.1. With the highest match being 99% (N1), all of these sequences were obtained from different study areas in Kenya, South Africa, Madagascar, Mauritius and the Balearic Islands (Onyango *et al*., 2015). The two specimens of *C. enderleini* had a 98% identity match and 0.0 E-value with the *C. enderleini* sequences found on GenBank (HQ447066.1). Some sequences were obtained in Reunion Islands and are unpublished data of Desvars *et al*. (2016). Sequences of Desvars *et al*. (2016) correlated with the unpublished data of Garros (2010) as well.

An affiliation of 88% and 89% and E-value of 0.0 was observed with published *C. similis* sequences (KT307844.1) by Harrup *et al*. (2016). These sequences of *C. similis* are from southern India and that could explain why the identity match is only 88% and 89%. Additionally, *C.* sp. #61 is closely related to *C. shivasi* (JX681734.1) with an identity percentage of 83% and undesirable E-values. Unplaced in a subgenus, *C. shivasi* belongs to the Immaculatus group (Bellis *et al*., 2013). These are Australian *Culicoides* species and have not yet been documented in southern Africa. Namibian species *C.* sp. #61 subgenus or group are undescribed. The BLAST results showed that these two species had very similar barcodes based on the MT-COI gene region. Identity match percentages of these species are in the low eighties, which is not a good theoretical match and may perhaps indicate ancestral descent or synonym species. This might also suggest a new subgenus group forming.

According to the BLAST search, *C. schultzei* (marked in grey; Table 2.7) did not relate to any *C. schultzei* sequences already in GenBank database. Two species sequences related 92% and one 88% to *C. oxystoma* (KT307836.1). Only one species, N21, had a 92% match with the sequences obtained from China (KF528694.1). All the *C. schultzei* specimens in this study except N21 were male specimens. The apicolateral process of *C. schultzei* separates this species clearly and easily from the other within the group morphologically.

All *C. subschultzei* (marked in grey; Table 2.7) BLAST results indicated a 92% to 93% match with *C. oxystoma* (KT307836.1) from southern India (published data) from Harrup *et al*. (2016). From results described above, *C. similis* also matched to *C. similis* species from southern India from the published data of Harrup *et al*. (2016). According to the results of *C. similis* and *C. subschultzei* a pattern formed and species collected in Namibia were found to have similar sequences to those

Table 2.7: Basic local alignment search tool results of sequences from Namibian *Culicoides* species. Compared with barcodes from GenBank (National Center for Biotechnology Information) database. Light grey highlighted cells indicate positive identification of morphologically identified species and reference species. Grey highlighted cells indicate negative identification of morphological identified species and reference species. Dark grey highlighted parts indicate identification match with another species. ID: identification.

SAMPLE ID	MORPHOLOGICAL ID	GEOGRAPHICAL ID	MOLECULAR ID (GenBank)	E-VALUE	IDENTITY MATCH	ACCESSION NUMBER
N*116**	*C.* sp. #61***	Windhoek	*Culicoides shivasi*	5,00E-155	83%	JX681734.1
N117	*C.* sp. #61	Windhoek	*Culicoides shivasi*	3,00E-162	83%	JX681734.1
N123	*C.* sp. #61	Okahandja	*Culicoides shivasi*	6,00E-154	83%	JX681734.1
N100	*C. enderleini*	Okahandja	*Culicoides enderleini*	0.0	98%	HQ447066.1
N101	*C. enderleini*	Okahandja	*Culicoides enderleini*	0.0	98%	HQ447066.1
N29	*C. eriodendroni*	Okahandja	*Nemophora metallica*	2,00E-148	84%	KX040182.1
N30	*C. eriodendroni*	Okahandja	*Nemophora metallica*	2,00E-163	84%	KX040182.1
N126	*C. eriodendroni*	Okahandja	*Taractrocera dolon*	6,00E-179	84%	KF391631.1
N24	*C. exspectator*	Okahandja	*Culicoides oxystoma*	9,00E-87	85%	KT307839.1
N25	*C. exspectator*	Okahandja	*Culicoides oxystoma*	5,00E-168	90%	KT307836.1
N26	*C. exspectator*	Okahandja	*Culicoides oxystoma*	2,00E-148	88%	KT307836.1
N161	*C. herero*	Windhoek	*Culicoides oxystoma*	2,00E-172	86%	KF528693.1

Table 2.7 (cont.): Basic local alignment search tool results of sequences from Namibian *Culicoides* species. Compared with barcodes from GenBank (National Center for Biotechnology Information) database.

N1	***C. imicola***	**Okahandja**	***Culicoides imicola***	**0.0**	**99%**	**KT945263.1**
N3	*C. imicola*	Okahandja	*Culicoides imicola*	0.0	93%	KT339721.1
N4	*C. imicola*	Okahandja	*Culicoides imicola*	0.0	94%	KT339721.1
N6	*C. imicola*	Okahandja	*Culicoides imicola*	0.0	93%	KT339716.1
N9	*C. imicola*	Okahandja	*Culicoides imicola*	0.0	93%	KT339720.1
N22	*C. leucostictus*	Okahandja	*Culicoides sonorensis*	0.0	87%	KR680744.1
N76	*C. leucostictus*	Okahandja	*Culicoides mesghali*	0.0	87%	KT307829.1
N97	*C. leucostictus*	Okahandja	*Culicoides oxystoma*	2,00E-163	86%	KT307835.1
N128	*C. leucostictus*	Okahandja	*Culicoides immaculatus*	0.0	87%	JX681720.1
N129	*C. leucostictus*	Okahandja	*Culicoides immaculatus*	0.0	87%	JX681720.1
N28	*C. nivosus*	Okahandja	*Culicoides peliliouensis*	0.0	90%	KT307854.1
N44	*C. nivosus*	Windhoek	*Culicoides peliliouensis*	0.0	88%	KT307854.1
N70	*C. nivosus*	Okahandja	*Culicoides peliliouensis*	0.0	89%	KT307854.1
N75	*C. nivosus*	Okahandja	*Culicoides peliliouensis*	0.0	89%	KT307854.1
N132	*C. pretoriensis*	Okahandja	*Culicoides mesghali*	0.0	86%	KT307832.1
N31	*C. punctithorax*	Okahandja	*Culicoides oxystoma*	0.0	93%	KT307836.1
N45	*C. pycnostictus*	Windhoek	*Culicoides sonorensis*	0.0	87%	KT794137.1
N108	*C. pycnostictus*	Windhoek	*Culicoides variipennis*	0.0	86%	KT794161.1
N127	*C. pycnostictus*	Okahandja	*Culicoides sonorensis*	0.0	87%	KT794137.1

Table 2.7 (cont.): Basic local alignment search tool results of sequences from Namibian *Culicoides* species. Compared with barcodes from GenBank (National Center for Biotechnology Information) database.

N151	***C. ravus***	**Okahandja**	***Culicoides oxystoma***	**5,00E-179**	**88%**	**KT307840.1**
N152	*C. ravus*	Okahandja	*Culicoides* sp.	0.0	88%	KM987986.1
N157	*C. ravus*	Windhoek	*Culicoides* sp.	1,00E-180	87%	KR695194.1
N158	*C. ravus*	Windhoek	*Culicoides oxystoma*	0.0	87%	KF528693.1
N159	*C. ravus*	Windhoek	*Culicoides oxystoma*	0.0	88%	KF528693.1
N21	*C. schultzei*	Okahandja	*Culicoides oxystoma*	0.0	92%	KF528694.1
N103	*C. schultzei*	Okahandja	*Culicoides oxystoma*	0.0	92%	KT307836.1
N104	*C. schultzei*	Okahandja	*Culicoides oxystoma*	0.0	88%	KT307836.1
N105	*C. schultzei*	Okahandja	*Culicoides oxystoma*	0.0	92%	KT307836.1
N78	*C. similis*	Okahandja	*Culicoides similis*	0.0	88%	KT307844.1
N124	*C. similis*	Okahandja	*Culicoides similis*	0.0	89%	KT307844.1
N11	*C. subschultzei*	Okahandja	*Culicoides oxystoma*	0.0	93%	KT307836.1
N13	*C. subschultzei*	Okahandja	*Culicoides oxystoma*	0.0	93%	KT307836.1
N14	*C. subschultzei*	Okahandja	*Culicoides oxystoma*	0.0	93%	KT307836.1
N16	*C. subschultzei*	Okahandja	*Culicoides oxystoma*	0.0	92%	KT307836.1
N19	*C. subschultzei*	Okahandja	*Culicoides oxystoma*	0.0	92%	KT307836.1
N93	*C. tropicalis*	Windhoek	*Culicoides* sp.	2,00E-142	83%	KM904453.1
N64	*C. tropicalis*	Windhoek	*Culicoides oxystoma*	0.0	88%	KT307839.1
N118	*C. tropicalis*	Windhoek	*Culicoides* sp.	1,00E-175	87%	KR686414.1

**N stands for Namibia*
***Number indicates the sample number after identification*
****Numbered species (#) indicates that this species is yet to be described.*

from southern India, all with E-values of 0.0. From the results it can further be derived that *C. oxystoma* is a relative of the *C. schultzei* and *C. subschultzei* specimens from Namibia. All three of these species are part of the subgenus group, Remmia and Schultzei groups. According to Boorman (1989), cited by Bakhoum *et al*. (2013), *C. schultzei* or the Schultzei group from northern Africa towards India, is spoken of as *C. oxystoma*.

Other species also showed analogous relations to *C. oxystoma*. Namibian *C. exspectator* showed an 85% (KT307839.1), 88% and 90% (KT307836.1) similarity to the unpublished sequences of *C. oxystoma* from southern India (Harrup *et al*., 2016), all of which have adverse E-values. Morphologically identified *C. herero* matched 86% with *C. oxystoma* (KF528693.1) and *C. punctithorax* matched 93% with *C. oxystoma* (KT307836.1) on a molecular level.

Culicoides pycnostictus had an 87% identity match with *C. sonorensis* (KT794159.1 and KT794137.1). However, *C. sonorensis* is placed in the *Monoculicoides* Khalaf subgenus, a member of the *C. variipennis* complex: which can explain the identity match of 86% to *C. variipennis*, whereas *C. pycnostictus* forms part of the *Beltranmyia* subgenus with an unspecified group. Furthermore, *C. tropicalis* sequences indicated relations with both *Culicoides* sp. (unpublished sequence; KM904453.1) and Herbert *et al*. (2016) (KR686414.1), where both sequences were obtained from Canadian specimens. This Namibian species also relates 87% to C*. oxystoma* (KT307839.1) species from Harrup *et al*. (2016). The species *C. ravus*, also matched to these two species but with different accession sequences: *C. oxystoma* (KT307837.1 and KF528693.1) and *Culicoides* sp. (KM987986.1 and KR695194.1). Sequences of *C. nivosus* also revealed lineage with *C. oxystoma* (KT307837.1 and KF528693.1) and *Culicoides* sp. (KM987986.1 and KR695194.1).

The one *C. pretoriensis* specimen matched 86% to unpublished *C. mesghalii* (KT307832.1) collected in southern India by Harrup *et al*. (2016). Four of the five *C. leucostictus* identity matches were different, with common relations of 87% to *C. sonorensis* (KR680744.1) sequenced by Herbert *et al*. (2016), two specimens matched 87% with *C. immaculatus* (JX681720.1) collected in Australia by Bellis *et al*. (2013), one matched 87% with *C. mesghalii* (KT307829.1) and 86% with *C. oxystoma* (KT307835.1), both of the latter being unpublished sequences from southern India (Harrup *et al*., 2016). In total, three different continents were identified as collection

sites of these species sequences. This might indicate synapomorphies between these species and regions.

The obtained sequences of *C. eriodendroni* shown in dark grey did not relate with any *Culicoides* species. Two species matched 84% with *Nemophora metallica* (KX040182.1) and one with *Taractrocera dolon* (KF391631.1). *Taractrocera dolon* is better known as the sandy-grass dart butterfly of the Hesperiidae family. It can be found in the Northern Territory, Queensland and New South Wales in Australia as well as Papua New Guinea. The moth species *Nemophora metallica* is from the family Adelidae and can be found in Europe. Namibian *C. eriodendroni* species might have a closer association with the moth and butterfly family than with Ceratopogonidae.

Researchers do not indicate in their papers where voucher specimens are housed or whether they even keep it at all. This prevents future studies from checking uncertain species identification and interpret discrepancies, contributing to the chaotic state of *Culicoides* taxonomy. Percentages of the results found are not ideal, but it can still be an indication of same descendants and species. Nevertheless, these identity matches with the published sequences are sufficient enough to state that they are essentially the same species as those found in the databases. Due to the regional differences within all of the species, inter-species evolution could have taken place in the area compound.

However, it can be said that the species in this study has a wide range of phenotypic variation. BLAST results did not provide identification with high confidence levels, due to the scarce database, but correlations between species and subgenus groups could be drawn. These results are a good indication that species from southern Africa and surrounding islands are closely related to a different species than expected from northern Africa and other parts of the world. Future research must be done on a larger scale to reduce the uncertainty of *Culicoides* species classification worldwide.

2.4.4. Alignment of sequences for the phylogenetic tree

The sequences in this study represented both those of previously described *Culicoides* species, including vector species of AHSV and novel DNA sequences. Sequences used in the final dataset for the Namibian *Culicoides* phylogenetic tree were between 503 and 620 bp in length and corresponded to nucleotide positions 514 to 1 399 of the MT-COI gene. All sequences were manually aligned and sequences that were too long

were deleted at the 5' or 3' ends. This analysis involved 64 nucleotide sequences, with some of the positions containing gaps and missing data were eliminated, to yield the most efficient alignment. The elimination of gaps and differences in sequence length would imply, that even if sequences are similar to one another they might be grouped apart. This notation can be seen in Figure 2.6. Reference sequences of *Culicoides* species that were found on GenBank and BOLD that are referred to in this study and that are used in Figure 2.6 can be found in Appendix B.

A neighbour-joining phylogenetic tree was constructed using MEGA 7. The phylogenetic tree in Figure 2.6 illustrates the relationship of *Culicoides* species collected in Namibia during April 2016 in Windhoek and Okahandja, with reference sequences of five published species. The species names in the phylogenetic tree were obtained through morphological identification. In this tree, an Anopheles gambiae (mosquito) isolate was chosen as an outgroup. The genus *Culicoides* and *Anopheles* falls within the same family (Ceratopogonidae).

Figure 2.6 shows three groups of *Culicoides* midges forming in the tree. Although three separate groups are formed, the genetic distances within the species are not significant. The scale in the tree is only 0.050, which indicates less than 1% genetic change, demonstrating that there is a common evolutionary ancestor and that they are descendant groups. This points towards an insignificant genetic change within the species in this study. Bootstrap values in the tree were good, with the majority of species groupings between 97% and 100%.

According to Meiswinkel (1996) classification of subgenus and groups, the phylogenetic tree can be discussed as follows. All the Schultzei group species in this study clustered together with two outlier species, namely *C. punctithorax* and *C. exspectator*. *Culicoides subschultzei* (N11, N16, N14, N13 and N19) grouped with the reference *C. subschultzei* isolate (KF682525.1) found in Senegal with a bootstrap of 100% (Bakhoum *et al*., 2013). An outlier of *C. punctithorax* (N31) could be detected within the *C. subschultzei* grouping. Although C*. punctithorax* subgenus or group is undescribed, it cannot be concluded that the species falls under the Schultzei group. According to the results, *C. subschultzei* and *C. enderleini* formed a monophyletic cluster. Reference species from GenBank and Namibian species of *C. enderleini* (KF682528.1, KF682473.1 and KF682479.1) from the published work of Bakhoum *et*

al. (2013) and KJ833701.1 sequences from Senegal (Sambou *et al*., 2015) grouped with each other, forming a clade.

Although *C. schultzei* and *C. expectator* do not fall under the same subgenus, one *C. expectator* specimen grouped with three *C. schultzei* specimens with a bootstrap of 100%; this points towards common ancestors. Theoretically, these species cannot group with each other and do not have a sibling relationship. As can be seen, *C. subschultzei*, *C. schultzei* and *C. enderleini* the close grouping of the mentioned species indicated that they were descendants for a common ancestor. This may have been due to the species belonging to the same subgenus, *Remmia*. A repetitive pattern of 100% bootstrap between two species that are not sibling species were seen again within the group of *C. tropicalis.* Namibian *C. schultzei* (N104) from the *Remmia* subgenus formed a group with *C. tropicalis* from the subgenus *Synhelea* Kieffer. A close relationship could be seen between *C. tropicalis*, *C. schultzei* and *C. exspectator* (N24). From sequences obtained, the other two *C. exspectator* (N25 and N26) specimens grouped together with a 100% bootstrap value in a more distant group.

Culicoides similis (N78 and N124) grouped with the reference species with a 97% bootstrap. The *C. similis* Namibian species presents a closer relationship with *C. leucostictus* than with other species within the Similis group. All four of the *C. nivosus* specimens grouped together with a 100% bootstrap value, indicating a similar genetic variation within the MT-COI gene. *Culicoides nivosus*, which is grouped morphologically with the *Beltranmyia* Vargas subgenus (Meiswinkel, 1996) did not cluster with other species of the group. *Culicoides pretoriensis* and one *C. leucostictus* specimen grouped together with a 100% bootstrap value, having a close relationship with *C. exspectator* and *C. nivosus*. Thus, it can be said that *C. pretoriensis* may not be morphologically similar, but rather that on molecular level they are closely related to *C. leucostictus*.

Four of the five *C. leucostictus* species formed a cluster with high bootstrap values, with a close relationship of 100% to *C. pretoriensis*. *Culicoides ravus* (N152, N159, N157, N158 and N151) are grouped together with good bootstrap values, forming a monophyletic cluster with *C. pycnostictus* and the only *C. herero* (N161) specimen. According to Figure 2.6, *C. herero* is closely related to these two species. In contrast to their subgenus classification, this can be an indication of their being common ancestral descendants. The *C. herero* sequence obtained in this study was a novel

sequence. Catchments of this species are very rare as indicated by Becker *et al*. (2012, 2013), Goffredo *et al*. (2015) and Liebenberg *et al*. (2016).

A separate cluster was formed for the *C. imicola* specimens and *C. bolitinos*. The former grouped together forming a monophyletic cluster with published sequences from GenBank. *Culicoides bolitinos* grouped within the cluster with *C. imicola*. These results exemplify the precision of the methodology used in this study, attributable to the groupings/clusters of the same subgenus and groups the formed. Finally, *C.* sp. #61 and *C. eriodendroni* formed the third separate cluster within the tree. No barcodes have yet been published on *C.* sp. #61 and *C. eriodendroni* and both these species remain unspecified. These two species appear to have a monophyletic relationship and are closely related to the Imicola group.

The neighbour-joining phylogenetic tree in Figure 2.7 compares sequences based on the *Culicoides* species collected in Namibia and nucleotide sequences available from BOLD and GenBank. Note that the sequences from the bioinformatics systems are not all Afrotropical *Culicoides*. Figure 2.6 is a simplified version of Figure 2.7 for illustration of the relationships of the Namibian specimens. Figure 2.7 provides a complete illustration of relationships between all published sequences. GenBank and BOLD sequences were included in the phylogenetic analysis for the assessment of morphological misidentification, misclassification or misinterpretation of species relationships. Thus, eliminating confusion of morphologically similar species.

The expected result of *C. imicola* grouping together, forming a monophyletic cluster with published sequences from GenBank was obtained. Within this tree, *C. imicola* was closely related to *C. bolitinos*, *C. loxodontis*, *C. pseudopallidipennis*, *C. tuttifrutti*, *C. kwagga*, *C. asiana* and *C. brevitarsis*. These species formed a distinct cluster within the tree. Sequences of Namibian *C. enderleini* grouped together with published *C. enderleini* (GenBank: KF682528.1, KF682473.1, KF682479.1 and KJ833701.1) sequences, with a bootstrap value of 100%. The *C. subschultzei* specimens also grouped with the reference published species from GenBank (KF682525.1) found in Senegal (Bakhoum *et al*., 2013), with an outlier of *C. punctithorax* in the grouping, as could also be seen in Figure 2.6.

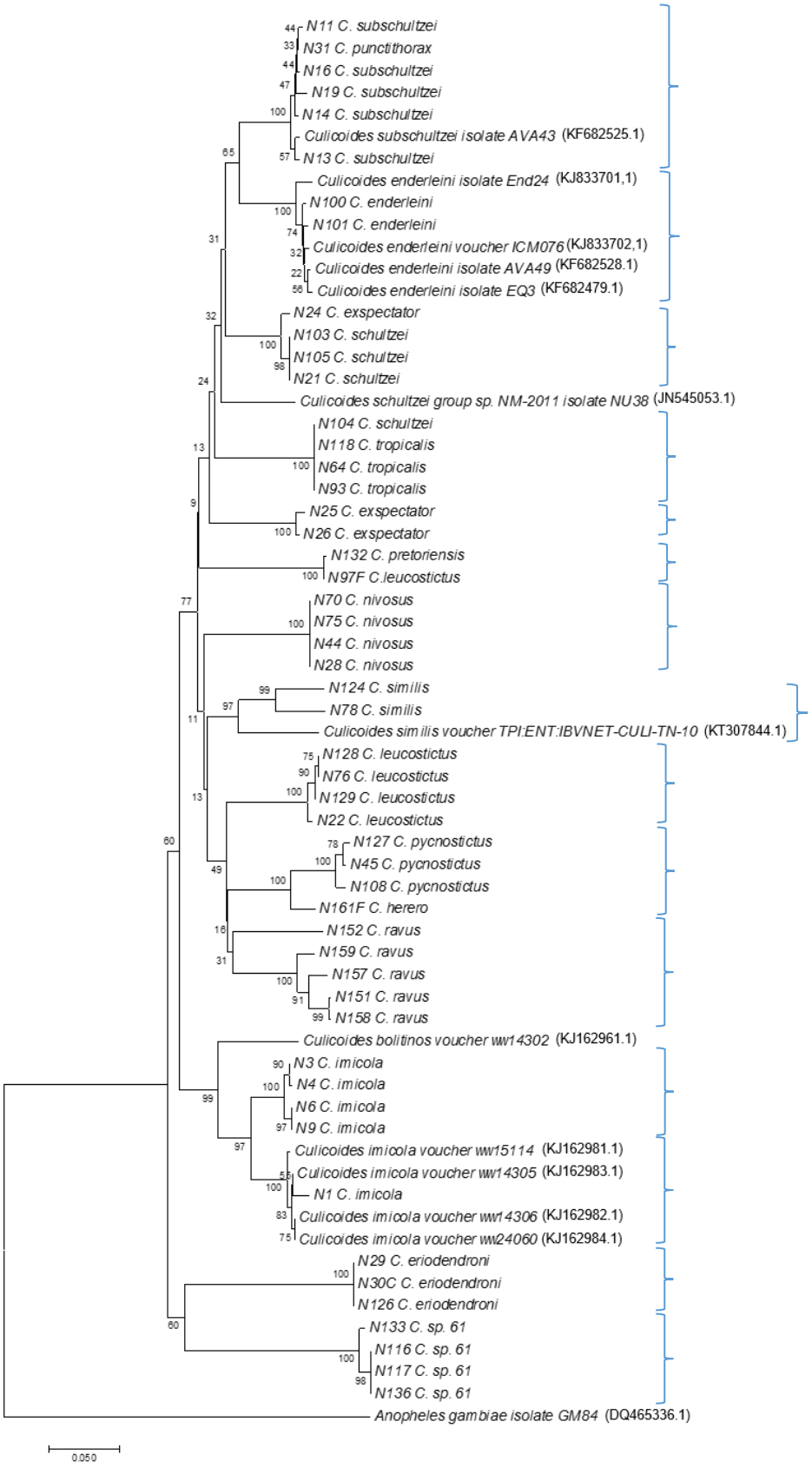


Figure 2.6: Neighbour-joining phylogenetic tree based on *Culicoides* species collected in Namibia during April 2016 in Windhoek and Okahandja. N: sample identification number of specimens collected in Namibia.

A close relationship was observed between *C. subschultzei* and *C. schultzei* with *C. nevelli* (BOLD: GBCER132-15) and *C. oxystoma* (GenBank: KF682533.1), as described and discussed in Section 2.4.3. This grouping indicated a positive morphological identification of species. In reference to Figure 2.7, all the *Remmia* subgenus species grouped together in this combined tree. From the tree, it is evident that Namibian *C. schultzei* formed a separate group from the reference sequenced species from Israel (GenBank: JN545053.1) published by Morag *et al*. (2012). An outlier can again be noticed, which includes *C. exspectator* (N24).

Culicoides similis specimens (N78 and N124) were identified correctly, according to the group formed with published *C. similis* (BOLD: CULIN010-14; GenBank: KT307844.1) with a bootstrap value of 84%. Unpredictably, however, *C. similis* was closely related to *C. pretoriensis* and one of the *C. leucostictus* specimens (N97) sequences. Results obtained by phylogenetic analysis demonstrated that all the *C. tropicalis* species grouped together. Noticeably, one *C. schultzei* specimen grouped with *C. tropicalis* with a bootstrap value of 100%. According to their alignment, these species were closely related to published sequenced *C. creouscularis* (BOLD: NGAAA832-14), *C. fagineus* and *C. truncorum*, forming a monophyletic cluster. The only *C. herero* specimen grouped together with *C. pycnostictus*, with a bootstrap of 99%, forming a relationship with *C. mulrennani* (BOLD: CNGBK397-14). These three species had a monophyletic relationship with sequenced species *C. furcillatus* (GenBank: KJ624083.1), *C. arakawae* (BOLD: GBCER008) and *C. odiatus* (BOLD: GBCER108-15).

Culicoides exspectator (N25 and N26) showing a bootstrap value of 100%, grouping with *C. japonicus* (BOLD: GBDP4698-08) and forming a monophyletic cluster with *C. nivosus*, *C. pelilioensis* (BOLD: CULIN033-14) and *C. dubius* (BOLD: GBDP4700-08). All the *C. nivosus* specimens aligned 100% with each other with a 100% bootstrap value. One of the *C. ravus* specimens (N152) falls within another cluster and not with the rest of the *C. ravus* specimens. According to the results, *C. ravus* had a close relationship with *C. kepongensis* (BOLD: CULIN057-14). Four of the five *C. leucostictus* specimens (N22, N76, N129 and N128) (bootstrap value of 100%) formed a monophyletic cluster with *C. immaculatus* (GenBank: JX681722.1), *C. shivasi* (GenBank: JX681735.1), collected in Australia and *C. subfagineus* (BOLD: GBDP8748-10, GenBank: GQ338927), collected in Spain. However, the *C. eriodendroni* specimens from Namibia grouped with *C. wansoni* (GenBank:

KJ833687.1) and *C. milnei* (BOLD: GBCER135-15), hence clustering with *C*. sp. #61. The specimens of the unspecified species *C.* sp. #61 showed a 100% resemblance to each other.

Interpretation of phylogenetic relationships within the *Culicoides* genus was done in recently published papers by using genomic methods (Ander *et al*., 2013; Augot *et al*., 2013; Bakhoum *et al*., 2013; Lassen *et al*., 2012; Linton *et al*., 2002; Morag *et al*., 2012; Schwenkenbecher *et al*., 2008). As described in literature, by using the MT-COI gene, useful sequences for phylogenetic analyses at species levels can be produced (Folmer *et al*., 1994). According to Ander *et al*. (2013), 95% of species studied can be distinguished by the use of MT-COI barcodes. Thus, MT-COI DNA barcoding can be the solution for problematic species identification. By comparing morphological and molecular data such as sequencing, more information of individual midges can be obtained.

Information obtained from the present study showed that the groupings and clusters of Namibian *Culicoides* midges were almost identical to those of Meiswinkel (1996). Different ancestral background from the other reference species that were not classified as Afrotropical species could have played a role in the groupings and classification within the tree in Figure 2.6. Theoretically, species in the same genus must have nearly identical partial gene sequences of a specific part of the genome. Nonetheless, this is not always the case practically.

Although some species formed distinct groups, sequences of the same species can vary according to this study. Some species grouped perfectly together with a high bootstrap value and some had an outlier of one or two dissimilar species. Thus, species that were morphologically identified as similar, in some cases did not group together. Subgenus group relationships were observed between the species in the two phylogenetic trees. However, formation of new groupings with species that do not fall in the same subgenus classification according to Meiswinkel (1996) were also observed.

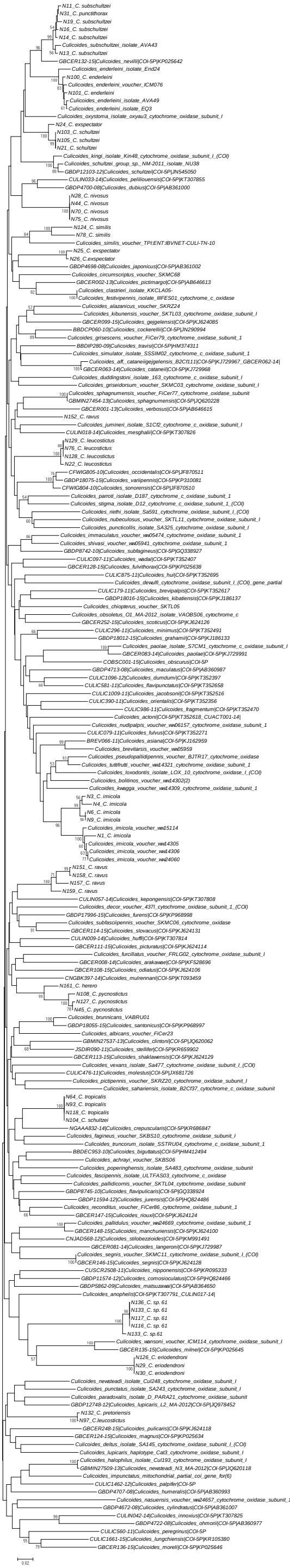


Figure 2.7: Neighbour-joining phylogenetic tree based on the comparison of *Culicoides* species collected in Namibia and nucleotide sequences available from BOLD and GenBank. N: sample identification number of specimens collected in Namibia.

Most of the species grouped with reference sequences from the studies of Bakhoum *et al*. (2013), Bellis *et al*. (2013), Harrup *et al*. (2016) and Sambou *et al*. (2015). Relationship-forming was observed with reference species from southern India, Australia and Senegal; the Schultzei group was studied by Bakhoum *et al*. (2013) in Senegal and sub-Saharan Africa. According to this study, genetic similarities occurred with the Oriental and Australian specimens and this indicated that differences from Afrotropical species were not that large. This pattern could also be seen in the present study.The geographical distribution of these midges can be the cause of some irregular groupings. Genetic mutation and evolution can take place in a specific community or area, leading to intra-species genetic changes (Ramel, 1998). Each area of collection had dissimilar environments, animal surroundings, life cycle conditions and feeding habits that are far removed from those of midges found in Namibia. The reference species used in both Figures 2.6 and 2.7 were all from different parts of the world.

As explained by Harrup *et al*. (2015), specific area assessments formed the basis of contributions to subgenera classification. Insufficient attempts to validate groupings with those from other areas have been seen in former studies. Gomulski *et al*. (2006) and Schwenkenbecher *et al*. (2009) both proposed polyphyletic occurrences in current subgenera, explaining that they descend from one or more common ancestor (Perrin *et al*., 2006), with other species possibly being synonymous in reference to morphological characteristics that are perceived in mounting evidence. In conclusion, *Culicoides* species from Namibia warrants further investigation because of their wide phenotypic variation. Some species occur from the Afrotropical regions to the Orient and Australia.

CHAPTER 3: DEVELOPMENT OF A SIMPLIFIED NUCLEIC ACID DIAGNOSTIC TOOL FOR THE DETECTION OF AHSV

3.1 METHODOLOGY FOR DIAGNOSTIC TOOL

For many years PCR-based methods were broadly used for detection and identification of viruses, bacteria and fungi due to their simplicity and reliability (Chan & Fox, 1999). Polymerase chain reaction (PCR) is one of the most used molecular diagnostic tools worldwide. Numerous forms of PCR have been developed, e.g. reverse-transcription PCR (RT-PCR), Multiplex PCR and Nested PCR (Ratcliff *et al*., 2007). The loop-mediated isothermal amplification (LAMP) method was designed by Notomi *et al*. (2000). This method has been used in recent years as a diagnostic tool for the detection and identification of viruses and other bacterial diseases.

3.2 LAMP PRINCIPLE

Loop-mediated isothermal amplification (LAMP) is a novel real-time amplification method developed by Notomi *et al*. (2000) and can amplify DNA/RNA under isothermal conditions rapidly (Bi *et al*., 2012), efficiently, with a high specificity (Notomi *et al*., 2000; Parida *et al*., 2004; Chen *et al*., 2008) and cost-effectively. The optimal isothermal conditions vary between 60 and 65ºC with incubation time varying from 15 to 60 minutes (Eiken Chemical Co Ltd, 2016; Notomi *et al*., 2000). This one-step method uses four specially designed primers that recognise six distinct regions on target sequences (Parida *et al*., 2004; Parida, 2008; Notomi *et al*., 2000).

Loop-mediated isothermal amplification (LAMP) primers are designed to be sensitive and specific. Primer design can be done by using the Primer Explorer Software (Net Laboratory, Japan). This programme allows the user to select specific target regions with GC-content, secondary structure formation and base composition. There are four types of primers, namely the Forward Inner Primer (FIP), Forward Outer Primer (F3), Backwards Inner Primer (BIP) and Backwards Outer Primer (B3). LAMP can be accelerated by using two loop primers: Forward Loop Primer (FLP) and Backwards Loop Primer (BLP). In so doing, the amplification time of the LAMP reaction is reduced (Nagamine *et al*., 2002).

The reaction is set up in one tube with DNA/RNA (sample), sets of primers, dTNPs, $Mg^{2+,}$ reverse transcriptase (RNA) and *Bst* DNA polymerase. The real-time LAMP (RT-LAMP) method is similar to LAMP, with the only difference that the uracil on the target RNA sequence will be transcribed into thymine (Eiken Chemical Co Ltd, 2016). LAMP

uses strand displacement activity (Chai *et al*., 2008; Parida, 2008) of DNA polymerase and uracil-forming primers (Figure 3.1). A large amount of target DNA is amplified (three-fold for every half cycle) according to Notomi *et al*. (2000). During LAMP, by-products such as magnesium pyrophosphates can build up causing turbidity (Dai *et al*., 2012; Parida, 2008). Measuring increased turbidity can be done in real-time to monitor LAMP product formation (Mori *et al*., 2001; Parida *et al*., 2004; Parida, 2008; Dai *et al*., 2012; Chen *et al*., 2008).

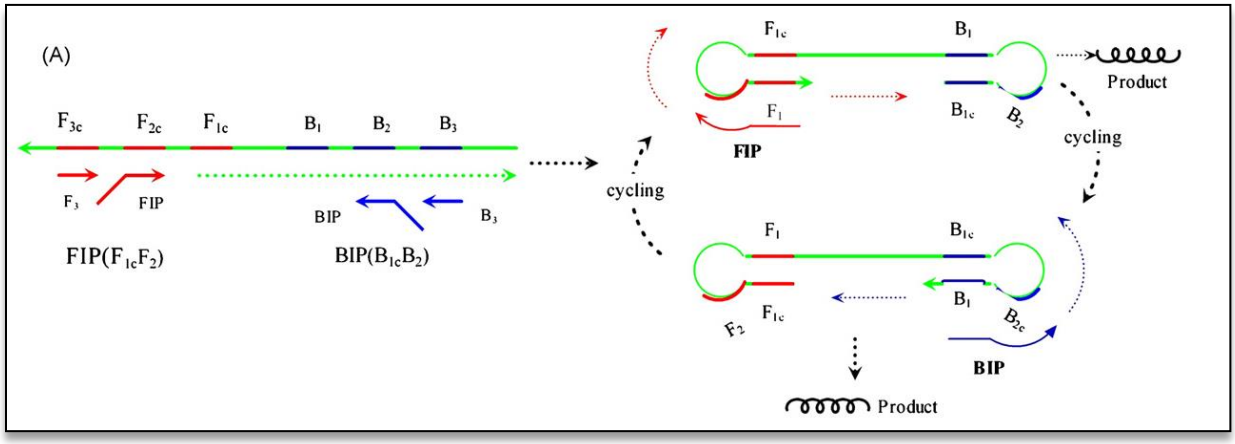


Figure 3.1: Illustration of strand displacement activity of DNA polymerase and uracil-forming primers (Source: Chai *et al*., 2008).

3.3 MECHANISMS OF LAMP

3.3.1 Non-cyclic step

At a constant temperature between 60 and 65ºC, a complimentary sequence of target DNA anneals to one of the LAMP primers (F3) (Figure 3.2). DNA polymerase with strand displacement activity initiates DNA synthesis where displacement and releasing of a single-stranded template DNA take place (Notomi *et al*., 2000; Ushikubo, 2004) (Figure 3.2a). Synthesis starts from the 3'-end of the F3e region of the target DNA annealing with F3 primer (Figure 3.2b). The FIP-linked complementary strand is released through strand displacement when, outside the FIP, the F3 primer anneals to the F3c region (Figure 3.2c). From the F3 primer and template DNA, a double strand is formed (Figure 3.2d). At the 5'-end of the released FIP-linked complementary strand (Figure 3.2d), a stem-loop structure is formed because of the complementary F1c and F1 regions.

Synthesis of complementary DNA takes place again at the 3'-end of the B3 and it anneals to the single-strand DNA produced as shown in Figure 3.2e. After this process

is completed, the loop structure of the DNA returns into a linear structure. DNA polymerase activity at the 3'-end, outside the BIP, anneals the B3 primer (Figure 3.2f). BIP complementary single strand is displaced and released through strand displacement DNA synthesis before DNA synthesis from the B3 primer (Figure 3.2g). Through these processes, double stranded DNA is produced (Figure 3.2h). A stem-loop structure is formed at each end (Figure 3.2i) through BIP-linked complementary strand displacement process (Figure 3.2g). This structure serves as the starting structure for the amplification cycle in the LAMP method (LAMP cycling).

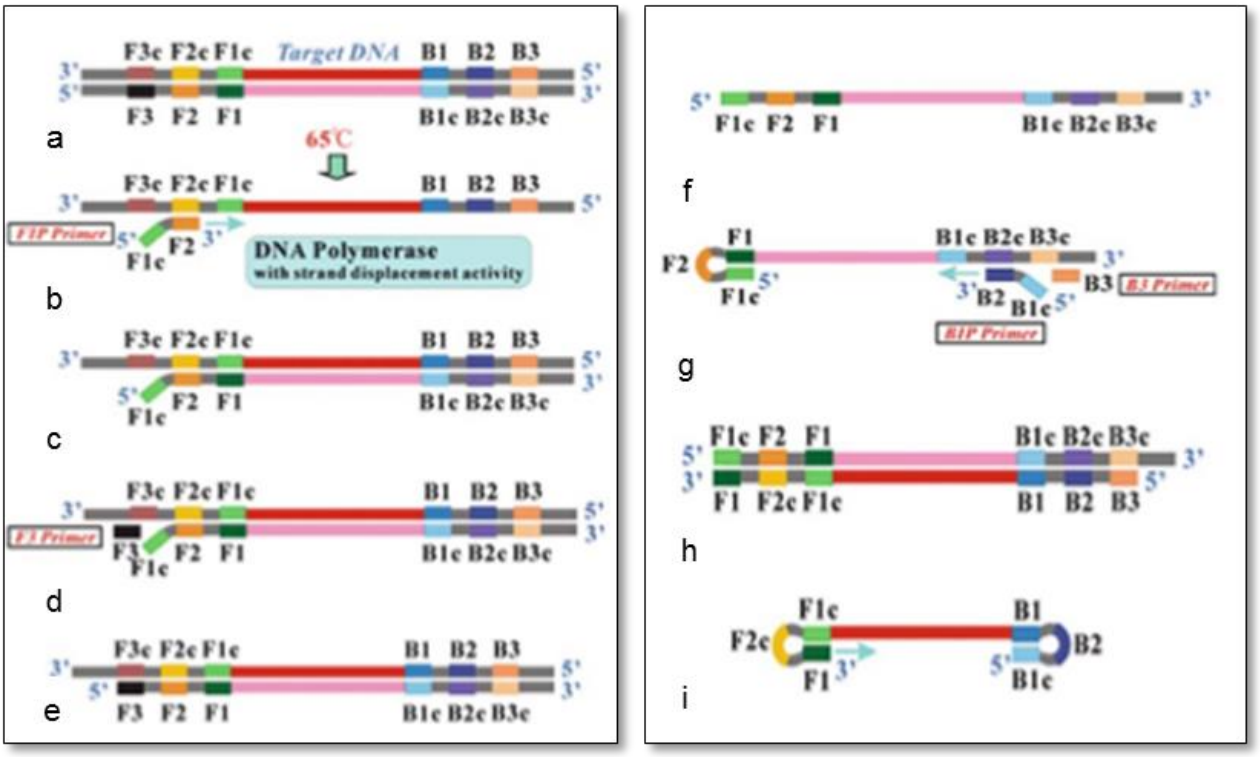


Figure 3.2: Non-cyclic steps of the loop-mediated isothermal amplification principle (Source: Eiken Chemical Co Ltd, 2016).

3.3.2 Cyclic amplification

A new stem-loop DNA is yielded through hybridisation of the inner primer, which initiates the displacement DNA synthesis from the stem-loop structure in Figure 3.3j. The previously synthesised strand is released through the FIP primer that anneals to the single-strand region of stem-loop DNA and primer strand displacement DNA synthesis. Because of the complementary B1c and B1 regions, the released single-strand forms a stem-loop at the 3'-end. The FIP-linked complementary strand is released through DNA synthesis, starting at the 3'-end of the B1 region using self-structure as a template (Figure 3.3k). At step m (Figure 3.3), both ends now have

complementary regions (F1–F1c and B1c–B1) and forms a turn-over structure of step k.

The B1-primed DNA strand is released through the annealing of BIP to the B2c region and primes strand displacement DNA synthesis. BIP anneals to the single-stranded B2c region, displacing the double-stranded DNA sequence through DNA synthesis and the structure shown in Figure 3.3l is produced. Numerous cised stem-loop DNAs with a number of alternately inverted repetitions of the target sequence are produced during this process.

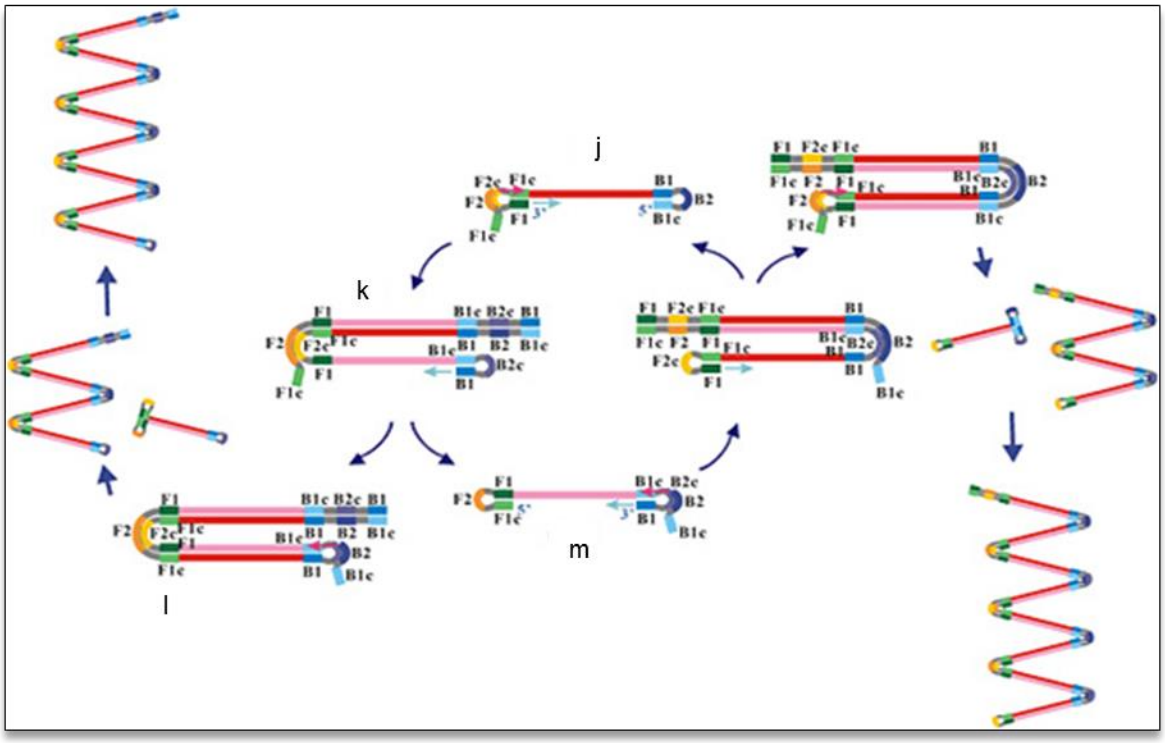


Figure 3.3: Cyclic steps of the loop-mediated isothermal amplification principle principle (Source: Eiken Chemical Co Ltd, 2016).

3.3.3 Applications of LAMP

Before LAMP was developed, nucleic acid sequence-based amplification (Compton, 1999, cited by Notomi *et al*., 2000; Cai *et al*., 2008; Parida, 2008), transcription-mediated amplification, rolling circle amplification (Cai *et al*., 2008), strand displacement amplification (Walker *et al*., 1992a, 1992b; Parida, 2008), self-sustained sequenced replication (Guatelli *et al*., 1990, cited by Notomi *et al*., 2000; Parida, 2008) and PCR (Perida, 2008) was first used as alternative methods to detect both human and veterinary pathogens.

The field of microbiology has applied the LAMP method on bacteria (Pan *et al*., 2011), viruses (Parida *et al*., 2004), fungi (Niessen & Vogel, 2010) and recently on plant pathogens (Dai *et al*., 2012). Previous studies have explored the various applications of LAMP (Table 3.1). Detection of viruses through the LAMP method is efficient because it can be implemented with limited sophisticated equipment. A simple aluminium heating block and/or water bath can be used in the place of a turbidity machine (Bi *et al*., 2012; Perida *et al*., 2008). A turbidity machine is a device specially designed for the LAMP method with a preset time and temperature setting. Detection of the amplification by-product can be done through monitoring the white turbidity formation, simultaneously. LAMP can be viewed as a low-cost method, with few requirements for special reagents and a temperature cyclic device.

Because of LAMP's simplicity and sensitivity, it can be used in field testing. A higher copy number can be detected by means of this method in accordance with previously mentioned methods. Almost all LAMP methods can amplify a target nucleic acid within an hour (Notomi *et al*., 2000; Parida *et al*., 2008). Visual detection of amplified product can be done in various ways. In a laboratory setup, gel electrophoresis can be used (Ma *et al*., 2010; Dai *et al*., 2012). Visual detection of amplified product can also be done by viewing LAMP tubes under a UV-light to identify any colour changes in the reaction by including fluorescent dye such as ethidium bromide (Parida, 2008), SYBR Green I (Poon *et al*., 2006) or calcein (Boehme *et al*., 2007). No colour change (orange) indicates negative results, whereas positive results are indicated by a change to bright green.

Table 3.1: Several investigations of the loop-mediated isothermal amplification (LAMP) technique have reported on its application in various fields.

Year	Publication	Notes
2000	Loop-mediated isothermal amplification of DNA (Notomi *et al*., 2000)	Hepatitis B virus (HBV)
2001	Loop-mediated isothermal amplification reaction using a non-denatured template (Nagamine *et al*., 2001/2002)	Non-denatured template
2003	Validation of the loop-mediated isothermal amplification method for single nucleotide polymorphism genotyping with whole blood (Iwasaki *et al*., 2003)	Single nucleotide polymorphism genotyping
2004	Real-time turbidimetry of LAMP reaction for quantifying template DNA (Mori *et al*., 2001, 2004)	Plasmid DNA of HBV and hepatitis C virus
2004	Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus (Hong *et al*., 2004)	Severe acute respiratory syndrome coronavirus
2004	Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus (Parida *et al*., 2004)	West Nile virus
2004	Rapid sexing of bovine preimplantation embryos using loop-mediated isothermal amplification (Hirayama *et al*., 2004)	Sexing of fertilised eggs in cow in vitro fertilisation
2006	Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification (Poon *et al*., 2006)	Malaria: *Plasmodium falciparum* DNA
2006	Rapid detection of norovirus from faecal specimens by real-time reverse transcription-loop mediated isothermal amplification assay (Fukuda *et al*., 2007)	Norovirus from faecal specimens
2007	Development of a loop-mediated isothermal amplification assay for rapid detection of BK virus (Bista *et al*., 2007)	BK virus (polyomavirus)

Table 3.1 (cont.): Several investigations of the loop-mediated isothermal amplification (LAMP) technique have reported on its application in various fields.

2007	Rapid diagnosis of H5N1 avian influenza virus infection by newly developed influenza H5 hemagglutinin gene-specific loop-mediated isothermal amplification method (Imai *et al*., 2007)	H5N1 avian influenza virus, A(H5N1)
2007	Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses (Yoda *et al*., 2007)	Norovirus detection
2007	Preliminary application and evaluation of loop-mediated isothermal amplification (LAMP) for detection of bovine theileriosis and trypanosomosis in Tanzania (Thekisoe *et al*., 2007)	Bovine theileriosis and trypanosomosis detection
2008	Development and evaluation of real-time loop-mediated isothermal amplification for hepatitis B virus DNA quantification: A new tool for HBV management (Cai *et al*., 2008)	Quantifying HBV
2008	African trypanosomiasis: Sensitive and rapid detection of the sub-genus Trypanozoon by loop-mediated isothermal amplification (LAMP) of parasite DNA (Njiru *et al*., 2008)	Human African trypanosomiasis (HAT) detection
2008	Development of reverse transcription loop-mediated isothermal amplification for rapid detection of H9 avian influenza virus (Chen *et al*., 2008)	H9 influenza virus detection
2008	Loop-mediated isothermal amplification assay for rapid detection of common strains of *Escherichia coli* (Hill *et al*., 2008)	Detection of *Escherichia coli* strains
2008	Discriminating between varicella-zoster virus vaccine and wild-type strains by loop-mediated isothermal amplification (Higashimoto *et al*., 2008)	Varicella-zoster virus and wild-type strains
2010	Evaluation of a loop-mediated isothermal amplification method as a tool for diagnosis of infection by the zoonotic simian malaria parasite *Plasmodium knowlesi* (Iseki *et al*., 2010)	Malaria: *Plasmodium knowlesi* diagnosis

Table 3.1 (cont.): Several investigations of the loop-mediated isothermal amplification (LAMP) technique have reported on its application in various fields.

2010	Development of a reverse transcription-loop-mediated isothermal amplification assay for detection of pandemic (H1N1) 2009 virus as a novel molecular method for diagnosis of pandemic influenza in resource-limited settings (Kubo *et al*., 2010)	Influenza A virus (H1N1) detection
2012	Development of a loop-mediated isothermal amplification assay for detection of *Phytophthora sojae* (Dai *et al*., 2012)	*Phytophthora sojae* plant pathogen detection
2012	A rapid loop-mediated isothermal amplification assay targeting hspX for the detection of *Mycobacterium tuberculosis* complex (Bi *et al*., 2012)	*Mycobacterium tuberculosis* detection
2013	Diagnostic accuracy of loop-amp *Trypanosoma brucei*: Detection kit for diagnosis of human African trypanosomiasis in clinical samples (Mitashi *et al*., 2013)	Diagnosis of HAT
2014	A loop-mediated isothermal amplification (LAMP) assay for early detection of *Schistosoma mansoni* in stool samples: A diagnostic approach in a murine model (Fernández-Soto *et al*., 2014)	Detection of *Schistosoma mansoni* in stool samples
2015	LAMP technology: Rapid identification of *Brucella* and *Mycobacterium avium* subsp. *paratuberculosis* (Trangoni *et al*., 2015)	*Brucella* and *Mycobacterium avium* subsp. *paratuberculosis* detection
2015	Development of reverse transcription loop mediated isothermal amplification assay for rapid detection of bluetongue viruses (Mohandas *et al*., 2015)	Bluetongue virus (BTV) detection
2015	The development of an accelerated reverse transcription loop mediated isothermal amplification for the serotype specific detection of bluetongue virus 8 in clinical samples (Mulholland *et al*., 2015)	BTV detection
2016	Reverse transcription loop-mediated isothermal amplification assays for rapid identification of eastern and western strains of bluetongue virus in India (Maan *et al*., 2016/2011)	BTV identification
2016	Development of a novel reverse transcription loop-mediated isothermal amplification assay for the rapid detection of African horse sickness virus (Fowler *et al*., 2016)	African horse sickness virus detection

3.3.4 AHSV and LAMP

Viruses can be detected through cell culture methods or more sensitive molecular techniques (Staggemeier *et al*., 2012). African horse sickness virus (AHSV) is identified through molecular diagnostic techniques, specifically. Identification techniques at present range from PCR-based methods such as RT-PCR, real-time RT-PCR (RT-qPCR), and serotype identification such as enzyme-linked immunosorbent assays (ELISAs). Direct isolation of viruses has been done through cell culture methods, using baby hamster kidney cells (BHK-21) and inoculation of new-born mice. Serotype-specific antibody detection of virus isolates can also be done through virus neutralisation (VN) and sequencing (OIE, 2016).

Serotype identification in virus assays has been done through ELISA, based on soluble AHSV antigen or a recombinant protein VP2 (Hamblin *et al*., 1990; Chuma *et al*., 1992; Laviada *et al*., 1992; Bremer *et al*., 1994; House *et al*., 1996; Maree & Paweska, 2005). A universal methodology for the detection and identification of AHSV is yet to be described (OIE, 2012). The use of PCR-based methods has been proposed by the OIE because of its high sensitivity and quickness (Fowler *et al*., 2016; OIE, 2015). The methodology for the detection of viral RNA usually consists of RT-qPCR (Miura *et al*., 2011). This method is not 100% accurate in indicating the presence of the infectious virus (OIE, 2012). Reverse-transcriptase qPCR of a different viral segment was recently described and used (Aguero *et al*., 2008; Fernandez-Pinero *et al*., 2009; Guthrie *et al*., 2013; Rodriquez-Sanchez *et al*., 2008; Quan *et al*., 2010); to ensure a more sensitive and specific identification, along with agarose gel-based RT-PCR (Laviada *et al*., 1997; Sailleau *et al*., 1997; Zientara *et al*., 1993, 1994, 1995). Real-time PCR (qPCR) and RT-qPCR methods are used to detect AHSV in *Culicoides* midges (Scheffer *et al*., 2011; De Waal *et al*., 2016). Although these methods are recommended, they must be executed in a laboratory. In consequence, samples must be transported from the collection area to a laboratory under suitable conditions.

Therefore, a rapid and more sensitive technique is desired to detect the virus without sophisticated equipment. The novel LAMP method might be a powerful diagnostics tool for the detection of AHSV. A LAMP assay has been developed for BTV, which is in the same genus (orbivirus) as AHSV (Mulholland *et al*., 2014; Mohandas *et al*., 2015; Maan *et al*., 2016). Most recent work done by Fowler *et al*. (2016) described a novel RT-LAMP assay for the detection of AHSV in horses for diagnostic purposes.

However, no LAMP method has been developed for the detection of AHSV in *Culicoides* specimens.

The aim of this chapter is to describe and discuss the development of a simplified diagnostic tool for the detection of AHSV in *Culicoides* midges.

The specific objectives included the following:

- To design specific primers for LAMP assays of AHSV.
- To optimise the RT-LAMP method for AHSV detection.
- To evaluate the assay with artificially infected *Culicoides* samples.

3.4 MATERIALS AND METHODS: DIAGNOSTIC TOOL

As mentioned above, these specially designed four primers (FIP, BIP, F3, B3) can recognise six distinct regions on target sequences (Parida *et al*., 2004; Parida, 2008, Notomi *et al*., 2000). LAMP can be accelerated by using two loop primers, namely FLP and BLP.

3.4.1 Primer design

LAMP primers are designed to be sensitive and specific. A set of four primers was designed, with the help of the LAMP primer design support software programme, PrimerExplorer V4 (https://primerexplorer.jp/e/). A reference sequence of AHSV isolate HS 02/07 structural protein VP4 gene, complete coding DNA sequence from GenBank (http://www.ncbi.nlm.nih.gov/nuccore/209167968?report=genbank), published by Fasina *et al*. (2008) (FJ196587.1), was used for designing the primers (Table 3.2). Primers were manufactured by Inqaba Biotech, South Africa. A stock solution was prepared to 100 µM, following the manufacturer's instructions; and diluted to 10 µM. Primers FIP and BIP were then diluted to 40 pmol/µl and primers F3 and B3 to 5 pmol/µl. A primer mix of the four primers was made up out of 40 µl of FIP and BIP, respectively and 5 µl of F3 and B3, respectively.

Table 3.2: The four primer sequences designed by PrimerExplorer V4 software for reverse-transcription loop-mediated isothermal amplification. AHSV: African horse sickness virus; B3: backwards outer primer; BIP: backwards inner primer; F3: forward outer primer; FIP: forward inner primer; Tm: melting temperature.

PRIMER NAME	PRIMER'S SEQUENCES (5'-3')		LENGTH (bp)	Tm
AHSV-LAMP-F3	GAATGGCGTGTGACACAT		18	57.13
AHSV-LAMP-B3	CCAATTAACGTTTCAAATCTTC		24	56.45
AHSV-LAMP-FIP	F1c	ACTTATGAACAAAAATCTATGCGCG	48	56.52
	F2	CAGTTGGAAAAATTGATCAATGG		60.49
AHSV-LAMP-BIP	B1c	AAGAGGAAAATCAGGTTGAAGGAT	49	55.99
	B2	CATACTCCCTTAATTCTTTTTTCAG		60.06

3.4.2 RT-LAMP and optimisation

3.4.2.1 RT-LAMP optimisation using an RNA amplification kit

For testing the applicability of the method and primers, an RNA amplification kit of Eiken Chemical Co. Ltd, Japan was used. According to the manufacturer, a typical amplification condition for RT-LAMP is 35 min at 62.5ºC. To establish an optimal amplification time and temperature for the designed primers, a gradient of each was tested. The reaction consisted of the following: 2.6 µl of primer mix, 12.5 µl 2x reaction mix, 1 µl of the virus (x10^7 copies dsRNA) and 1 µl of enzyme mix and filled up to 25 µl with 8 µl dH_2O. Incubation conditions testing different temperatures and times were as follows: temperatures of 57ºC, 60ºC and 62.5ºC were performed for 60 min, 75 min and 90 min respectively.

3.4.2.2 Standard protocol for RT-LAMP

According to Notomi *et al*. (2000), optimal isothermal conditions for LAMP can vary between 60 and 65ºC, with incubation time differing from 15 to 60 min (Eiken Chemical Co Ltd, Japan). The OptiGene Ltd (UK) commercial kit was used for testing the samples in this study, because it is less expensive than the RNA amplification kit of Eiken Chemical Co. Ltd. According to OptiGene Ltd., UK, RNA amplification is usually optimal at 63ºC. RT-LAMP was done with the use of two outer (B3 and F3) two inner primers (FIP and BIP). An Isothermal Master Mix kit and AMV Reverse Transcriptase (OptiGene Ltd, UK) were used for LAMP. The Alpha Cycler 1 PCRmax detection system (PCR max, UK) was used for amplification of target sequences.

Each reaction comprised a total volume of 25 µl: 0.5 µl of F3 and B3 primer (5 pmol/µl), 2 µl of FIP and BIP primers (40 pmol/µl), 15 µl Isothermal Master Mix (OptiGene Ltd, UK), 1 µl virus (x10^7 copies dsRNA), 0.25 µl AMV reverse transcriptase and filled up to 25 µl with dH_2O. For visualisation of amplification, 1 µl of fluorescent dye (Eiken Chemical Co Ltd, Japan) was added to the reaction mixture. A one-step protocol for cycling conditions was used according to results obtained after optimisation (3.4.2.1), which included the following: (i) 62.5ºC for 90 min and (ii) an inactivation (enzyme) step at 80ºC for 5 min.

3.4.2.3 Visualisation of amplification

Amplified product was confirmed using 1.0% (w/v) gel electrophoresis and fluorescent dye (Eiken Chemical Co Ltd, Japan) and 3 µl LAMP product was mixed with 2 µl of

loading dye (6X Orange Loading Dye, Fermentas, USA) with GelRed (1000x) (Biotium, USA) and loaded into the 1.0% agarose gel. Buffer used was 1x TAE (20 mM acetic acid, 100 mM EDTA, 40 mM Tris at pH 8.0) gel electrophoresis buffer. A 1 kb molecular weight marker (1 kb, O'GeneRuler, Fermentas, USA) was used to estimate the molecular weight. Conditions for gel electrophoresis were set at 80 V for 45 min in a Mini Sub-cell GT and Power-Pac (Bio-Rad, USA). The Bio-Rad Chemidoc MP (Bio-Rad, US) was used to capture images of gels. To test field samples, eye assessment could be done through turbidity or fluorescence checks (Parida, 2008). The reaction tube with previously added fluorescent dye was viewed under a UV-light to identify any colour changes.

3.4.3 Sensitivity of novel RT-LAMP

A serial dilution ranging from 10^7 copies/µl to 10^3 copies/µl was done. The Isothermal Master Mix kit and AMV Reverse Transcriptase (OptiGene Ltd, UK) were used for RT-LAMP. For amplification of target sequences, the Alpha Cycler 1 PCRmax, detection system (PCR max, UK) was used. Each reaction comprised a total volume of 25 µl: 0.5 µl of F3 and B3 primer (5 pmol/µl), 2 µl of FIP and BIP primers (40 pmol/µl), 15 µl Isothermal Master Mix (OptiGene Ltd, UK), 1 µl of virus dilution ($x10^3$/µl, $x10^4$/µl, $x10^5$/µl and $x10^6$/µl, dsRNA), 0.25 µl AMV Reverse Transcriptase, filled up to 25 µl with dH_2O. A one-step protocol for cycling conditions was used according to results found after optimisation, which included the following steps: (i) 62.5ºC for 90 min and (ii) with an inactivation (enzyme) step at 80ºC for 5 min.

3.4.4 Evaluation of RT-LAMP sensitivity for in-field testing

Sensitivity assessments for the RT-LAMP method to be applied under field conditions was performed through direct and indirect (RNA extraction) amplification of *Culicoides* midges under simulated field conditions in the laboratory.

3.4.4.1 Artificial infection of Culicoides midges with AHSV

Live specimens of *Culicoides* midges were collected at OVI. These midges were artificially infected with AHSV4 as described by Venter *et al*. (1991). Before feeding, *Culicoides* were kept at favourable conditions for a 24-hour period. These conditions included temperature (23.5ºC), relative humidity (50–70%), no exposure to daylight (-1%) and no intake of water or nutrients.

AHSV4 isolates (10^5 TCID50/ml) were mixed with fresh defibrinated sheep blood, as described by Venter and Paweska (2007). Midges were fed through a one-day old chicken-skin membrane for 30–45 min. After feeding, engorged specimens were separated and identified on a chill table that ensures that the midges stay immobilised (Venter & Paweska, 2007). Bright red colouring of the abdomen indicated that fresh blood had been ingested (Figure 3.4). These blood-engorged midges were stored at -80ºC. Day-0 infected midges were used for both direct and indirect amplification. Thus, the virus concentration was still low with no time to replicate within the vector.

Figure 3.4: Example of a fully engorged *Culicoides* female midge (source: Liebenberg, 2016) indicated by the arrow.

3.4.4.2 Direct amplification of AHSV infected Culicoides midges

RT-LAMP was done on field samples as explained in Section 3.4.2.2. One (day 0) *Culicoides* midge fed with AHSV-infected blood was used in the place of 1 µl RNA 10^7 copies/µl to test in-field catchment sensitivity. The *Culicoides* midge was homogenised with a 3 mm stainless steel ball for 2x 1 min at 50 rpm in 20 µl of dH_2O. The reaction comprised a total volume of 25 µl: 0.5 µl of F3 and B3 primers (5 pmol/µl), 2 µl of FIP and BIP primers (40 pmol/µl), 15 µl Isothermal Master Mix (OptiGene Ltd, UK), 5 µl of homogenised midge in dH_2O, 0.25 µl AMV Reverse Transcriptase (OptiGene Ltd, UK) and filled up to 25 µl with dH_2O. Cycling conditions were as follows: (i) 62.5ºC for 90 min and (ii) inactivation step at 80ºC for 5 min. Amplified RNA product was confirmed using 1.0% (w/v) gel electrophoresis.

3.4.4.3 Indirect amplification of AHSV through viral RNA extraction from infected Culicoides midges

RNA was extracted from a whole infected *Culicoides* midge. First the midge was homogenised in TRIzol® LS reagent (Invitrogen, USA) with 3 mm stainless steel beads for 2x 1 min at 50 rpm in a TissueLyser (Qiagen, Germany). A centrifuge step allowed the sample to be split into three phases. The upper aqueous phase, containing the RNA, was removed according to the manufacturer's instructions (ThermoFischer Scientific, 2015), followed by RNA isolation with the use of the Qiagen Rneasy® MinElute® Cleanup Kit (Qiagen, Germany) according to the manufacturer's instructions. RT-LAMP was done on field samples as explained in Section 3.4.2.2 and the amplified RNA product was confirmed using 1.0% (w/v) gel electrophoresis.

3.5 RESULTS AND DISCUSSION

The aim of this chapter is to describe and discuss the development of a simplified nucleic acid diagnostic tool for the detection of AHSV in *Culicoides*. Four specific primers of the VP4 region of the gene were designed. RT-LAMP was done and optimisation followed. Lastly, the LAMP assay was evaluated with infected *Culicoides*.

3.5.1 Primer design

In this study, an RT-LAMP assay for AHSV was developed and optimised with a set of four specifically designed primers using the reference sequence of AHSV-VP4 gene published by Fasina *et al*. (2008) (Table 3.2). LAMP primers were designed with the use of LAMP primer design support software programme, specifically intended for the LAMP method.

Primers from the conserved complete protein coding sequence of VP4 were selected from the study of Potgieter *et al*. (2009). The bp length of the two outer primers, F3 and B3, ranged from 18 to 24 bp and that of the two inner primers, FIP and BIP, ranged from 48 to 49 bp. The melting temperature varied from 55 to 60ºC, which is good, according to the Eiken Genome website (http://loopamp.eiken.co.jp/e/lamp/primer.html), for AT-rich primer regions. As can be seen in Figure 3.5, the primer sequences were chosen at the beginning of the sequence, with a distance of 119 bp between the 5'-ends of F2 and B2. The distance between F2 and F3 was 21 bp and that between B2 and B3 was 32 bp. These distances were close to the desired bp distances between primer regions as described on the Eiken Genome website (http://loopamp.eiken.co.jp/e/lamp/primer.html).

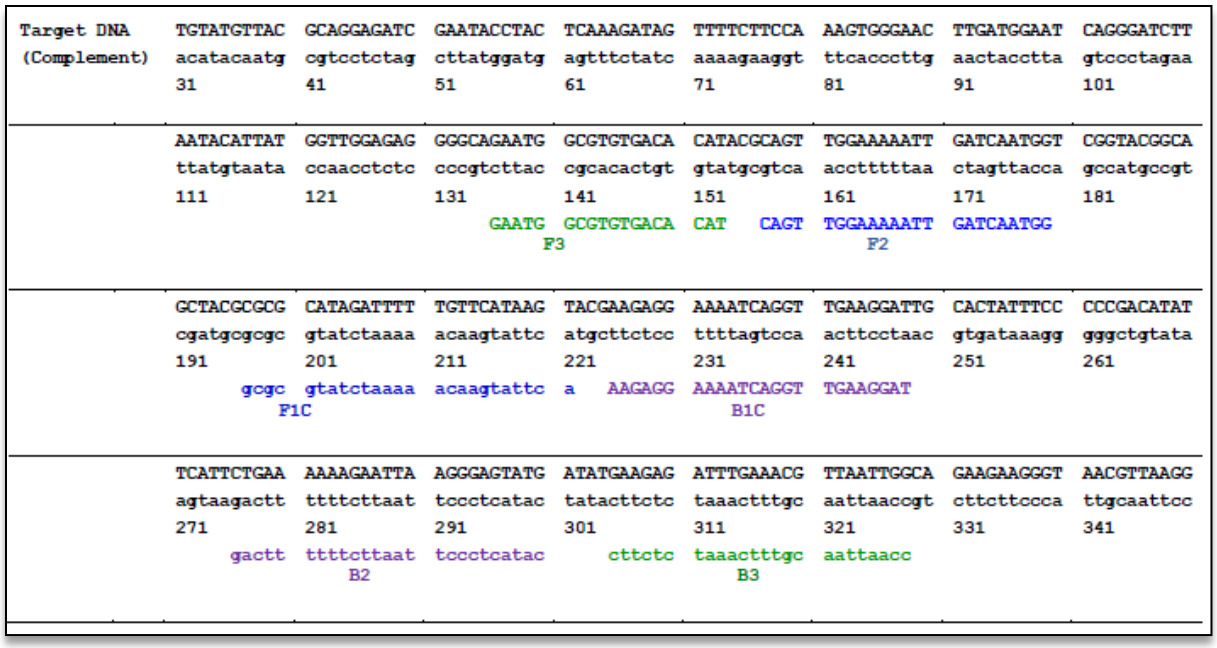


Figure 3.5: Positioning of partial sequence of African horse sickness-virus viral protein 4 (AHSV-VP4) gene used for primer design in this study.

According to the PrimerExplorer V4 software, the primer set chosen was the best option for this region of the gene. Theoretically, the primers should give effective amplification. A full description of the primer sets designed can be found in Appendix C.

3.5.2 Optimisation of RT-LAMP

3.5.2.1 Optimisation of amplification method

A test was carried out at different temperatures and time intervals to obtain the optimal reaction parameters for amplification. A gradient temperature reaction was run with temperatures ranging from 57 to 65ºC, with reaction time variables of 60 min, 75 min and 90 min, varying from the standard protocol. This test included a negative control. This was done to determine the optimal incubation temperature and time for the AHSV RT-LAMP reaction. Figure 3.6 is a demonstration of the optimisation test performed, according to the method described in Section 3.4.2.1. A visible smear of bands in lane 9 can be seen (Figure 3.6), with no primer-dimers detected below 100 bp.

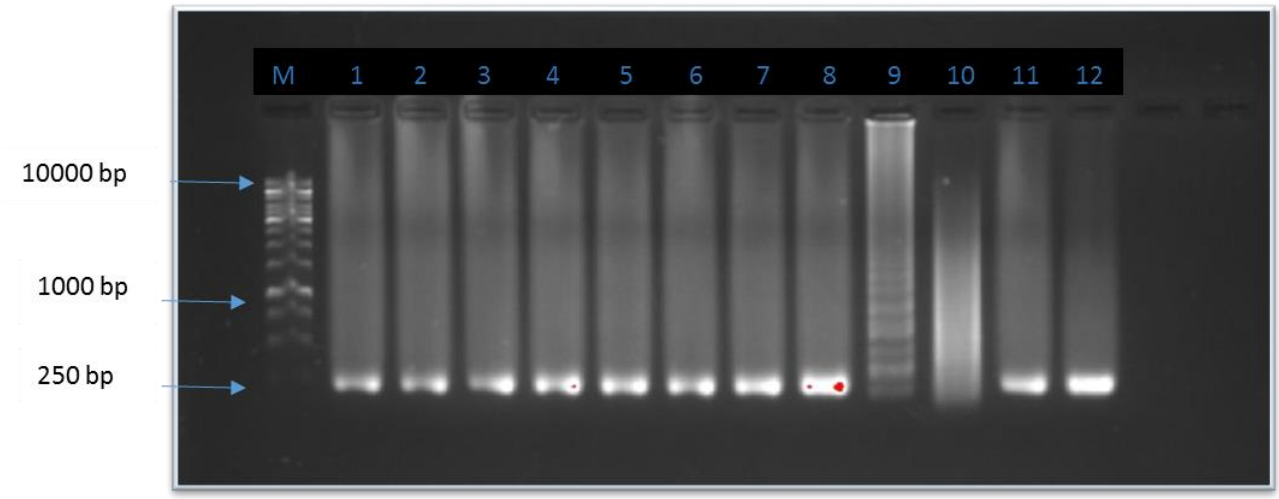


Figure 3.6: Optimisation of African horse sickness virus (10^7 copies) reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay and 1% agarose gel electrophoresis of RT-LAMP products produced at different parameters. From left to right: lane M, 1 kb molecular marker; lane 1, 62.5ºC for 60 min; lane 2, 60ºC for 60 min; lane 3, 57ºC for 60 min; lane 4, negative control (60 min); lane 5, 62.5ºC for 75 min; lane 6, 60ºC for 75 min; lane 7, 57ºC for 75 min; lane 8, 60ºC negative control (75 min); lane 9, 62.5ºC for 90 min; lane 10, 60ºC for 90 min; lane 11, 57ºC for 90 min; lane 12. negative control (90 min).

Thus, the RT-LAMP reaction could be regarded as successful at those specific variables (62.5ºC for 90 min). The optimal temperature for AHSV to amplify was therefore found to be 62.5ºC with a detection time of 90 min. Amplification of the desired multiple size bands was shown, indicating accurate primer set concentration and cyclic conditions for the AHSV RT-LAMP assay. A lighter smear was also detected in lane 10 (Figure 3.6). This shows non-optimal parameter conditions for this specific primer set. Lanes 4, 8 and 12 was the non-template control sample for the specific time variables; primer-dimers can be seen. Lanes 1–3, 5–7, 11 and 12 show smears with primer-dimers detected below 100 bp. To achieve high sensitivity for the target sequence in this study, RT-LAMP reaction time was extended. A 90 min reaction time at 62.5ºC for the RT-LAMP assay was used, followed by heat inactivation at 80ºC for 5 min (Figure 3.6). A smear of bands was detected at different sizes, indicating positive strand displacement activity (Chai *et al*., 2008; Parida, 2008).

3.5.2.2 AHSV amplification using optimised RT-LAMP method

A one-step, single-tube RT-LAMP assay was developed for the detection of the AHSV-VP4 gene with specifically designed primers. Amplification could be detected through agarose gel electrophoresis of the virus strain. Amplicons were seen as multiple bands on the gel (Figure 3.7a) and the results could be confirmed by visualisation of the fluorescence under UV-light (Figure 3.7b). RT-LAMP reaction was done based on the method described in Section 3.4.2.2. The specific RT-LAMP assay produced multiple bands of diverse sizes on agarose gel electrophoresis (Figure 3.7). RT-LAMP products consisted of several inverted-repeat structures, thus forming numerous bands. Primers were successfully designed for the development of an RT-LAMP method to specifically detect AHSV-VP4. Band patterns on agarose gel electrophoresis verified RT-LAMP-specific primer amplification. According to the principle of RT-LAMP reaction, the results gave a clear signal of amplification, thus indicating that the VP4 strain of AHSV could be successfully amplified using the RT-LAMP method and primer sets.

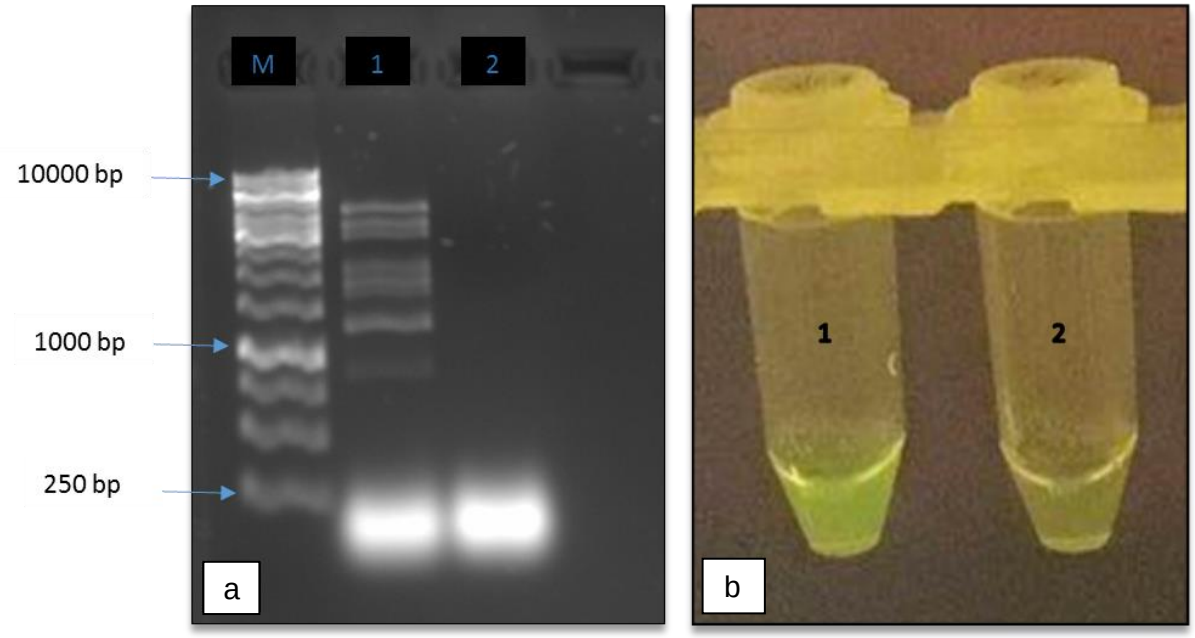


Figure 3.7: Amplified virus (10^7 copies) product by reverse-transcription loop-mediated isothermal amplification assay, visualised by means of 1% agarose gel electrophoresis and direct ultraviolet-light detection. A: From left to right: lane M, 1 kb molecular marker; lane 1, x10^7 virus; lane 2, non-template control. B: Tube 1: x10^7 copies of virus tested positive (green colour); tube 2: non-template control tested negative (no colour change).

Clear primer-dimer bands in Figure 3.7a are demonstrated, indicating low annealing temperature or high concentration of primers. Although these limitations can be overcome by changing the physical-chemical characteristics, it can reduce the efficiency of the reaction. Primer design can contribute to the development of primer-dimer. During the primer design step, it is important to make sure that complementary and special sequences at the primer's ends are eliminated. These sequences can form primer-dimers during the RT-LAMP reaction. This could have been be the case in this study, as the primers used were first-time designed primers. Therefore, the primers can be re-designed with these shortcomings in mind in future studies.

The findings in this study suggested that the assay has the specificity to detect the AHSV-VP4 gene. Even though this RT-LAMP reaction needed 90 min for amplification, it was less time than required for a RT-PCR. Previous studies (Hong *et al*., 2004; Parida *et al*., 2004; Poon *et al*., 2006; Bista *et al*., 2007; Imai *et al*., 2007; Yoda *et al*., 2007; Chen *et al*., 2008; Cai *et al*., 2008) these studies concluded that the shorter time frame that RT-LAMP requires (17–60 min) is considerably better than that of RT-PCR (3–4 hours). Other advantages of RT-LAMP over RT-PCR are that no expensive equipment is required for the former and because of turbidity naked-eye evaluation, no fluorogenic probes and primers are needed. Nonetheless, the sensitivity of this assay could be evaluated further because of the size of bands formed in Figure 3.7a.

3.5.2.3 Sensitivity of the RT-LAMP

Serial dilution of AHSV was done from $x10^6$ to $x10^3$ copies per µl to validate the detection limit of the RT-LAMP method developed. This could also have been an indication of the sensitivity of the reaction. Figure 3.8 illustrates the serial dilution at desired cyclic conditions. All the dilutions gave negative results, with primer-dimers perceived in all lanes on the agarose gel.

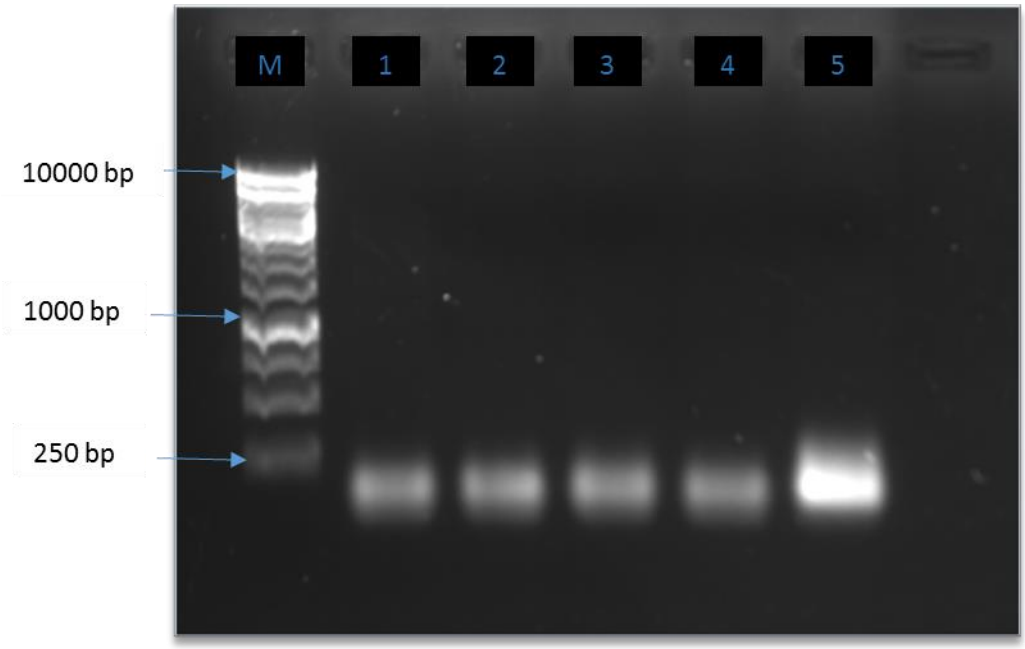


Figure 3.8: Serial dilution series of African horse sickness virus from 10^6 to 10^3 copies/µl were amplified including a non-template control to determine detection limit of the reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method. Detection of the LAMP products was confirmed by 1% agarose gel electrophoresis. From left to right: lane M, 1 kb molecular marker; lane 1, x10^6/µl; lane 2, x10^5/µl; lane 3, x10^4/µl; lane 4, x10^3/µl; lane 5, non-template control.

From Figure 3.8, it is clear that the lower copy numbers of the virus cannot be detected through RT-LAMP (lane 1-4) as no bands have formed. Expected results for serial dilutions would have involved bands forming from 10^6 to 10^3 of the x10 dilution due to the sensitivity and specificity of the LAMP method. Errors could have occurred prior to or during assay preparation.

No clear conclusion could be made of the desired detection limit for this assay. Nonetheless, large-scale analysis must be done to prove its reliability and sensitivity of this assay. By referring to the study of Chen *et al*. (2008), an RT-PCR dilution series of AHSV can be done to compare sensitivity to AHSV-VP4 RT-LAMP. In this case, RT-PCR should be kept in mind for future studies.

3.5.3 Evaluation of assay sensitivity by comparing indirect and direct amplification of AHSV-infected *Culicoides* midges

After *Culicoides* midges were infected with AHSV, the blood-engorged specimens were used for direct amplification of *Culicoides* midges using an RT-LAMP assay together with an indirect viral RNA extraction from the midges as described in Section 3.4.3 (Figure 3.9).

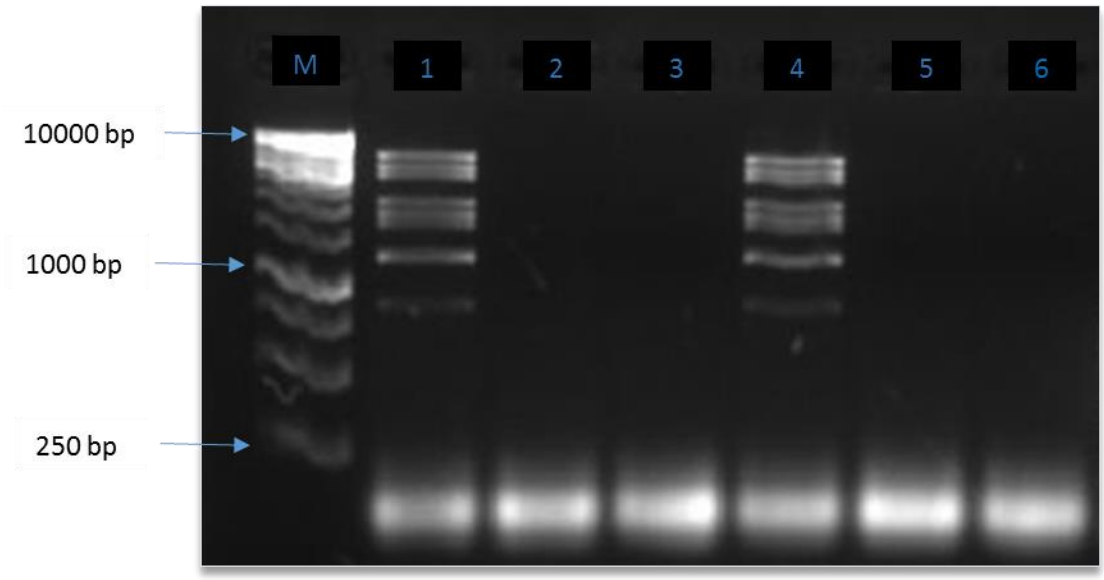


Figure 3.9: Reverse-transcription loop-mediated isothermal amplification of African horse sickness virus RNA (10^7 copies) amplified product, homogenised infected *Culicoides* midges and viral RNA extracted from blood-engorged *Culicoides*, with visualising by means of 1% agarose gel electrophoresis. Lanes 1–3 were amplified together, as were lanes 4–6. Lane M, 1 kb molecular marker; lane 1, x10^7 virus; lane 2, one *Culicoides* midge homogenised in PCR water; lane 3, non-template control of the reaction; lane 4, x10^7 virus; lane 5, 1 ul of extracted RNA; lane 6, non-template control of reaction.

The method of RT-LAMP with live-virus was adequate to detect AHSV because of its high efficiency to amplify viral RNA without RNA extraction, as shown by the results of this study. Day-0 old infected midges were used in this study. The RT-LAMP of the virus (x10^7) in lanes 1 and 4 was more sensitive than in the indirect method of RNA extraction before RT-LAMP was performed and the direct method, where no detection of infected *Culicoides* midges could be observed, as can be seen in lane 5 (Figure 3.9).

The RNA extraction evaluation was repeated in triplicate, delivering negative results for all three amplifications. Viral RNA extraction from infected midges might have been the problem. Further research could be conducted to evaluate the results and to state whether this is the case or not. Nevertheless, during the study of Nakao *et al*. (2010), when extracted DNA from *Amblyomma variegatum* (tropical bont tick) was included in the newly developed LAMP assay, the LAMP assay was inhibited. Investigations showed that *Amblyomma* tick tissue contains certain elements that can inhibit PCR reactions. To some extent, *Culicoides* midges may also carry inhibitory factors that may influence RT-LAMP reactions. These elements cannot always be removed during the purification step of RNA extraction. Consequently, creating a less sensitive RT-LAMP to inhibitory factors compared to RT-PCR. Even though this may be the case in this study, RT-LAMP can have a higher sensitivity than that of conventional PCR as described by Dahlenborg *et al*. (2001), Ding *et al*. (2013), and Farooq *et al*. (2015). The fact that day 0 midges were used can also play a big role. The virus has not yet multiplied in the vector at this stage and the titre in the midge is very low.

Direct amplification of the infected midge, using only one *Culicoides* specimen, was also done in triplicate. Pool sizes of 1, 25 and 50 midges were tested. No previous work was done based on AHSV-VP4 RT-LAMP and therefore no parallels could be made with reference studies. However, a study was done based on AHSV-VP7 gene that represented all nine serotypes of the virus (Fowler *et al*., 2016). According to the results found in their study, RT-LAMP test of AHSV-VP4 appears to be efficient. Moreover, results seen on the agarose gel in the studies of Chen *et al*. (2008), Fukuda *et al*. (2007), Kubo *et al*. (2010), Parida *et al*. (2004) and Yoda *et al*. (2007), also using RT-LAMP for virus RNA amplification, can be used to compare band formation, indicating positive reactions. However, the reliability of this assay should be investigated further. The RT-LAMP assay for AHSV should be more sensitive with visualisation of a more distinct smears of bands.

In this study, the band sizes visualised by agarose gel electrophoresis were mostly larger than reference agarose gel results and more compacted, indicating a less sensitive amplification. As previously described, no extracted viral RNA could be detected and no direct detection from the artificially infected *Culicoides* specimens could be seen. More intense evaluation can be done by testing different (larger) pool

sizes of infected *Culicoides* midges in reference to De Waal *et al*. (2016) and the RT-qPCR can be substituted by the AHSV RT-LAMP assay.

To facilitate the use of this assay in the field, collecting *Culicoides* specimens can confirm the appropriateness of the use of RT-LAMP to detect viral RNA in clinical specimens. On the other hand, eliminating the nucleic acid extraction step would also be a step closer to developing a fully direct amplification. By establishing a viable RT-LAMP assay, observation of AHSV can be done through this tool to prevent epidemics. In conclusion, an initial development stage of specific RT-LAMP assay in this study is an indication of a new simple, specific and highly sensitive assay for the clinical detection of AHSV especially in developing countries. The possibility was raised that this assay will be useful for the detection of the live virus.

CHAPTER 4: CONCLUSION

The *Culicoides* genus is of importance to medical and veterinary science because of its role as biological vector in pathogens (Harrup *et al*., 2015). AHS is one of the diseases transmitted by *Culicoides* that has severe economic impacts. There is potential for developing molecular methods that can be used as standard protocols for the identification of *Culicoides* and the rapid detection of AHSV. An extensive amount of time passes before a diagnosis of AHSV can be made with the use of present methods of detection. By the time the infected horse or other animals are diagnosed, they will be too weak to recover and in severe cases already diseased. Because of the mortality rate of AHS, fast detection of the virus is crucial. In order to develop a toolkit for the identification of *Culicoides* vectors from Namibia and detection of AHSV, two objectives were set. A conclusion on the outcome of these objectives is given below.

4.1. The establishment of *Culicoides* barcodes of the MT-COI gene (Chapter 2)

Species identification was done morphologically and on the molecular level. The wing picture atlas of Afrotropical *Culicoides* (Meiswinkel, 1996) was used to distinguish between species morphologically. Nineteen different *Culicoides* species of Namibia were identified. The DNA extraction method was effective, with only three species not being able to be amplified and sequenced with the MT-COI gene. Useful sequences for phylogenetic analyses at species and other taxonomic levels were produced, through the sequencing of the 5'-region (Rebijith *et al*., 2012) of the MT-COI gene (Ander *et al*., 2013).

Previous sequences of six species found on GenBank were compared with the same sequences from Namibia. Three species showed a close identity match with previously published reference sequences. From the 16 different species, 11 were novel DNA sequences, includes *C.* sp. #61, *C. eriodendroni*, *C. exspectator*, *C. herero*, *C. leucostictus*, *C. nivosus*, *C. pretoriensis*, *C. punctithorax*, *C. pycnostictus*, *C. ravus* and *C. tropicalis*. Barcodes of these Namibian species were successfully obtained. Phylogenetic analysis showed that some species formed distinct groups, but some sequences of the same species varied, highlighting the importance this study.

Limited molecular studies have been done on morphological and phylogenetic characterisation of different *Culicoides* species in Namibia. Sequence data are also

significantly lacking from Namibia and morphological tools have been used until recently to identify specimens. Future studies on *Culicoides* are crucial for both morphological and phylogenetic classification. Until all the species or area-specific compound species have been compared on molecular and morphological levels, classification of *Culicoides* is likely to remain in disarray.

In this study, morphological identification and classification of species correlated well with the molecular analysis, with three outlier species. However, based on these results, the methodology used could efficaciously be applied for the identification of *Culicoides* species and in future AHSV vector identification. It was concluded that the degree of barcode divergence was significant for the majority of taxa. Approaches used to identify and classify *Culicoides* species did not contradict each other and a clear resemblance could been seen. Therefore, methods used to establish DNA barcodes were successful.

4.2 Developing a simplified diagnostic tool for the detection of AHSV in *Culicoides* (Chapter 3)

Primers were successfully designed for the development of the RT-LAMP method to specifically detect AHSV-VP4. Optimisation of the novel RT-LAMP method was done and the first development stage of a specific one-step, single-tube AHSV-VP4 RT-LAMP assay could be seen in this study. The development might be an indication of a new simple, specific and highly sensitive assay for the clinical detection of AHSV in *Culicoides* especially in developing countries. Furthermore, RNA extraction from the virus and direct amplification of an infected midge delivered negative results. Day-0 midges were used for RNA extraction and direct amplification with low virus titre. According to results, x 10^7 copies of the virus could be detected with the use of this novel RT-LAMP method, with the indirect and direct amplification being unsuccessful. Sensitivity and reliability of this assay should be further evaluated on account of its low detection limit. The limited literature available with matching research objectives and methodology makes evaluation challenging. This assay can be developed further to a point where in-field testing and detection of AHSV in *Culicoides* can be made within minutes. Future expectation for the AHSV-specific RT-LAMP assay would be for used in-field testing and laboratory use for the early detection and identification of AHSV. Thus, a novel LAMP method can be developed to overcome the inadequacies of current AHSV-detection methods. The development of a more cost-effective, sensitive

and accurate in-field method, for which fewer sophisticated instruments and reagents are needed, is crucial today to prevent extensive financial losses as a result of the disease.

The results from this study are the first step in the development of a diagnostic toolkit for the identification of *Culicoides* as AHSV vectors and a simplified diagnostic tool for AHSV detection in *Culicoides*. Identifying possible AHSV vectors through molecular techniques would be helpful to prevent the distribution of these species in the surrounding environment. By establishing a viable RT-LAMP assay, observation in-field of AHSV can be done by the use of this tool. The importance of developing such a toolkit involves the promptness of obtaining results before endemic and epidemic outbreaks transpire and fatality statistics increase. No effective treatment is available for AHS, but this toolkit can help to control the virus in a specific area by making possible the necessary steps to prevent the spread of the disease.

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APPENDIX A

Sequences of Namibian *Culicoides* specimens:

***C. enderleini*_N100**

TTTAGAATT-CTTA-TTCG----AGCTG-AATTAGGTCA-----TCCTGGAGCA---TTA-
ATCGGCAATGACCAAATTTATAAT--GTAATTGTCACTGCC-CATGCTT-
TTATTATAATTTTTTTTTAT-AATTATACCTATTA-TA-ATTGGGGGAT--
TTGGTAATTGACTTG---TGCCTTTAAT--
ACTAGGGGCTCCTGATATAGCTTTTCCTCGAATGAATAATATAAGTTTTTGATTA
TT----ACCTCCTTCTTTATCTTTATTATTAATTAGAA--
GTTTAGTGGAAAATGGAGCAGGAA--
CTGGGTGAACAGTTTATCCTCCTTTATCAGC-TAATGTATCTCATGCCGG-----
AGCTTCTGTAGATTTAGCAATTTT--TTCAT--TACATTTA--GCTGGTATT-
TCTTCAATTCTAGGGGCAGTTAATTT-CATTA-
CAACTATTATTAATATACGGTCAAACGGAATT---
TCATTTGATCGAATACCATTATTTGTATGATCAGTATTAA-TTACTGC-TA-
TTCTTCT---TTTATTA----TC-ATTACCAGT-TTTAGCCGGGG--CTATCACTA-
TATTATTAACTGATCGTAATATCAACACTTCCTTTTTT

***C. enderleini*_N101**

GATT-CTTA-TTCG----AGCTG-AATTAGGTCA-----TCCTGGAGCA---TTA-
ATTGGCAATGACCAAATTTATAAT--GTAATTGTCACTGCC-CATGCTT-
TTATTATAATTTTTTTTTAT-AATTATACCTATTA-TA-ATTGGGGGAT--
TTGGTAATTGACTTG---TGCCTTTAAT--
ACTAGGGGCTCCTGATATAGCTTTTCCTCGAATGAATAATATAAGTTTTTGATTA
TT----ACCTCCTTCTTTATCTTTATTATTAATTAGAA--
GTTTAGTGGAAAATGGAGCAGGAA--
CTGGGTGAACAGTTTATCCTCCTTTATCAGC-TAATGTATCTCATGCCGG-----
AGCTTCTGTAGATTTAGCAATTTT--TTCAT--TACATTTA--GCTGGTATT-
TCTTCAATTCTAGGGGCAGTTAATTT-CATTA-
CAACTATTATTAATATACGGTCAAACGGAATT---
TCATTTGATCGAATACCATTATTTGTGTGATCAGTATTAA-TTACTGC-TA-
TTCTTCT---TTTATTA----TC-ATTGCCAGT-TTTAGCCGGGG--CTATCACTA-
TATTATTAACTGATCGTAATATCAACACTTCCTTTTTT

***C. schultzei*_N103**

TAAGTATC-CTTA-TTCG----AGCAG-AATTAGGTCA-----TCCAGGTGCT---TTA-
ATTGGTAACGACCAGATTTATAAT--GTCATTGTTACTGCA-CATGCTT-
TTATTATAATTTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TACCTTTAAT--
ATTAGGAGCCCCAGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGATTA
CT----ACCACCTTCTTTATCTTTATTATTAATTAGAA--
GCTTAGTAGAAAATGGGGCAGGAA--
CAGGATGAACTGTTTACCCTCCTTTATCTGC-TAATGTTTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCTATTTT--TTCTT--TACATTTA--GCCGGTATT-
TCTTCTATTTTAGGAGCTGTTAATTT-CATTA-
CTACAATTATTAATATGCGATCAAATGGAATT---

TCATTTGACCGAATACCTTTATTTGTATGATCTGTATTAA-TTACAGC-AA-
TTCTTTT---ATTATTA----TC-TTTGCCAGT-TTTAGCTGGAG--CTATTACTA

***C. schultzei*_N104**

TCGTTAAGAATT-CTTA-TTCG----AGCAG-AATTAGGGCA-----CCCAGGAGCT---
TTA-ATTGGAAATGACCAAATTTATAAT--GTAATTGTTACAGCT-CATGCCT-
TTATTATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATCGGGGGAT--
TTGGAAATTGACTAG---TTCCTTTAAT--
ATTAGGGGCCCCAGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGAATA
TT----ACCCCCTTCTTTATCCTTATTATTAATTAGAA--
GCCTTGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTATACCCCCCACTTTCAGC-AAATGTTTCTCATGCAGG-----
AGCATCTGTTGATTTAGCAATTTT--TTCAT--TACATTTA--GCTGGAATC-
TCTTCAATTTTAGGGGCAGTAAATTT-TATTA-
CTACTATTATTAATATACGGTCAAATGGAATT---
TCATTTGACCGTATACCTTTATTTGTTTGATCTGTTTTAA-TCACAGC-TA-
TTCTTCT---TTTACTT----TC-ACTTCCAGT-ATTAGCTGGAG--CTATCACTA-
TACTTTTAACTGACCGTAACATCAATACTTCATTTTTT

***C. schultzei*_N105**

TAGTATC-CTTA-TTCG----AGCAG-AATTAGGTCA-----TCCAGGTGCT---TTA-
ATTGGTAACGACCAGATTTATAAT--GTCATTGTTACTGCA-CATGCTT-
TTATTATAATTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TACCTTTAAT--
ATTAGGAGCCCCAGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGATTA
CT----ACCACCTTCTTTATCTTTATTATTAATTAGAA--
GCTTAGTAGAAAATGGGGCAGGAA--
CAGGATGAACTGTTTACCCTCCTTTATCTGC-TAATGTTTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCTATTTT--TTCTT--TACATTTA--GCCGGTATT-
TCTTCTATTTTAGGAGCTGTTAATTT-CATTA-
CTACAATTATTAATATGCGATCAAATGGAATT---
TCATTTGACCGAATACCTTTATTTGTATGATCTGTATTAA-TTACAGC-AA-
TTCTTTT---ATTATTA----TC-TTTGCCAGT-TTTAGCTGGAG--CTATTACTA-
TATTATTAACAGACCGTAATATTAATACTTCATTTTT-

***C. pysnostictus*_N108**

TCTCTAAGTATT-TTAA-TTCG----AGCAG-AATTAGGGCA-----CCCTGGAGCT---
TTA-ATTGGAAATGACCAAATTTATAAT--GTTATTGTTACAGCC-CATGCTT-
TCGTTATAATTTTTTTTAT-AGTAATGCCTATTA-TA-ATTGGAGGAT--
TTGGAAATTGATTAG---TACCTCTTAT--
ATTAGGAGCTCCTGATATAGCTTTCCCGCGAATAAATAATATAAGTTTCTGAATA
CT----ACCACCATCTCTGTCTTTACTTTTAATTAGCA--
GTCTAGTTGAAAATGGAGCTGGAA--
CCGGTTGAACTGTTTATCCCCCTCTTTCTGC-TAATGTTTCCCATGCTGG-----
AGCCTCTGTAGACTTAGCTATCTT--TTCCC--TTCATTTA--GCTGGTATT-
TCTTCTATTTTAGGGGCGGTTAATTT-TATTA-

CTACTATCATTAATATACGATCTAATGGTATT---
ACATTTGACCGAATACCTTTATTTGTTTGATCAGTTCTTA-TTACTGC-TA-
TTTTACT---GTTACTT----TC-TTTACCTGT-ACTCGCCGGAG--CTATCACTA-
TACTTCTAACTGACCGAAACATTAATACCTCTTTCTTT

***C. subschultzei*_N11**

CTTTAGAATT-CTTA-TTCG----AGCAG-AATTAGGACA-----TCCTGGTGCT---TTA-
ATTGGTAATGACCAAATTTATAAT--GTTATTGTTACTGCC-CATGCTT-
TTATTATAATTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TTCCTTTAAT--
ATTAGGAGCCCCAGATATGGCTTTTCCACGAATAAATAATATAAGTTTTTGATTA
TT----ACCCCCTTCTTTATCTTTATTATTAATTAGAA--
GACTAGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTTTATCCTCCTTTATCTGC-AAATGTTTCTCATGCCAG-----
AGCTTCTGTAGATTTAGCAATTTT--CTCTT--TACATTTA--GCTGGAATT-
TCTTCCATTTTAGGGGCTGTTAATTT-TATTA-
CTACTATTATTAATATACGATCAAATGGTATT---
TCATTTGATCGTATACCATTATTTGTATGATCTGTTTTAA-TTACTGC-TA-
TTCTTTT---ATTACTT----TC-TTTACCTGT-TTTAGCCGGAG--CTATTACTA-
TACTATTAACAGATCGTAATATTAACACCTCATTTTT

***C*. sp. #61_N116**

AAGAATT-TTAA-TTCG----ACTTG-AATTAAGCCA-----CCCAGGCTCT---CTT-
ATTGGTAATGACCAAATTTACAAT--GTAATTGTTACTGCC-CATGCTT-
TTGTAATAATTTTTTTCAT-AGTTATACCAATTA-TA-ATTGGAGGAT--
TCGGAAATTGATTAG---TTCCCCTTAT--
ATTAGGAGCTCCTGATATAGCTTTCCCCCGAATAAATAATATAAGATTTTGACTA
TT----ACCCCCAGCTTTATCCCTTCTCCTTATAAGAA--
GACTAGTTGAATCAGGCGCAGGCA--
CAGGATGAACAGTCTACCCCCCTCTCTCCTC-CAACATCGCCCATACAGG-----
AGCTTCTGTAGATTTAGCTATTTT--TTCCT--TACATTTA--GCGGGAATT-
TCTTCAATTTTAGGGGCAATTAATTT-TATTA-
CTACAATTTATAATATACGATTGTCCGGTTTAT--CTTAT-
GATCAAATCCCCCTTTTTGTTTGATCAATTTTTA-TTACTGC-TT-TTCTATT---
GCTTCTT----TC-TCTCCCAGT-ACTAGCAGGTG--CAATTACTA-
TACTATTAACAGATCGTAATATTAATACTTCTTTTTT

***C*. sp. #61_N117**

GAATT-TTAA-TTCG----ACTTG-AATTAAGCCA-----CCCAGGCTCT---CTT-
ATTGGTAATGACCAAATTTACAAT--GTAATTGTTACTGCC-CATGCTT-
TTGTAATAATTTTTTTCAT-AGTTATACCAATTA-TA-ATTGGAGGAT--
TCGGAAATTGATTAG---TTCCCCTTAT--
ATTAGGAGCTCCTGATATAGCTTTCCCCCGAATAAATAATATAAGATTTTGACTA
TT----ACCCCCAGCTTTATCCCTTCTCCTTATAAGAA--
GACTAGTTGAATCAGGCGCAGGCA--
CAGGATGAACAGTCTACCCCCCTCTCTCCTC-CAACATCGCCCATACAGG-----

AGCTTCTGTAGATTTAGCTATTTT--TTCCT--TACATTTA--GCGGGAATT-
TCTTCAATTTTAGGGGCAATTAATTT-TATTA-
CTACAATTTATAATATACGATTGTCCGGTTTAT--CTTAT-
GATCAAATCCCCCTTTTTGTTTGATCAATTTTTA-TTACTGC-TT-TTCTATT---
GCTTCTT----TC-TCTCCCAGT-ACTAGCAGGTG--CAATTACTA-
TACTATTAACAGATCGTAATATTAATACTTCTTTTTTT

***C. tropicalis*_N118**

AAGAATT-CTTA-TTCG----AGCAG-AATTAGGGCA-----CCCAGGAGCT---TTA-
ATTGGAAATGACCAAATTTATAAT--GTAATTGTTACAGCT-CATGCCT-
TTATTATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATCGGGGGAT--
TTGGAAATTGACTAG---TTCCTTTAAT--
ATTAGGGGCCCCAGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGAATA
TT----ACCCCCTTCTTTATCCTTATTATTAATTAGAA--
GCCTTGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTATACCCCCCACTTTCAGC-AAATGTTTCTCATGCAGG-----
AGCATCTGTTGATTTAGCAATTTT--TTCAT--TACATTTA--GCTGGAATC-
TCTTCAATTTTAGGGGCAGTAAATTT-TATTA-
CTACTATTATTAATATACGGTCAAATGGAATT---
TCATTTGACCGTATACCTTTATTTGTTTGATCTGTTTTAA-TCACAGC-TA-
TTCTTCT---TTTACTT----TC-ACTTCCAGT-ATTAGCTGGAG--CTATCACTA-
TACTTTTAACTGACCGTAACATCAATACTTCATTTTTT

***C. similis*_N124**

GACCTCATTAAGTATC-CTTA-TTCG----GGCTG-AATTAGGCCA-----
CCCGGGGGCA---TTA-ATTGGAAATGATCAAATTTATAAT--
GTTATTGTTACTGCC-CATGCTT-TTGTTATAATTTTTTTTAT-AGTTATGCCAATTA-
TA-ATCGGAGGAT--TTGGTAATTGACTTG---TCCCTTTAAT--
GCTTGGGGCACCAGATATAGCGTTTCCACGTATAAATAATATAAGTTTTTGAATA
TT----GCCCCCTTCTTTATCATTATTATTAATCAGTA--
GATTAGTTGAAAATGGAGCCGGGA--
CAGGTTGAACTGTATACCCGCCTTTATCAGC-AAATGTCTCTCACGCAGG-----
CGCATCAGTAGACCTAGCTATTTT--TTCCT--TACACTTA--GCCGGAATT-
TCATCAATTTTAGGGGCAGTAAATTT-TATTA-
CTACAATTATTAATATACGATCTAATGGGATT---
ACTTTTGACCGAATACCCTTATTTGTTTGATCCGTTTTAA-TTACAGC-TA-
TTTTACT---TTTACTA----TC-TTTACCCGT-TTTAGCAGGAG--CAATTACTA-
TACTTTTAACGGATCGTAATATTAATACTTCTTTTTTT

***C. eriodendroni*_N126**

CTTTA-GAATT-TTAA-TTCG----AATAG-AATTAAGTCA-----TCCTGGATCT---TTA-
ATTGGAAATGATCAAATTTATAAT--ACAATTGTAACTGCT-CATGCTT-
TCATTATAATTTTTTTTAT-AGTTATACCTATTA-TA-ATTGGAGGAT--
TTGGTAATTGATTAG---TTCCTCTAAT--
ATTAGGGGCCCCAGACATAGCTTTCCCCCGAATAAATAACCTAAGTTTTTGATTT
TT----ACCCCCTTCAATCTCTCTCCTCCTCTTAAGAA--

GCCTAATCGAGAATGGCGCTGGAA--
CTGGCTGAACAGTTTACCCTCCCCTATCTAA-TAATATATTTCACCCTGG-----
AGCTTCTGTCGATTTAACAATTTT--TTCTT--TACATTTA--GCAGGAATC-
TCCTCAATTTTAGGAGCAATTAATTT-TATTA-
CAACTATTATTAACATACGCATAATTAATATAC--AATTT-
GATCAAATATCTTTATTTACTTGATCAGTTCTTA-TTACAGC-TA-TCCTATT---
ATTACTT----TC-TTTACCTGT-ATTAGCAGGGG--CAATTACTA-
TACTACTTACTGACCGAAATTTTAATACTTCATTTTTT

***C. pycnostictus*_N127**

TTCTTTAAGTATT-TTAA-TTCG----AGCAG-AATTAGGGCA-----CCCTGGAGCT---
TTA-ATTGGCAATGACCAAATTTATAAT--GTTATTGTTACAGCC-CATGCTT-
TCGTTATAATTTTTTTTAT-AGTAATGCCTATTA-TA-ATTGGAGGAT--
TTGGAAATTGATTAG---TACCTCTTAT--
ACTAGGAGCTCCTGATATAGCTTTCCCACGAATAAATAATATAAGTTTCTGAATA
CT----ACCACCATCTCTGTCTTTACTTTTAATTAGCA--
GTCTAGTTGAAAATGGAGCTGGAA--
CCGGTTGAACTGTTTATCCCCCTCTTTCTGC-TAATGTTTCCCATGCTGG-----
GGCCTCAGTAGACTTAGCTATCTT--TTCCC--TTCATTTA--GCTGGTATT-
TCTTCTATTTTAGGAGCAGTTAATTT-TATTA-
CTACTATCATTAATATACGATCTAATGGTATT---
ACATTTGACCGAATACCTTTATTTGTTTGATCAGTTCTTA-TTACTGC-TA-
TTTTACT---GTTACTT----TC-TTTACCTGT-ACTTGCCGGAG--CTATCACTA-
TACTTTTAACTGACCGAAACATTAATACCTCTTTCTTT

***C. leucostictus*_N128**

AGAATC-CTTA-TTCG----AGCAG-AATTAGGTCA-----TCCAGGTGCT---CTG-
ATCGGTAACGATCAAATTTATAAT--GTAATTGTTACAGCA-CATGCAT-
TTGTGATAATTTTCTTTAT-AGTAATGCCTATTA-TA-ATCGGGGGAT--
TTGGTAATTGACTAG---TGCCACTAAT--
ATTAGGAGCCCCAGATATAGCTTTCCCCCGGATAAATAATATAAGTTTCTGAATA
CT----ACCCCCTTCTCTTTCTTTATTATTAATTAGTA--
GTTTAGTAGAAAATGGAGCAGGAA--
CAGGATGAACGGTTTATCCTCCCTTGTCAGC-TAATGTATCTCATGCTGG-----
TGCTTCAGTGGACTTAGCAATTTT--TTCTC--TTCATTTA--GCTGGTATT-
TCTTCTATTTTAGGAGCAGTAAATTT-TATCA-
CAACAATTATTAATATACGATCAAATGGGGTT---
ACTTTCGACCGAATACCTTTATTTGTCTGATCAGTTTTTA-TTACAGC-AA-
TTCTTCT---TCTCCTT----TC-TTTACCAGT-TTTAGCTGGTG--CGATTACAA-
TACTATTAACAGATCGAAACATTAATACCTCATTTTT

***C. leucostictus*_N129**

TTAAGAATC-CTTA-TTCG----AGCAG-AATTAGGTCA-----TCCAGGTGCT---CTG-
ATCGGTAACGATCAAATTTATAAT--GTAATTGTTACAGCA-CATGCAT-
TTGTGATAATTTTCTTTAT-AGTAATGCCTATTA-TA-ATCGGGGGAT--
TTGGTAATTGACTAG---TGCCACTAAT--

ATTAGGAGCCCCAGATATAGCTTTCCCCCGAATAAATAATATAAGTTTCTGAATA
CT----ACCCCCTTCTCTTTCTTTATTATTAATTAGTA--
GTTTAGTAGAAAATGGAGCAGGAA--
CAGGATGAACGGTTTATCCTCCCTTGTCAGC-TAATGTATCTCATGCTGG-----
TGCTTCAGTGGACTTAGCAATTTT--TTCTC--TTCATTTA--GCTGGTATT-
TCTTCTATTTTAGGAGCAGTAAATTT-TATCA-
CAACAATTATTAATATACGATCAAATGGGGTT---
ACTTTCGACCGAATACCTTTATTTGTCTGATCAGTTTTTA-TTACAGC-AA-
TTCTTCT---TCTCCTT----TC-TTTACCAGT-TTTAGCTGGTG--CGATTACAA-
TACTATTAACAGATCGAAACATTAATACCTCATTTTT

***C. subschultzei*_N13**

TTAAGAATT-CTTA-TTCG----AGCAG-AATTAGGACA-----CCCTGGTGCT---TTA-
ATTGGTAATGACCAAATTTATAAT--GTTATTGTTACTGCC-CATGCTT-
TTATTATAATTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TTGGAAATTGACTTG---TTCCTTTAAT--
ATTAGGAGCCCCAGATATGGCTTTTCCACGAATAAATAATATAAGTTTTTGATTA
TT----ACCCCCTTCTTTATCTTTATTATTAATTAGAA--
GATTAGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTTTATCCTCCTTTATCCGC-AAATGTCTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCAATTTT--TTCTT--TACATTTA--GCTGGAATT-
TCTTCCATTTTAGGGGCTGTTAATTT-TATTA-
CTACTATTATTAATATACGATCAAATGGTATT---
TCATTTGATCGTATACCATTATTTGTGTGATCTGTTTTAA-TTACTGC-TA-
TTCTTTT---ATTACTT----TC-TTTACCTGT-TTTAGCCGGAG--CTATTACTA-
TACTATTAACAGATCGTAATATTAACACCTCATTTTTT

***C. pretoriensis*_N132**

TTTAAGCCTT-TTAA-TTCG----AATTG-AATTAGGCCA-----ACCAGGAGCC---TTT-
ATTGGAAATGACCAAATTTATAAT--GTTCTTGTTACTGCC-CATGCTT-
TTGTAATAATTTTTTTTAT-AGTTATACCTATTA-TA-ATTGGGGGAT--
TTGGGAATTGATTAG---TCCCTTTAAT--
ATTAGGGGCTCCTGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGAATG
TT----ACCCCCCTCTCTTACTTTACTGTTAATTAGAG--
GACTAGTGGAAAATGGGGCTGGAA--
CAGGTTGAACAGTTTATCCTCCTTTATCTTC-TAATATTTCTCACGCAGG-----
GGCATCAGTAGATTTAGCAATTTT--TTCTT--TACATCTG--GCTGGTATT-
TCTTCCATTTTGGGGGCAGTTAATTT-TATTA-
CAACAATTATTAATATGCGAGCTAATGGAATTA--CATTT-
GATCGTATGCCTTTATTTGTTTGATCTGTTCTAA-TTACTGC-TG-TTTTACT---
ATTATTA----TC-ATTACCTGT-TTTAGCTGGAG--CTATTACTA-
TACTTCTTACAGATCGAAATATTAATACTTCTTTTTTT

***C. subschultzei*_N14**

AAGAATT-CTTA-TTCG----AGCAG-AATTAGGACA-----TCCTGGTGCT---TTA-
ATTGGTAATGACCAAATTTATAAT--GTTATTGTTACTGCC-CATGCTT-

TTATTATAATTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TTCCTTTAAT--
ATTAGGAGCCCCAGATATGGCTTTTCCACGAATAAATAATATAAGTTTTTGATTA
TT----ACCCCCTTCTTTATCTTTATTATTAATTAGAA--
GATTAGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTTTATCCTCCTTTATCTGC-AAATGTTTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCAATTTT--CTCTT--TACATTTA--GCTGGAATT-
TCTTCCATTTTAGGGGCTGTTAATTT-TATTA-
CTACTATTATTAATATACGATCAAATGGTATT---
TCATTTGATCGTATACCATTATTTGTATGATCTGTTTTAA-TTACTGC-TA-
TTCTTTT---ATTACTT----TC-TTTACCTGT-TTTAGCCGGAG--CTATTACTA-
TACTATTAACAGATCGTAATATTAACACCTCATTTTT

***C. ravus*_N151**

CTTTAAGAATT-TTAA-TTCG----AGCAG-AATTAGGACA-----CCCTGGAGCT---TTA-
ATTGGTAATGACCAAATTTATAAT--GTTATTGTAACCGCC-CATGCTT-
TTATTATAATTTTTTTTAT-AGTAATACCTATTA-TA-ATTGGAGGAT--
TTGGAAACTGATTAG---TCCCATTAAT--
GCTTGGAGCCCCTGATATAGCTTTCCCTCGAATAAATAATATAAGTTTTTGAATA
TT----GCCTCCTTCTCTTTCTCTATTATTAATCAGAA--
GTTTAGTAGAAAACGGTGCAGGGA--
CTGGATGAACTGTTTACCCCCCTCTTTCAGC-CAATGTATCTCATGCCGG-----
AGCTTCTGTAGATTTAGCCATTTT--TTCTC--TGCATTTA--GCAGGTATT-
TCTTCTATTTTAGGAGCAGTAAATTT-TATTA-
CTACTATTATTAATATACGGTCTAATGGAATT---
ACATTTGACCGAATACCTCTTTTTGTCTGATCGGTATTAA-TTACCGC-TA-
TCCTTCT---TCTATTA----TC-TCTTCCTGT-GTTAGCAGGAG--CAATTACTA-
TACTTTTAACAGACCGAAATATTAATACTTCCTTTTTT

***C. ravus*_N152**

TTAGAATT-CTCA-TTCG----AGCTG-AATTAGGTCA-----TCCTGGCGCC---TTG-
ATTGGAAATGATCAAATTTACAAT--GTAATTGTTACAGCA-CATGCAT-
TCATTATAATTTTTTTTAT-AGTAATACCTATTA-TG-ATCGGAGGTT--
TTGGTAATTGACTAG---TTCCATTAAT--
ATTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGAATA
CT----CCCCCCTTCTCTTTCTTTATTATTAATTAGCA--
GCCTCGTAGAAAATGGTGCAGGAA--
CCGGTTGAACTGTTTATCCCCCTTTATCTGC-AAATGTTTCACATGCTGG-----
AGCTTCAGTTGATTTAGCTATTTT--TTCCC--TTCATTTA--GCAGGTATT-
TCTTCTATTTTAGGAGCAGTAAATTT-TATTA-
CTACAATTATTAATATACGGTCTAACGGTATT---
ACTTTTGACCGAATACCTTTATTTGTGTGATCGGTATTAA-TTACTGC-CA-
TTCTTCT---TCTTTTA----TC-CCTACCAGT-TTTAGCCGGAG--CAATTACTA-
TACTTTTAACTGATCGAAACATTAATACATCTTTTTT

***C. ravus*_N157**

GGACTTCTTTAAGAATT-TTAA-TTCG----AGCAG-AATTAGGACA-----
CCCCGGGGCT---TTA-ATTGGTAATGACCAAATTTATAAT--
GTTATTGTAACCGCC-CATGCTT-TTATTATAATTTTTTTTAT-AGTAATACCTATTA-
TA-ATTGGAGGAT--TTGGAAACTGATTAG---TTCCATTGAT--
GCTTGGAGCCCCTGATATAGCTTTCCCTCGAATAAATAATATAAGTTTTTGAATA
TT----GCCCCCTTCTCTTTCTCTATTATTAATCAGAA--
GTTTAGTAGAAAACGGTGCAGGGA--
CTGGATGAACTGTTTACCCCCCTCTGTCAGC-CAATGTATCTCATGCCGG-----
AGCTTCTGTAGATTTAGCAATTTT--TTCCC--TCCATTTA--GCAGGTATT-
TCTTCTATTTTAGGAGCAGTAAATTT-TATTA-
CTACTATTATTAATATGCGGTCTAATGGAATT---
ACATTTGACCGAATACCTCTTTTTGTCTGATCAGTATTAA-TTACTGC-TA-
TTCTTCT---TCTATTA----TC-TCTTCCTGT-CTTAGCAGGAG--CAATTACTA-
TACTTTTAACAGACCGAAATATTAATACTTCCTTTTT

***C. ravus*_N158**

GGACTTCTTTAGAATT-TTAA-TTCG----AGCAG-AATTAGGACA-----
CCCTGGAGCT---TTA-ATTGGTAATGACCAAATTTATAAT--GTTATTGTAACCGCC-
CATGCTT-TTATTATAATTTTTTTTAT-AGTAATACCTATTA-TA-ATTGGAGGAT--
TTGGAAACTGATTAG---TCCCATTAAT--
GCTTGGAGCCCCTGATATAGCTTTCCCTCGAATAAATAATATAAGTTTTTGAATA
TT----GCCTCCTTCTCTTTCTCTATTATTAATCAGAA--
GTTTAGTAGAAAACGGCGCAGGGA--
CTGGATGAACTGTTTACCCCCCTCTTTCAGC-CAATGTATCTCATGCCGG-----
AGCTTCTGTAGATTTAGCCATTTT--TTCTC--TGCATTTA--GCAGGTATT-
TCTTCTATTTTAGGAGCAGTAAATTT-TATTA-
CTACTATTATTAATATACGGTCTAATGGAATT---
ACATTTGACCGAATACCTCTTTTTGTCTGATCGGTATTAA-TTACCGC-TA-
TCCTTCT---TCTATTA----TC-TCTTCCTGT-GTTAGCAGGAG--CAATTACTA-
TACTTTTAACAGACCGAAATATTAATACTTCCTTTTTT

***C. ravus*_N159**

CTTCTTTAGAATT-TTAA-TTCG----AGCAG-AATTAGGACA-----CCCTGGAGCT---
TTA-ATTGGTAATGACCAAATTTATAAT--GTTATTGTAACTGCC-CATGCTT-
TTATTATAATTTTTTTTAT-AGTAATACCTATTA-TA-ATTGGGGGGT--
TTGGAAACTGATTAG---TTCCATTAAT--
GCTTGGGGCCCCCGATATAGCTTTCCCTCGAATAAATAATATAAGTTTTTGAATA
TT----GCCCCCTTCTCTTTCTTTATTATTAATCAGAA--
GTTTAGTAGAAAACGGAGCAGGGA--
CTGGATGAACTGTTTACCCCCCTCTTTCAGC-CAATGTATCTCATGCTGG-----
AGCTTCTGTAGATTTAGCAATTTT--TTCTC--TACATTTA--GCAGGTATT-
TCTTCTATTTTAGGGGCAGTAAATTT-TATTA-

CTACTATTATTAATATACGATCTAATGGAATT---
ACATTTGACCGAATACCTCTTTTTGTCTGATCAGTATTAA-TTACCGC-TA-
TCCTTCT---TCTATTA----TC-TCTCCCTGT-ATTAGCAGGAG--CAATTACTA-
TACTTTTAACAGACCGAAATATTAATACTTCCTTTTT

***C. subschultzei*_N16**

TAGAATT-CTTA-TTCG----AGCAG-AATTAGGACA-----TCCTGGTGCT---TTA-
ATTGGTAATGACCAAATTTATAAT--GTTATTGTTACTGCC-CATGCTT-
TTATTATAATTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TTCCTTTAAT--
ATTAGGAGCCCCAGATATGGCTTTTCCACGAATAAATAATATAAGTTTTTGATTA
TT----ACCCCCTTCTTTATCTTTATTATTAATTAGAA--
GACTAGTAGAAAATGGAGCAGGAA--
CTGGTTGAACAGTTTATCCTCCTTTATCTGC-AAATGTTTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCAATTTT--CTCTT--TACATTTA--GCTGGAATT-
TCTTCCATTTTAGGGGCTGTTAATTT-TATTA-
CTACTATTATTAATATACGATCAAATGGTATT---
TCATTTGATCGTATACCATTATTTGTATGATCTGTTTTAA-TTACTGC-TA-
TTCTTTT---ATTACTT----TC-TTTACCTGT-TTTAGCCGGAG--CTATTACTA-
TACTATTAACAGATCGTAATATTAACACCTCATTTTTT

***C.* sp. #61_N136**

T-TTAA-TTCG----ACTTG-AATTAAGCCA-----CCCAGGCTCT---CTT-
ATTGGTAATGACCAAATTTACAAT--GTAATTGTTACTGCC-CATGCTT-
TTGTAATAATTTTTTTCAT-AGTTATACCAATTA-TA-ATTGGAGGAT--
TCGGAAATTGATTAG---TTCCCCTTAT--
ATTAGGAGCTCCTGATATAGCTTTCCCCCGAATAAATAATATAAGATTTTGACTA
TT----ACCCCCAGCTTTATCCCTTCTCCTTATAAGAA--
GACTAGTTGAATCAGGCGCAGGCA--
CAGGATGAACAGTCTACCCCCCTCTCTCCTC-CAACATCGCCCATACAGG-----
AGCTTCTGTAGATTTAGCTATTTT--TTCCT--TACATTTA--GCGGGAATT-
TCTTCAATTTTAGGGGCAATTAATTT-TATTA-
CTACAATTTATAATATACGATTGTCCGGTTTAT--CTTAT-
GATCAAATCCCCCTTTTTGTTTGATCAATTTTTA-TTACTGC-TT-TTCTATT---
GCTTCTT----TC-TCTCCCAGT-ACTAGCAGGTG--CAATTACTA-
TACTATTAACAGATCGTAATATTAATACTTCTTTTTTTGACCCAGCAGGAGGGGG
AGACCCAATTTTATATCAACATTTATTTTGA

***C.* sp. #61_N133**

GAATT-TTAA-TTCG----ACTTG-AATTAAGCCA-----CCCAGGCTCT---CTT-
ATTGGTAATGACCAAATTTACAAT--GTAATTGTTACTGCC-CATGCTT-
TTGTAATAATTTTTTTCAT-AGTTATACCAATTA-TA-ATTGGAGGAT--
TCGGAAATTGATTAG---TTCCCCTTAT--
ATTAGGAGCTCCTGATATAGCTTTCCCCCGAATAAATAATATAAGATTTTGACTA
TT----ACCCCCAGCTTTATCCCTTCTCCTTATAAGAA--
GACTAGTTGAATCGGGCGCAGGCA--

CAGGATGAACAGTCTACCCCCCTCTTTCCTC-CAACATCGCCCATACAGG-----
AGCTTCTGTAGATTTAGCTATTTT--TTCCT--TACATTTA--GCAGGAATT-
TCTTCAATTTTAGGGGCAATTAATTT-TATTA-
CTACAATTTATAATATACGATTGTCCGGTTTAT--CTTAT-
GATCAAATCCCCCTTTTTGTTTGATCAATTTTTA-TTACTGC-TT-TTCTATT---
GCTTCTT----TC-TCTCCCAGT-ATTAGCAGGTG--CAATTACTA-
TACTATTAACAGATCGTAATATTAATACTTCTTTTTTTGACCCAGCAGGAGGGGG
AGACCCAATTTTATATCAACATTTATTTTGA

***C. herero*_N161**

CTCTCTTTAGTATTCTTAA-TTCG----AGCAG-AATTAGGGCA-----CCCTGGAGCT---
TTA-TT--GTAATGACCAAATTTATAAT--GTTATTGTTACAGCC-CATGCTT-
TCGTTATAATTTTTTTTAT-AGTAATGCCTATTA-TA-ATTGGAGGAT--
TTGGAAATTGATTAG---TACCTCTTAT--
ACTAGGAGCTCCTGATATAGCTTTCCCGCGAATAAATAATATAAGTTTCTGAATA
CT----ACCACCTTCTCTGTCTTTATTTTTAATTAGAA--
GTTTAGTTGAAAATGGAGCTGGAA--
CCGGTTGAACTGTTTACCCCCCTCTTTCTGC-TAATGTTTCTCATGCTGG-----
AGCCTCAGTAGACTTAGCTATTTT--TTCCT--TTCATTTA--GCCGGAATT-
TCTTCTATTTTAGGAGCAGTTAATTT-TATTA-
CTACTATTATTAATATACGATCTAATGGAATT---
ACTTTTGACCGAATACCTTTATTTGTTTGATCAGTTTTAA-TTACTGC--A-
TTTTACTT--GTTACTT----TC-TTTACCTGT-ACTAHCAGGAG--CTATCACTA-
TACTTTTAACTGACCGTAACATTAATACCTC

***C. subschultzei*_N19**

TTAGAATT-CTTA-TTCG----AGCAG-AATTAGGACA-----TCCTGGTGCT---TTA-
ATTGGTAATGACCAAATTTATAAT--GTTATTGTTACTGCC-CATGCTT-
TTATTATAATTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TTCCTTTAAT--
ATTAGGAGCTCCAGATATGGCTTTTCCACGGATAAATAATATAAGTTTTTGATTA
TT----ACCCCCCTCTTTATCTTTATTATTAATTAGAA--
GACTAGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTTTATCCTCCTTTATCTGC-AAATGTTTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCAATTTT--CTCTT--TACATTTA--GCTGGAATT-
TCTTCCATTTTAGGGGCTGTTAATTT-TATTA-
CTACTATTATTAATATACGATCAAATGGTATT---
TCATTTGATCGTATGCCATTATTTGTATGATCTGTTTTAA-TTACTGC-TA-
TTCTTTT---ATTACTT----TC-TTTACCTGT-TTTAGCCGGAG--CTATTACTA-
TACTATTAACAGATCGTAATATTAACACCTCATTTTTT

***C. schultzei*_N21**

A----AGCAGGAATTAGGTCA-----TCCAGGTGCT---TTA-
ATTGGTAACGACCAGATTTATAAT--GTCATTGTTACTGCA-CATGCTT-
TTATTATAATTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TACCTTTAAT--

ATTAGGAGCCCCAGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGATTA
CT----ACCACCTTCTTTATCTTTATTATTAATTAGAA--
GCTTAGTAGAAAATGGGGCAGGAA--
CAGGATGAACTGTTTACCCTCCTTTATCTGC-TAATGTTTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCTATTTT--TTCTT--TACATTTA--GCCGGTATT-
TCTTCTATTTTAGGAGCTGTTAATTT-CATTA-
CTACAATTATTAATATGCGATCAAATGGAATT---
TCATTTGACCGAATACCTTTATTTGTATGATCTGTATTAA-TTACAGC-AA-
TTCTTTT---ATTATTA----TC-TTTGCCAGT-TTTAGCTGGAG--CTATTACTA-
TATTATTAACAGACCGTAATATTAATACTTCATTTTTT

***C. leucostictus*_N22**

TGGAACTTCTTTAAGAATC-CTTA-TTCG----AGCAG-AATTAGGCCA-----
TCCAGGTGCT---CTG-ATCGGTAACGATCAAATTTATAAT--
GTAATTGTTACAGCA-CATGCAT-TTGTGATAATTTTCTTTAT-
AGTAATACCTATTA-TA-ATCGGGGGAT--TTGGTAATTGACTAG---TGCCACTAAT-
-
ATTAGGAGCCCCAGATATAGCTTTCCCCCGAATAAATAATATAAGTTTCTGAATA
CT----ACCCCCTTCTCTTTCTTTATTATTAATTAGTA--
GTTTAGTAGAAAATGGAGCAGGAA--
CAGGATGAACAGTCTATCCTCCCTTGTCAGC-TAATGTATCTCATGCTGG-----
TGCTTCAGTGGACTTAGCAATTTT--TTCTC--TTCATTTA--GCTGGTATT-
TCTTCTATTTTAGGAGCAGTAAATTT-TATCA-
CAACAATTATTAATATACGATCAAATGGGATT---
ACTTTCGACCGAATACCTTTATTTGTCTGATCAGTTTTTA-TTACAGC-GA-
TTCTTCT---TCTCCTT----TC-TTTACCAGT-TTTAGCTGGTG--CGATTACGA-
TACTATTAACAGATCGAAACATTAATA

***C. exspectator*_N24**

TTAAGTATC-CTTA-TTCG----AGCAG-AATTAGGTCA-----TCCAGGTGCT---TTA-
ATTGGTAACGACCAGATTTATAAT--GTCATTGTTACTGCA-CATGCTT-
TTATTATAATTTTTTTTAT-AATTATACCAATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TACCTTTAAT--
ATTAGGAGCCCCAGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGATTA
CT----ACCACCTTCTTTATCTTTATTATTAATTAGAA--
GCTTAGTAGAAAATGGGGCAGGAA--
CAGGATGAACTGTTTACCCTCCTTTATCTGC-TAATGTTTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCTATTTT--TTCTT--TACATTTA--GCCGGTATT-
TCTTCTATTTTAGGAGCAGTTAATTT-CATTA-
CTACAATTATTAATATGCGATCAAATGGAATT---
TCATTTGACCGAATACCCTTATTTGTATGATCTGTATTAA-TTACAGC-AA-
TTCTATT---ATTATTA----TC-TTTACCAGT-TTTAGCAGGAG--CTATTACTA-
TACTATTAACAGATCGTAATATTAATACTTCATTTTTGATCT

***C. exspectator*_N25**

GGTCA-----TCCTGGTGCT---TTA-ATTGGTAATGATCAAATTTATAAT--
GTTATTGTGACAGCT-CATGCTT-TTATTATAATTTTTTTTAT-AGTTATACCAATTA-
TA-ATTGGAGGAT--TTGGAAATTGATTAG---TACCTTTAAT--
ACTAGGAGCACCAGATATAGCTTTTCCTCGTATAAATAATATAAGTTTTTGAATAT
T----ACCTCCCTCATTATCATTACTATTGATTAGCA--
GCCTTGTAGAGAATGGGGCAGGAA--
CAGGATGAACAGTTTACCCTCCTCTTTCTGC-TAATGTTTCTCATGCAGG-----
AGCTTCAGTAGATTTAGCAATTTT--TTCTT--TGCATTTA--GCTGGAATT-
TCTTCAATCTTAGGGGCAGTAAATTT-TATTA-
CAACAATTATTAATATACGTTCTAATGGAATTT--CTTT-
CGACCGAATACCCCTATTTGTTTGATCTGTATTAA-TTACAGC-AA-TTTTATT---
ACTTCTT----TC-TTTGCCCGT-CTTAGCAGGTG--CGATTACTA-
TACTTTTAACAGATCGTAATA

***C. exspectator*_N26**

AGTATC-CTTA-TTCG----TGCAG-AATTAGGTCA-----TCCTGGTGCT---TTA-
ATTGGTAATGATCAAATTTATAAT--GTTATTGTGACAGCT-CATGCTT-
TTATTATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATTGGAGGAT--
TTGGAAATTGATTAG---TACCTTTAAT--
ACTAGGAGCACCAGATATAGCTTTTCCTCGTATAAATAATATAAGTTTTTGAATAT
T----ACCCCCCTCATTATCATTACTATTGATTAGCA--
GCCTTGTAGAGAATGGGGCAGGAA--
CAGGATGAACAGTTTACCCTCCTCTTTCTGC-TAATGTTTCTCATGCAGG-----
AGCTTCAGTAGATTTAGCAATTTT--TTCTT--TGCATTTA--GCTGGAATT-
TCTTCAATCTTAGGGGCAGTAAATTT-TATTA-
CAACAATTATTAATATACGTTCTAATGGAATTT--
CTTTTCGACCGAATACCTCTATTTGTTTGATCTGTATTAA-TTACAGC-AA-
TTTTATT---ACTTCTT----TC-TTTACCCGT-CTTAGCAGGTG--CGATTACTA-
TACTTTTAACAGATCGTAATATTAATACATCTTTTTT

***C. nivosus*_N28**

GAGCAGGTATATAAGAATT-CTTA-TTCG----AGCTG-AATTAGGACA-----
CCCGGGAGCA---TTA-ATTGGAAATGATCAAATTTATAAT--GTAATTGTTACTGCA-
CATGCTT-TCGTAATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATTGGTGGAT--
TTGGCAATTGATTAG---TACCTCTTAT--
ATTAGGAGCCCCTGACATGGCTTTCCCTCGTATAAATAATATAAGATTTTGAATA
TT----ACCCCCCTCTTTATCCTTACTATTAATTAGTA--
GACTTGTAGAAAATGGAGCAGGCA--
CTGGATGAACTGTTTATCCACCCTTATCAGC-TAATATTTCTCATGCCGG-----
AGCTTCAGTAGATTTAGCTATTTT--TTCCC--TTCATTTA--GCCGGTATC-
TCCTCCATTCTAGGCGCTGTAAATTT-TATTA-
CAACAATTATTAATATACGATCAAATGGAATC---
ACTTTTGATCGAATACCTTTATTCGTTTGATCAGTTCTTA-TTACAGC-GA-
TTTTATT---ACTTTTA----TC-TTTACCTGT-TTTAGCAGGAG--CTATCACTA-
TATTACTTACAGATCGAAATAT

***C. eriodendroni*_N29**

GATATTGGTACTTTATATTTTATCTTTGGAATTTGGTCAGGAATAATTGGCTCTTC
TTTAAGAATT-TTAA-TTCG----AATAG-AATTAAGTCA-----TCCTGGATCT---TTA-
ATTGGAAATGATCAAATTTATAAT--ACAATTGTAACTGCT-CATGCTT-
TCATTATAATTTTTTTTAT-AGTTATACCTATTA-TA-ATTGGAGGAT--
TTGGTAATTGATTAG---TTCCTCTAAT--
ATTAGGGGCCCCAGACATAGCTTTCCCCCGAATAAATAACCTAAGTTTTTGATTT
TT----ACCCCCTTCAATCTCTCTCCTCCTCTTAAGAA--
GCCTAATCGAGAATGGCGCTGGAA--
CTGGCTGAACAGTTTACCCTCCCCTATCTAA-TAATATATTTCACCCTGG-----
AGCTTCTGTCGATTTAACAATTTT--TTCTT--TACATTTA--GCAGGAATC-
TCCTCAATTTTAGGAGCAATTAATTT-TATTA-
CAACTATTATTAACATACGCATAATTAATATAC--AATTT-
GATCAAATATCTTTATTTACTTGATCAGTTCTTA-TTACAGC-TA-TCCTATT---
ATTACTT----TC-TTTACCTGT-ATTAGCAGGGG--CAATTACTA-
TACTACTTACTGATCGAAATTTTAATACTTCA

***C. imicola*_N3**

-TTTAAGAATA-TTAA-TTCG----TCTAG-AATTAAGTCA-----CCCAGGTTCT---TTA-
ATTGGTAATGATCAAATTTATAAT--GTAATTGTTACAGCT-CATGCTT-
TTGTAATAATTTTTTTTAT-AGTAATACCTATTA-TA-ATTGGAGGAT--
TTGGAAATTGGTTAG---TTCCATTAAT--
ATTAGGCGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGAATAT
T----ACCTCCTTCTATTACTCTTCTTTTATTAAGAA--
GATTAGTAGAAAATGGGGCAGGAA--
CAGGATGAACTGTTTATCCTCCATTATCGGC-TAATGTTTCTCATGCTGG-----
AGCTTCAGTTGATTTAGCTATTTT--TTCTT--TGCATTTA--GCCGGTATT-
AGTTCAATTTTAGGTGCTGTAAATTT-TATTA-
CAACAATTATTAATATACGTCCTATTGGAATA---
ACTATAGATCGAATGCCTTTATTTGTTTGATCAGTTTTTA-TTACAGC-TA-
TTTTATT---ATTATTA----TC-ATTGCCTGT-ATTAGCAGGAG--CTATTA----------
ACAGATCGAAATAT

***C. eriodendroni*_N30**

ATAAAGATATTGGTACTTTATATTTTATCTTTGGAATTTGGTCAGGAATAATTGGC
TCTTCTTTAAGAATT-TTAA-TTCG----AATAG-AATTAAGTCA-----TCCTGGATCT---
TTA-ATTGGAAATGATCAAATTTATAAT--ACAATTGTAACTGCT-CATGCTT-
TCATTATAATTTTTTTTAT-AGTTATACCTATTA-TA-ATTGGAGGAT--
TTGGTAATTGATTAG---TTCCTCTAAT--
ATTAGGGGCCCCAGACATAGCTTTCCCCCGAATAAATAACCTAAGTTTTTGATTT
TT----ACCCCCTTCAATCTCTCTCCTCCTCTTAAGAA--
GCCTAATCGAGAATGGCGCTGGAA--
CTGGCTGAACAGTTTACCCTCCCCTATCTAA-TAATATATTTCACCCTGG-----
AGCTTCTGTCGATTTAACAATTTT--TTCTT--TACATTTA--GCAGGAATC-

TCCTCAATTTTAGGAGCAATTAATTT-TATTA-
CAACTATTATTAACATACGCATAATTAATATAC--AATTT-
GATCAAATATCTTTATTTACTTGATCAGTTCTTA-TTACAGC-TA-TCCTATT---
ATTACTT----TC-TTTACCTGT-ATTAGCAGGGG--CAATTACTA-
TACTACTTACTGATCGAAATTTTAATACTTCA

***C. punctithorax*_N31**

AGGAACTTCTTTAAGAATT-CTTA-TTCG----AGCAG-AATTAGGACA-----
TCCTGGTGCT---TTA-ATTGGTAATGATCAAATTTATAAT--GTTATTGTTACTGCC-
CATGCTT-TTATTATAATTTTTTTTAT-AATTATACCTATTA-TA-ATTGGAGGAT--
TCGGAAATTGACTTG---TTCCTTTAAT--
ATTAGGAGCCCCAGATATGGCTTTTCCACGAATAAATAATATAAGTTTTTGATTA
TT----ACCCCCTTCTTTATCTTTATTATTAATTAGAA--
GACTAGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTTTATCCTCCTTTATCTGC-AAATGTTTCTCATGCCGG-----
AGCTTCTGTAGATTTAGCAATTTT--CTCTT--TACATTTA--GCTGGAATT-
TCTTCCATTTTAGGGGCTGTTAATTT-TATTA-
CTACTATTATTAATATACGATCAAATGGTATT---
TCATTTGATCGTATACCATTATTTGTATGATCTGTTTTAA-TTACTGC-TA-
TTCTTTT---ATTACTT----TC-TTTACCTGT-TTTAGCCGGAG--CTATTACTA-
TACTATTAACAGATCGTAATATTAACACCTCAT

***C. imicola*_N4**

TA-TTAAATTCG----TCTAG-AATTAAA----------AG---------A-
ATTGGTAATGATCAAATTTATAAT--GTAATTGTTACAGCT-CATGCTT-
TTGTAATAATTTTTTTTAT-AGTAATACCTATTA-TA-ATTGGAGGAT--
TTGGAAATTGGTTAG---TTCCATTAAT--
ATTAGGCGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGAATAT
T----ACCTCCTTCTATTACTCTTCTTTTATTAAGAA--
GATTAGTAGAAAATGGGGCAGGAA--
CAGGATGAACTGTTTATCCTCCATTATCGGC-TAATGTTTCTCATGCTGG-----
AGCTTCAGTTGATTTAGCTATTTT--TTCTT--TGCATTTA--GCCGGTATT-
AGTTCAATTTTAGGTGCTGTAAATTT-TATTA-
CAACAATTATTAATATACGTCCTATTGGAATAC--
ACTATAGATCGAATGCCTTTATTTGTTTGATCAGTTTTTA-TTACG---------TATT---
ATT--------TC-ATTGCCTGT-ATTAGCAGG----C----------ACAGATCGAAATATTA

***C. nivosus*_N44**

CTTCCCTAAGAATT-CTTA-TTCG----AGCTG-AATTAGGACA-----CCCGGGAGCA---
TTA-ATTGGAAATGATCAAATTTATAAT--GTAATTGTTACTGCA-CATGCTT-
TCGTAATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATTGGTGGAT--
TTGGCAATTGATTAG---TACCTCTTAT--
ATTAGGAGCCCCTGACATGGCTTTCCCTCGTATAAATAATATAAGATTTTGAATA
TT----ACCCCCCTCTTTATCCTTACTATTAATTAGTA--
GACTTGTAGAAAATGGAGCAGGCA--
CTGGATGAACTGTTTATCCACCCTTATCAGC-TAATATTTCTCATGCCGG-----

AGCTTCAGTAGATTTAGCTATTTT--TTCCC--TTCATTTA--GCCGGTATC-
TCCTCCATTCTAGGCGCTGTAAATTT-TATTA-
CAACAATTATTAATATACGATCAAATGGAATC---
ACTTTTGATCGAATACCTTTATTCGTTTGATCAGTTCTTA-TTACAGC-GA-
TTTTATT---ACTTTTA----TC-TTTACCTGT-TTTAGCAGGAG--CTATCACTA-
TATTACTTACAGATCGAAATATTAACACATCTTTCTTT

***C. pycnostictus*_N45**

CAAAATTTATAAT--GTTATTGTTACAGCC-CATGCTT-TCGTTATAATTTTTTTTAT-
AGTAATGCCTATTA-TA-ATTGGAGGAT--TTGGAAATTGATTAG---TACCTCTTAT--
ATTAGGGGCTCCTGATATAGCTTTCCCGCGAATAAATAATATAAGTTTCTGAATA
CT----ACCACCATCTCTGTCTTTACTTTTAATTAGCA--
GTCTAGTTGAAAATGGAGCTGGAA--
CCGGTTGAACTGTTTATCCCCCTCTTTCTGC-TAATGTTTCCCATGCTGG-----
GGCCTCAGTAGACTTAGCTATCTT--TTCCC--TTCATTTA--GCTGGTATT-
TCTTCTATTTTAGGAGCAGTTAATTT-TATTA-
CTACTATTATTAATATACGATCTAATGGTATT---
ACATTTGACCGAATACCTTTATTTGTTTGATCAGTTCTTA-TTACTGC-TA-
TTTTACT---GTTACTT----TC-TTTACCTGT-ACTTGCCGGAG--CTATTACTA-
TACTTTTAACTGACCGAAACATTAATACCTCTTTCTT

***C. imicola*_N6**

TAAGAATA-TTAA-TTCG----TCTAG-AATTAAGTCA-----CCCAGGTTCT---TTA-
ATTGGTAATGATCAAATTTATAAT--GTAATTGTTACAGCT-CATGCTT-
TTGTAATAATTTTTTTTAT-AGTAATACCTATTA-TA-ATTGGAGGAT--
TTGGAAATTGGTTAG---TTCCATTAAT--
ATTAGGTGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGAATAT
T----ACCTCCTTCTATTACTCTTCTTTTATTAAGAA--
GATTAGTAGAAAATGGGGCAGGAA--
CAGGATGAACTGTTTATCCCCCATTATCGGC-TAATGTTTCTCATGCTGG-----
AGCTTCAGTTGATTTAGCTATTTT--TTCTT--TACATTTA--GCCGGTATT-
AGTTCAATTTTAGGTGCTGTAAATTT-TATTA-
CAACAATTATTAATATACGTCCTATAGGAATA---
ACTATAGATCGAATGCCTTTATTTGTTTGATCAGTTTTTA-TTACAGC-TA-
TTTTATT---ATTATTA----TC-ATTGCCTGT-ATTAGCAGGAG--CTATTACAA-
TATTATTAACAGATCGAAATATTAATACTTCTTTTTT

***C. tropicalis*_N64**

CGTTAAGAATT-CTTA-TTCG----AGCAG-AATTAGGGCA-----CCCAGGAGCT---
TTA-ATTGGAAATGACCAAATTTATAAT--GTAATTGTTACAGCT-CATGCCT-
TTATTATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATCGGGGGAT--
TTGGAAATTGACTAG---TTCCTTTAAT--
ATTAGGGGCCCCAGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGAATA
TT----ACCCCCTTCTTTATCCTTATTATTAATTAGAA--
GCCTTGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTATACCCCCCACTTTCAGC-AAATGTTTCTCATGCAGG-----

AGCATCTGTTGATTTAGCAATTTT--TTCAT--TACATTTA--GCTGGAATC-
TCTTCAATTTTAGGGGCAGTAAATTT-TATTA-
CTACTATTATTAATATACGGTCAAATGGAATT---
TCATTTGACCGTATACCTTTATTTGTTTGATCTGTTTTAA-TCACAGC-TA-
TTCTTCT---TTTACTT----TC-ACTTCCAGT-ATTAGCTGGAG--CTATCACTA-
TACTTTTAACTGACCGTAACATCAATACTTCATTTTT

***C. nivosus*_N70**

ACTTCCCTAAGAATT-CTTA-TTCG----AGCTG-AATTAGGACA-----CCCGGGAGCA-
--TTA-ATTGGAAATGATCAAATTTATAAT--GTAATTGTTACTGCA-CATGCTT-
TCGTAATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATTGGTGGAT--
TTGGCAATTGATTAG---TACCTCTTAT--
ATTAGGAGCCCCTGACATGGCTTTCCCTCGTATAAATAATATAAGATTTTGAATA
TT----ACCCCCCTCTTTATCCTTACTATTAATTAGTA--
GACTTGTAGAAAATGGAGCAGGCA--
CTGGATGAACTGTTTATCCACCCTTATCAGC-TAATATTTCTCATGCCGG-----
AGCTTCAGTAGATTTAGCTATTTT--TTCCC--TTCATTTA--GCCGGTATC-
TCCTCCATTCTAGGCGCTGTAAATTT-TATTA-
CAACAATTATTAATATACGATCAAATGGAATC---
ACTTTTGATCGAATACCTTTATTCGTTTGATCAGTTCTTA-TTACAGC-GA-
TTTTATT---ACTTTTA----TC-TTTACCTGT-TTTAGCAGGAG--CTATCACTA-
TATTACTTACAGATCGAAATATTAACACATCTTTCTTT

***C. nivosus*_N75**

CCCTAAGAATT-CTTA-TTCG----AGCTG-AATTAGGACA-----CCCGGGAGCA---
TTA-ATTGGAAATGATCAAATTTATAAT--GTAATTGTTACTGCA-CATGCTT-
TCGTAATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATTGGTGGAT--
TTGGCAATTGATTAG---TACCTCTTAT--
ATTAGGAGCCCCTGACATGGCTTTCCCTCGTATAAATAATATAAGATTTTGAATA
TT----ACCCCCCTCTTTATCCTTACTATTAATTAGTA--
GACTTGTAGAAAATGGAGCAGGCA--
CTGGATGAACTGTTTATCCACCCTTATCAGC-TAATATTTCTCATGCCGG-----
AGCTTCAGTAGATTTAGCTATTTT--TTCCC--TTCATTTA--GCCGGTATC-
TCCTCCATTCTAGGCGCTGTAAATTT-TATTA-
CAACAATTATTAATATACGATCAAATGGAATC---
ACTTTTGATCGAATACCTTTATTCGTTTGATCAGTTCTTA-TTACAGC-GA-
TTTTATT---ACTTTTA----TC-TTTACCTGT-TTTAGCAGGAG--CTATCACTA-
TATTACTTACAGATCGAAATATTAACACATCTTTCTT

***C. leucostictus*_N76**

TAGATC-CTTA-TTCG----AGCAG-AATTAGGTCA-----TCCAGGTGCT---CTG-
ATCGGTAACGATCAAATTTATAAT--GTAATTGTTACAGCA-CATGCAT-
TTGTGATAATTTTCTTTAT-AGTAATGCCTATTA-TA-ATCGGGGGAT--
TTGGTAATTGACTAG---TGCCACTAAT--
ATTAGGAGCCCCAGATATAGCTTTCCCCCGGATAAATAATATAAGTTTCTGAATA
CT----ACCCCCTTCTCTTTCTTTATTATTAATTAGTA--

GTTTAGTAGAAAATGGAGCAGGAA--
CAGGATGAACGGTTTATCCTCCCTTGTCAGC-TAATGTATCTCATGCTGG-----
TGCTTCAGTGGACTTAGCAATTTT--TTCTC--TTCATTTA--GCTGGTATT-
TCTTCTATTTTAGGAGCAGTAAATTT-TATCA-
CAACAATTATTAATATACGATCAAATGGGGTT---
ACTTTCGACCGAATACCTTTATTTGTCTGATCAGTTTTTA-TTACAGC-AA-
TTCTTCT---TCTCCTT----TC-TTTACCAGT-TTTAGCTGGTG--CGATTACAA-
TACTATTAACAGATCGAAACATTAATACCTCATTTTT

***C. similis*_N78**

TTAAGTATC-CTTA-TTCG----AGCTG-AATTAGGCCA-----CCCAGGAGCA---TTA-
ATTGGGAATGATCAAATTTATAAT--GTTATTGTTACTGCC-CATGCTT-
TTGTTATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATCGGAGGAT--
TTGGTAATTGACTTG---TCCCTTTAAT--
GCTTGGAGCACCAGATATAGCATTCCCGCGTATGAATAACATAAGTTTTTGAATA
TT----ACCCCCTTCTTTATCATTATTATTAATCAGCA--
GATTAGTTGAAAATGGGGCCGGAA--
CAGGTTGAACTGTTTATCCACCTTTATCGGC-AAATGTCTCTCATGCAGG-----
TGCCTCAGTAGACCTAGCTATTTT--TTCCT--TACACCTA--GCGGGGATT-
TCATCAATTTTAGGAGCAGTAAATTT-TATTA-
CTACAATTATTAATATACGATCTAATGGAATT---
ACTTTTGACCGAATACCCTTATTTGTTTGGTCCGTTTTAA-TTACAGC-TA-
TTTTACT---TTTACTT----TC-TTTACCTGT-TTTAGCAGGAG--CAATTACTA-
TACTTTTAACGGATCGTAATATTAATACTTCTTTTTTT

***C. imicola*_N9**

TTAAGAATA-TTAA-TTCG----TCTAG-AATTAAGTCA-----CCCAGGTTCT---TTA-
ATTGGTAATGATCAAATTTATAAT--GTAATTGTTACAGCT-CATGCTT-
TTGTAATAATTTTTTTTAT-AGTAATACCTATTA-TA-ATTGGAGGAT--
TTGGAAATTGGTTAG---TTCCATTAAT--
ATTAGGTGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGAATAT
T----ACCTCCTTCTATTACTCTTCTTTTATTAAGAA--
GATTAGTAGAAAATGGGGCAGGAA--
CAGGATGAACTGTTTATCCCCCATTATCGGC-TAATGTTTCTCATGCTGG-----
AGCTTCAGTTGATTTAGCTATTTT--TTCTT--TACATTTA--GCCGGTATT-
AGTTCAATTTTAGGTGCTGTAAATTT-TATTA-
CAACAATTATTAATATACGTCCTATAGGAATA---
ACTATAGATCGAATGCCTTTATTTGTTTGATCAGTTTTTA-TTACAGC-TA-
TTTTATT---ATTATTA----TC-ATTGCCTGT-ATTAGCAGGAG--CTATTACAA-
TATTATTAACAGATCGAAATATTAATACTTCTTTTTT

***C. tropicalis*_N93**

TAAGAATT-CTTA-TTCG----AGCAG-AATCCGGGCA-----CCCAGGAGCT---TTA-
ATTGGAAATGACCAAATTTATAAT--GTAATTGTTACAGCT-CATGCCT-
TTATTATAATTTTTTTTAT-AGTTATACCAATTA-TA-ATCGGGGGAT--
TTGGAAATTGACTAG---TTCCTTTAAT--

ATTAGGGGCCCCAGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGAATA
TT----ACCCCCTTCTTTATCCTTATTATTAATTAGAA--
GCCTTGTAGAAAATGGGGCAGGAA--
CTGGTTGAACAGTATACCCCCCACTTTCAGC-AAATGTTTCTCATGCAGG-----
AGCATCTGTTGATTTAGCAATTTT--TTCAT--TACATTTA--GCTGGAATC-
TCTTCAATTTTAGGGGCAGTAAATTT-TATTA-
CTACTATTATTAATATACGGTCAAATGGAATT---
TCATTTGACCGTATACCTTTATTTGTTTGATCTGTTTTAA-TCACAGC-TA-
TTCTTCT---TTTACTT----TC-ACTTCCAGT-ATTAGCTGGAG--CTATCACTA-
TACTTTTAACTGACCGTAACATCAATACTTCATTTTTT

***C. leucostictus*_N97**

TAGCCTT-TTAA-TTCG----AATTG-AATTAGGCCA-----ACCAGGAGCC---TTT-
ATTGGAAATGACCAAATTTATAAT--GTTCTTGTTACTGCC-CATGCTT-
TTGTAATAATTTTTTTTAT-AGTTATACCTATTA-TA-ATTGGGGGAT--
TTGGGAATTGATTAG---TCCCTTTAAT--
ATTAGGGGCTCCTGATATAGCTTTCCCTCGTATAAATAATATAAGTTTTTGAATG
TT----ACCCCCCTCTCTTACTTTACTGTTAATTAGAG--
GACTAGTGGAAAATGGGGCTGGAA--
CAGGTTGAACAGTTTATCCTCCTTTATCTTC-TAATATTTCTCACGCAGG-----
GGCATCAGTAGATTTAGCAATTTT--TTCTT--TACATCTG--GCTGGTATT-
TCTTCCATTTTGGGGGCAGTTAATTT-TATTA-
CAACAATTATTAATATGCGAGCTAATGGAATTA--CATTT-
GATCGAATGCCTTTATTTGTTTGATCTGTTCTAA-TTACTGC-TG-TTTTACT---
ATTATTA----TC-ATTACCTGT-TTTAGCTGGAG--CTATTACTA-
TACTTCTTACAGATCGAAATATTAATACTTCTTTTTTTGACCCCGCTGGAGGAGG
GGACCCAA

BLAST results of Namibian *Culicoides* specimens

Sample ID	Description	Max score	Total score	Query cover	E value	Identity	Accession
N116	Culicoides shivasi voucher ww08191 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	558	558	99%	5,00E-155	83%	JX681734.1
N117	Culicoides shivasi voucher ww08191 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	582	582	97%	3,00E-162	83%	JX681734.1
N123	Culicoides shivasi voucher ww08191 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	555	555	99%	6,00E-154	83%	JX681734.1
N100	Culicoides enderleini voucher CIRAD:ENDER-EQ3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	821	821	71%	0.0	98%	HQ447066.1
N101	Culicoides enderleini voucher CIRAD:ENDER-EQ3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	741	741	76%	0.0	98%	HQ447066.1
N29	Nemophora metallica voucher BC ZSM Lep 37567 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	536	536	96%	2,00E-148	84%	KX040182.1
N30	Nemophora metallica voucher BC ZSM Lep 37567 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	586	586	98%	2,00E-163	84%	KX040182.1
N126	Taractrocera dolon voucher 11ANIC-08346 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	638	638	98%	6,00E-179	84%	KF391631.1
N24	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-6 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	331	331	66%	9,00E-87	85%	KT307839.1

N25	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	601	601	98%	5,00E-168	90%	KT307836.1
N26	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	536	536	100%	2,00E-148	88%	KT307836.1
N161	Culicoides oxystoma voucher JHKM5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	616	616	99%	2,00E-172	86%	KF528693.1
N1	Culicoides imicola isolate 1000610 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	832	832	100%	0.0	99%	KT945263.1
N3	Culicoides imicola isolate 1000584 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	774	774	97%	0.0	93%	KT339721.1
N4	Culicoides imicola isolate 1000584 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	660	660	86%	0.0	94%	KT339721.1
N6	Culicoides imicola isolate 1000579 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	970	970	99%	0.0	93%	KT339716.1
N9	Culicoides imicola isolate 1000583 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	922	922	96%	0.0	93%	KT339720.1
N76	Culicoides mesghalii voucher TPI:ENT:IBVNET-CULI-TN-23 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	680	680	100%	0.0	87%	KT307829.1
N97	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	586	586	98%	2,00E-163	86%	KT307835.1
N128	Culicoides immaculatus voucher ww05931 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	760	760	100%	0.0	87%	JX681720.1

N22	Culicoides sonorensis voucher BIOUG08067-A09 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	656	656	99%	0.0	87%	KR680744.1
N129	Culicoides immaculatus voucher ww05931 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	749	749	100%	0.0	87%	JX681720.1
N28	Culicoides peliliouensis voucher TPI:ENT:IBVNET-CULI-TN-34 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	701	701	94%	0.0	90%	KT307854.1
N44	Culicoides peliliouensis voucher TPI:ENT:IBVNET-CULI-TN-34 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	787	787	98%	0.0	88%	KT307854.1
N70	Culicoides peliliouensis voucher TPI:ENT:IBVNET-CULI-TN-34 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	800	800	98%	0.0	89%	KT307854.1
N75	Culicoides peliliouensis voucher TPI:ENT:IBVNET-CULI-TN-34 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	754	754	97%	0.0	89%	KT307854.1
N132	Culicoides mesghalii voucher TPI:ENT:IBVNET-CULI-TN-20 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	669	669	98%	0.0	86%	KT307832.1
N31	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	861	861	100%	0.0	93%	KT307836.1
N45	Culicoides sonorensis isolate c5 cytochrome oxidase subunit I gene, partial cds; mitochondrial	664	664	97%	0.0	87%	KT794137.1
N108	Culicoides variipennis isolate 24 cytochrome oxidase subunit I gene, partial cds; mitochondrial	658	658	84%	0.0	86%	KT794161.1
N127	Culicoides sonorensis isolate c5 cytochrome oxidase subunit I gene, partial cds; mitochondrial	721	721	97%	0.0	87%	KT794137.1
N151	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-7 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	649	649	99%	0.0	88%	KT307840.1

N159	Culicoides oxystoma voucher JHKM5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	654	654	98%	0.0	88%	KF528693.1
N158	Culicoides oxystoma voucher JHKM5 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	654	654	93%	0.0	87%	KF528693.1
N152	Culicoides sp. BOLD:ACC1144 voucher BIOUG03410-D04 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	765	765	95%	0.0	88%	KM987986.1
N157	Culicoides sp. BOLD-2016 voucher BIOUG07795-D04 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	643	643	93%	1,00E-180	87%	KR695194.1
N21	Culicoides oxystoma voucher JHKM6 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	732	732	97%	0.0	92%	KF528694.1
N103	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	817	817	100%	0.0	92%	KT307836.1
N104	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	702	702	99%	0.0	88%	KT307836.1
N105	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	812	812	98%	0.0	92%	KT307836.1
N124	Culicoides similis voucher TPI:ENT:IBVNET-CULI-TN-10 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	822	822	98%	0.0	89%	KT307844.1
N78	Culicoides similis voucher TPI:ENT:IBVNET-CULI-TN-10 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	675	675	100%	0.0	88%	KT307844.1
N11	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	835	835	98%	0.0	93%	KT307836.1

N13	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	848	848	99%	0.0	93%	KT307836.1
N14	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	837	837	99%	0.0	93%	KT307836.1
N16	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	825	825	99%	0.0	92%	KT307836.1
N19	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	796	796	98%	0.0	92%	KT307836.1
N93	Culicoides sp. BOLD:ABY1424 voucher BIOUG08030-H05 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	516	516	98%	2,00E-142	83%	KM904453.1
N64	Culicoides oxystoma voucher TPI:ENT:IBVNET-CULI-TN-6 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	704	704	99%	0.0	88%	KT307839.1
N118	Culicoides sp. BOLD-2016 voucher BIOUG07215-F09 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	627	627	98%	1,00E-175	87%	KR686414.1

APPENDIX B

Assession number	Author	Specie	Country
KT945263.1	Onyango *et al*., 2015	*C. imicola*	South Africa
KT339721.1	Onyango *et al*., 2015	*C. imicola*	South Africa
KT339716.1	Onyango *et al*., 2015	*C. imicola*	South Africa
KT339720.1	Onyango *et al*., 2015	*C. imicola*	South Africa
KT307836.1	Harrup *et al*., 2016 (Unpublished)	*C. oxystoma*	southern India
KF528694.1	He *et al*., 2013 (Unpublished)	*C. oxystoma*	-
KT307839.1	Harrup *et al*., 2016 (Unpublished)	*C. oxystoma*	southern India
KT307844.1	Harrup *et al*., 2016 (Unpublished)	*C. similis*	southern India
JX681734.1	Bellis *et al*., 2013	*C. shivasi*	Australia
KF528693.1	He *et al*., 2013 (Unpublished)	*C. oxystoma*	-
KT794159.1	Shults, 2016 (Unpublished)	*C. sonorensis*	Texas
KT794137.1	Shults, 2016 (Unpublished)	*C. sonorensis*	Texas
KT307837.1	Harrup *et al*., 2016 (Unpublished)	*C. oxystoma*	southern India
KT307829.1	Harrup *et al*., 2016 (Unpublished)	*C. oxystoma*	southern India
KT307835.1	Harrup *et al*., 2016 (Unpublished)	*C. oxystoma*	southern India
KT307832.1	Harrup *et al*., 2016 (Unpublished)	*C. mesghalii*	southern India
KM904453.1	Dewaard *et al*., 2015 (Unpublished)	*C*. sp.	-
KM987986.1	Dewaard *et al*., 2015 (Unpublished)	*C*. sp.	-
KR686414.1	Herbert *et al*., 2016	*C*. sp.	Canadian
KR695194	Herbert *et al*., 2016	*C*. sp.	Canadian
KR680744.1	Herbert *et al*., 2016	*C. sonorensis*	Canadian
KF528693	He *et al*., 2013 (Unpublished)	*C. oxystoma*	-
KF391631.1	Herbert *et al*., 2013	*Taractrocera dolon*	-

KF682525.1	Bakhoum *et al*., 2014	*C. subschultzei*	Senegal
KF682528.1	Bakhoum *et al*., 2014	*C. enderleini*	Senegal
KF682473.1	Bakhoum *et al*., 2014	*C. enderleini*	Senegal
KF682479.1	Bakhoum *et al*., 2014	*C. enderleini*	Senegal
KF682533.1	Bakhoum *et al*., 2014	*C. oxystoma*	Senegal
KJ624083.1	Sarvasova *et al*., 2014	*C. furcillatus*	Slovakia
KJ833701.1	Sambou *et al*., 2015	*C. enderleini*	Senegal
KX040182.1	Mutanen *et al*., 2016	*Nemophora metallica*	European
JX681720.1	Bellis *et al*., 2013	*C. immaculatus*	Australian
JX681722.1	Bellis *et al*., 2013	*C. immaculatus*	Australian
JX681735.1	Bellis *et al*., 2013	*C. shivasi*	Australian
KJ833687.1	Sambou *et al*., 2015	*C. wansoni*	Senegal
HQ447066.1	Desvars *et al*., 2016	*C. enderleini*	Reunion Island
GQ338927	Pages *et al*., 2009	*C. subfagineus*	-

APPENDIX C

LOCUS FJ196587 1978 bp RNA linear VRL 21-MAY-2009

DEFINITION African horsesickness virus isolate HS 02/07 structural protein VP4 gene, complete cds.

TITLE	African horsesickness virus isolate HS 02/07 structural protein VP4
ACCESSION	FJ196587
VERSION	FJ196587.1 GI:209167968
KEYWORDS	.
SOURCE	African horse sickness virus
ORGANISM	African horse sickness virus
	Viruses; dsRNA viruses; Reoviridae; Sedoreovirinae; Orbivirus.
REFERENCE	1 (bases 1 to 1978)
AUTHORS	Fasina,F., Potgieter,A.C., Ibironke,A., Bako,B., Bwala,D. and Kumbish,P.
TITLE	First Report of an Outbreak of African Horsesickness Virus Serotype 2 in the Northern Hemisphere
JOURNAL	J. Equine Vet. Sci. 28 (3), 167-170 (2008)
REFERENCE	2 (bases 1 to 1978)
AUTHORS	Potgieter,A.C., Page,N.A., Liebenberg,J., Wright,I.M., Landt,O. and van Dijk,A.A.
TITLE	Improved strategies for sequence-independent amplification and sequencing of viral double-stranded RNA genomes
JOURNAL	J. Gen. Virol. 90 (PT 6), 1423-1432 (2009)
PUBMED	19264638
REFERENCE	3 (bases 1 to 1978)
AUTHORS	Potgieter,A.C., Fasina,F. and van der Sluis,R.
TITLE	Direct Submission
JOURNAL	Submitted (08-SEP-2008) Virology, ARC-Onderstepoort Veterinary Institute, 100 Old Soutpan Road, Pretoria, Gauteng 0110, South Africa
COMMENT	GenBank Accession Numbers FJ196584-FJ196593 represent the complete genome of African horsesickness virus isolate HS 02/07.

FEATURES Location/Qualifiers

source 1..1978

/organism="African horse sickness virus"

/mol_type="genomic RNA"

/serotype="2"

/isolate="HS 02/07"

/host="horse"

/db_xref="taxon:40050"

/country="Nigeria"

CDS 12..1940

/codon_start=1

/product="structural protein VP4"

/protein_id="ACI41993.1"

/db_xref="GI:209167969"

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RRVTLRKSFGNMLRAYAFQHVTILHGSEAETLSYADPKRHVVKGQPKAAPMYHPD
KWWRDVDDGPTDKKLVSMLDYIIYSADEVYYVGCGDLKTLEQFASRDRKRFDRIR
WICIDPIAPETSYANVKIVKEKVVSARDLKHYLMRDEVERLLIWDVSADGLKGTIEWE
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ORIGIN

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121 ggttggagag gggcagaatg gcgtgtgaca catacgcagt tggaaaaatt gatcaatggt

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1201 acggtgatga gagaacgaga gcggatctct tttacttaac gaatatgcgg aatgctgcgt

1261 ttatgcatga tgtatatcga gtagttgaaa aaagttttat ttctaccttg tgggtttcga

1321 gcaggcagaa tttcacatat gatgatgttc cggttaatag gaattttatt acattacgct

1381 tttcaaagaa gaatcgacga gtgctcgacg ggaatggagc gattttgttt ttgatgtggc

1441 agcatccaaa ggattttcca aaaactacga actatgaccc cagttgggcg gagaactatg

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1621 ctgatatctt gaagaaattg ggcttggacg tatcaggtca tctatttatt tgtttgatgt

1681 caaattcata tgttgctgat cttgattggt ggtttcgcat gatcctagaa tggtcagtta

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